JOURNAL OF CHROMATOGRAPHY LIBRARY - volume 55

bioaffinity chromatography

second, completely revised edition

Jaroslava Turková

ELSEVIER

JOURNAL OF CHROMATOGRAPHY LIBRARY — volume 55

bioaffinity chromatography

Second, Completely Revised Edition

This Page Intentionally Left Blank

JOURNAL OF CHROMATOGRAPHY LIBRARY — volume 55

bioaffinity chromatography

Second, Completely Revised Edition

Jaroslava Turková

Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences
and
Institute of Pathological Physiology, Faculty of Medicine,
Charles University, Prague, Czech Republic

ELSEVIER
Amsterdam — London — New York — Tokyo 1993

ELSEVIER SCIENCE PUBLISHERS B.V.
Sara Burgerhartstraat 25
P.O. Box 211, 1000 AE Amsterdam, The Netherlands

Library of Congress Cataloging-in-Publication Data

Turková, Jaroslava.
Bioaffinity chromatography / Jaroslava Turková.
p. cm. -- (Journal of chromatography library ; v. 55)
Includes bibliographical references and index.
ISBN 0-444-89030-0 (acid-free paper)
1. Affinity chromatography. I. Title. II. Series.
QP519.9.A35T873 1993
574.19'28--dc20 93-11627
CIP

ISBN 0-444-89030-0

This book is printed on acid-free paper.

Printed in The Netherlands

Contents

Chapter 7

This Page Intentionally Left Blank

To my daughters,

Radka and Vlaďka with their husbands,

my effective helpers

This Page Intentionally Left Blank

PREFACE

In the rapidly developing field of bioaffinity chromatography, where new findings are published in a vast range of journals, there is an urgent need to be able to offer the most up-to-date coverage of information. Reprints of papers, lectures presented at symposia on affinity chromatography, and valuable discussions with my friends and colleagues have considerably helped me in studying the increased accessibility and stability of active sites of biologically active compounds attached to solid supports in bioadsorbents. I have also taken advantage of the work of many polymer chemists in Prague who have prepared many different activated solid supports for my study. I wish to cordially thank all my friends and colleagues who made the preparation of this book much easier than anticipated.

This book would never have been written without the support of my friends, Dr. Otakar Mikeš, author of many chapters and the books "High-performance Liquid Chromatography of Biopolymers and Biooligomers" (Part A and B, Elsevier, 1988) and Dr. Karel Macek, also an author of many chapters and chief editor of the Journal of Chromatography, Biomedical Applications (Elsevier, Amsterdam). I am profoundly grateful to them.

At present there are far more than 5000 references in bibliographic reviews dealing with the isolation, determination or removal of biologically active substances using bioaffinity chromatography. Therefore, this book cannot provide a comprehensive bibliographic review. All types of substances have been represented by selecting 40% of the studied materials. Tables dealing with low- and high-performance and large-scale bioaffinity chromatography include information on biologically active substances with their affinants, solid supports and methods of coupling. I am greatly indebted to Dr. Bedřich Meloun, who has checked the separation of substances according to their types.

I considered it necessary to have the manuscript read by other persons in order to eliminate possible errors and oversights that might easily have occurred. In this respect special thanks are due to my daughter, Dr. Radomíra Vaňková (Institute of Expe-

rimental Botany, Academy of Sciences of the Czech Republic, Prague), who read the whole manuscript and pointed out shortcomings and made preliminary linguistic corrections. I am also grateful to Dr. Zdeněk Havlas (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague) for critically reading and commenting on the chapter about the theory of bioaffinity chromatography. Beyond this, I am indebted to my son-in-law, Dr. Tomáš Vaněk (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague), who helped with the final preparation of the manuscript. I am also grateful to Dr. J. Cirýn, Mrs. D. Dundrová, Mrs. E. Schauerová and Mrs. H. Talacková for re-drawing the figures.

Prague, Czech Republic Jaroslava Turková

Chapter 1

Introduction

The rapid growth of industrial output and the population explosion lie beneath many social problems throughout the world. The management of biomolecules could be a key strategic factor in the amelioration of such problems. Fig. 1.1 is taken from the article of Sjödahl (1989) concerning his investigation of biotechnology in Japan. The Japanese government, academia and industry have perceived the potential of state-of-the-art biomolecule management, and are gearing up for the future business of applying the new biology. They appear to be pursuing the goal of a logical management of life, as is shown in this figure. As human knowledge of biological function advances, so does the range of potential products. Advances in biotechnology, biochemistry and pharmacology are increasingly dependent on bioaffinity chromatography, which is a very important and indispensable separation technique for the isolation and characterization of specific biomacromolecules (Anspach et al., 1989).

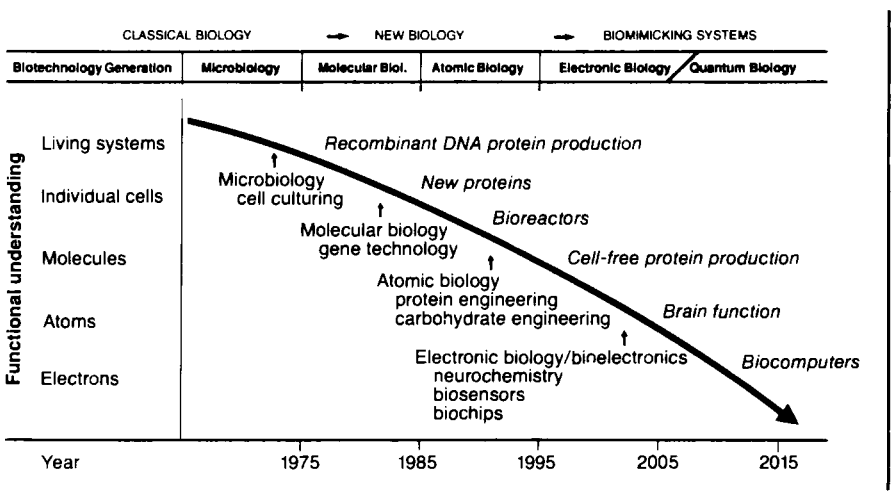


Fig. 1.1 The relationship between knowledge of biological systems and the development of new product areas. Reproduced with permission from J. Sjödahl, Trends Biotechnol., 7(1989) 144 - 147.

Macromolecules such as proteins, polysaccharides and nucleic acids differ only negligibly in their physico-chemical properties within the individual groups, and their isolation on the basis of these differences, for example by ion-exchange chromatography, gel filtration or electrophoresis, is therefore difficult and time consuming. Consequently, their activity decreases considerably during the isolation procedure owing to denaturation, cleavage, enzymatic hydrolysis, etc. One of the most characteristic properties of these biological macromolecules is their ability to bind other molecules reversibly. For example, active and regulatory sites of enzymes form complexes with substrates, inhibitors, cofactors or effectors; antibodies bind antigens against which they were prepared; and nucleic acids such as messenger RNA hybridize with complementary DNA, etc. The formation of biospecific, dissociable complexes of biological macromolecules can serve as a basis for their purification, determination, removal of impurities, or for the study of biospecific complex formation as this depends on the microenvironment in both solid and liquid phases.

The fundamental principle of bioaffinity chromatography consists in the utilization of the exceptional property of biologically active substances to form stable, specific and reversible complexes. If one of the components of the complex is immobilized, a specific sorbent is formed for the second component of the complex; under the assumption, of course, that all of the conditions necessary for the formation of this complex are maintained. The binding sites of the immobilized substances must retain good steric accessibility even after their binding to the solid carrier, and they must not be deformed. The first examples of specific sorbents prepared by covalent bonding to a solid support were immobilized antigens (Campbell et al., 1951). The methods developed for the attachment of antigens and antibodies to solid supports were used immediately for the preparation of immobilized enzymes; at the same time, the earlier method of binding enzymes to cellulose by means of an azide bond (Michael and Ewers, 1949) began to be used for the preparation of immunosorbents. The parallel development of the two branches based on the use of the bonds of biologically active substances to solid carriers is best revealed from the first review papers: "Reactive Polymers and Their Use for the Preparation of Antibody and Antibodies" by Manecke (1962), "Water-insoluble Deri-

vatives of Enzymes, Antigens and Antibodies" by Silman and Katchalski (1966) and "The Chemistry and Use of Cellulose Derivatives for the Study of Biological Systems" by Weliky and Weetall (1965). This simultaneous development of the two branches took place after the discovery of better carriers and the elaboration of methods of bonding that permitted the preservation of those properties which the immobilized substances possessed in solutions.

Their common development contributed substantially to the introduction of enzyme engineering. According to Wingard (1972), this specialization consists in the production, isolation, purification, immobilization and utilization of enzymes in various types of reactors. However, in nature enzymes are produced by organisms for their own use: in regulated metabolic processes their low stability, narrow specificity and strictly defined requirements concerning the reaction medium are inherently connected with their function. Most native enzymes are not suitable for application in the chemical or pharmaceutical industry and their modification is therefore desirable. One frequently used modification consists in their stabilization and suitable immobilization. The use of immobilized enzymes for the study of the influence of various methods of immobilization for many protein affinity ligands is advantageous, because the stability of immobilized proteins can be observed by the simple determination of enzymatic activity. Discussions on the stabilization of trypsin by use of multipoint attachment are given in Section 4.3.2, and on its stabilization by hydrophilization of the nonpolar surface in Section 4.3.3. Coupling of diazotized 4-amino-N'-(D-galactopyranosyl)-benzenesulphonohydrazide to tyrosine residues or coupling of D-galactose to lysin residues on the surface of trypsin allows the stabilization and immobilization of galactosylated proteins through their carbohydrate moieties (Turková et al., 1992). The advantage of galactose lays in the possibility of its enzymatic activation by galactose oxidase. The resulting aldehyde groups are suitable for the attachment of glycoproteins to hydrazide derivatives of supports (Petkov et al., 1990).

Biospecific interactions *in vivo* not only play an important role in the activity of biologically active compounds, they also provide accessibility of active sites and confer a desirable stability. Oriented immobilization of affinity ligands *in vitro* through anti-

bodies or carbohydrates as a tool for stabilization and good steric accessibility (e.g. Turková et al. 1988, 1990; Turková, 1990) is in detail discussed in Sections 4.3.4, 6.2.2 and 10.3.

Ohlson et al. (1989) have described the great improvement of bioaffinity chromatography resulting from a change-over from soft gel supports to small and hard particles used in high-performance bioaffinity chromatography (HPLBAC). A comparison of HPLBAC with soft gel bioaffinity chromatography (BAC) is shown in Fig. 1.2. Mechanically stable, rigid particles, with small and uniform sizes, provide high flow rates with good mass transfer characteristics, giving an overall high operational adsorption capacity.

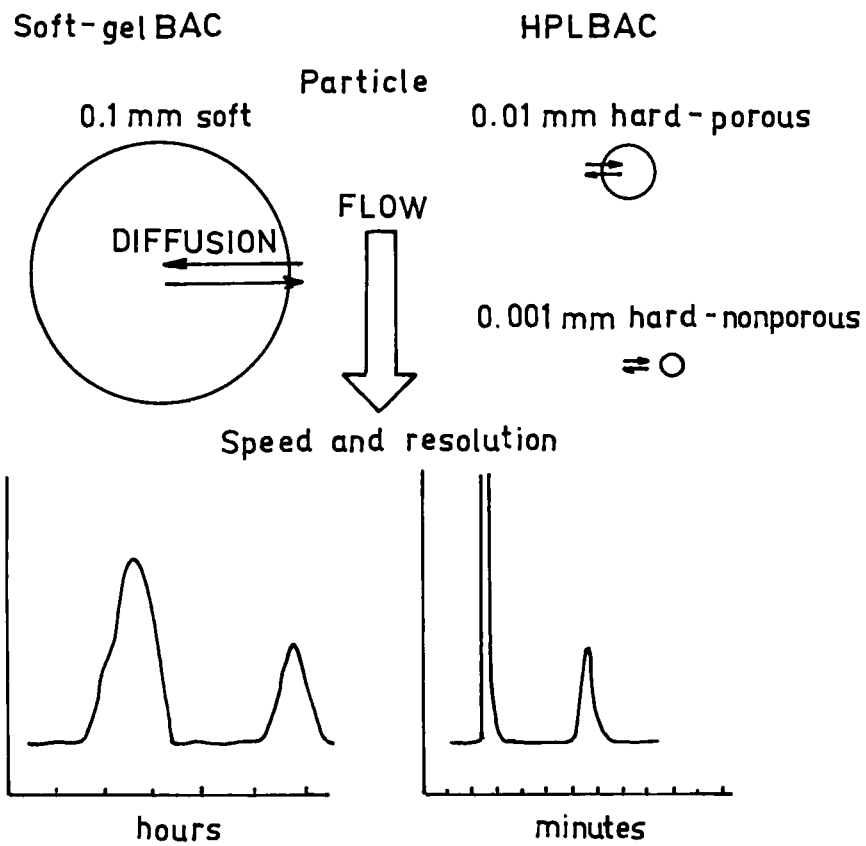


Fig. 1.2. A comparison of bioaffinity chromatographies using soft gel or porous and nonporous small hard particles.

Anspach et al. (1989) have demonstrated much more favourable mass transport and adsorption/desorption kinetic behaviour with nonporous supports. Monodisperse, narrow-sized particles (0.7-, 1.5- and 2.1-μm) have relatively low surface areas compared to commonly used porous silicas and permit shorter contact times between the sample and the stationary phase surface during the chromatographic run. Nonporous matrices exhibit improved mass and biological recoveries and higher accessibility of immobilized ligand than equivalent porous bioaffinity supports. The porous, highly porous and nonporous solid supports are described in detail in Section 4.1.1.

In contrast to BAC with large particles of soft gel, the use of HPLBAC with a high back-pressure necessitates special, costly equipment. However, the integration with high-performance equipment does afford sensitive detection and ease of operation. In preparative applications, the speed can also mean an improvement in the quality of the biomolecules being isolated, especially for fragile compounds that tend to denature during chromatography. Furthermore, peak height can often be used to give quantitative estimates of sample components. HPLBAC can be useful in assessing important molecular characteristics such as affinity, kinetics and molecular targets of interaction. The screening of possible effectors in receptor studies using HPLBAC can be of importance in drug design. In analytical biotechnology HPLBAC offers new ways of monitoring biomolecules in complex mixtures, potentially allowing the on-line analysis of bioreactors and downstream processing. In the large-scale preparation of biomolecules one cost-effective and useful example that can be given is the use of highly automated systems containing batch operation in adsorption and chromatography in elution (Ohlson et al., 1989).

Theoretical analyses of bioaffinity chromatography have important implications for the design of preparative-scale separations. The application of a theoretical description of nonlinear chromatography to the performance of necessary physicochemical measurements of thermodynamic and kinetic constants has been described by Wada et al. (1987).

In the study of the effect of the microenvironment on the specific interactions the method of affinity chromatography has begun to play an important role. As is described

in detail in Chapter 14, it is possible, for example, to use a column of a support with a bonded inhibitor and to displace the specifically sorbed enzyme with solutions of its inhibitors at various concentrations. The elution volumes can be used to determine the dissociation constants of the enzyme with both the bonded and the dissolved inhibitor. When the same inhibitor is used for the binding on a solid support and for the elution of the specifically sorbed enzyme, information can be obtained on the effect of the environment on complex formation from the differences, if any, in the dissociation constants determined. As specific interactions play a very important role in most of the processes that take place in nature, the development of a simple method for the determination of dissociation constants of complexes is undoubtedly of great importance.

Despite the simple concept and the many successful applications of bioaffinity chromatography, problems are often connected with rigidity, physical shape, reusability etc. of the biospecific matrix. The recent introduction of prepacked, commercially available HPLBAC columns by Beckman and Pierce has probably established the direction of future developments in supports for HPLBAC. These columns contain activated support material to which proteins or ligands can be attached by simply pumping the desired material through the column in a cyclic mode. The column is ready for use following a brief wash to remove unreacted material. The user is thus spared all the problems inherent in HPLBAC column packing and activation of the support. This approach permits a considerable saving in time at the expense of losing control over the degree of activation of the phase and the type of phase used (Groman and Wilchek, 1987). Another solution is the use of an activated membrane. The extremely simple use of an activated nylon membrane as an affinity adsorbent described by Huse et al. (1990), is discussed in detail in Section 5.6.

The aim of this book is to complement my first edition of "Affinity Chromatography" published by Elsevier in 1978. A bibliographic review presents the use of bioaffinity chromatography in the processing of more than five thousand compounds, extended by characteristic data such as the supports used, the affinity ligands and the spacers. However, at the present time the determined number of book's pages does not permit

to set out all examples. I have also tried to summarize briefly the principles that must be taken into consideration for the successful use of bioaffinity chromatography. Particular attention will be focused on the solid supports used in bioaffinity chromatography, the principles of affinity ligand immobilization, the use of bioaffinity chromatography in the evaluation of biospecific complex formation, and the effects of the carrier.

REFERENCES

Anspach, F.B., Wirth, H.J., Unger, K.K., Stanton, P., Davies, J.R. and Hearn, T.W., Anal. Biochem., 179 (1989) 171-181.

Campbell, D.H., Luescher, E.L. and Lerman, L.S., Proc. Nat. Acad. Sci. U.S.A., 37 (1951) 575-578.

Groman, E.V. and Wilchek, M., Trends Biotechnol., 5 (1987) 220-224.

Huse, K., Himmel, M., Gärtner, G., Kopperschläger, G. and Hofmann, E., J. Chromatogr., 502 (1990) 171-177.

Manecke, G., Pure Appl. Chem., 4 (1962) 507-520.

Micheel, F. and Ewers, J., Makromol. Chem., 3 (1949) 200-209.

Ohlson, S., Hansson, L., Glad, M., Mosbach, K. and Larsson, P.O., Trends Biotechnol., 7 (1989) 179-186.

Petkov, L., Sajdok, J., Rae, K., Šůchová, M., Káš, J. and Turková, J., Biotechnol. Techniques, 4 (1990) 25-30.

Silman, I.H. and Katchalski, E., Annu. Rev. Biochem., 35 (1966) 873-908.

Sjödahl, J., Trends Biotechnol., 7 (1989) 144-147.

Turková, J., Technology Today, 2 (1990) 82-85.

Turková, J., Beneš, M.J., Vohnik, S., Helusová, S. and Tichá, M., J. Chromatogr., 597 (1992) 19-27.

Turková, J., Fusek., M., Šťovíčková, J. and Králová, Z., Makromol. Chem., Macromol. Symp., 17 (1988) 241-256.

Turková, J., Petkov, L., Sajdok, J., Káš, J. and Beneš, M.J., J. Chromatogr., 500 (1990) 585-593.

Wade, J.L., Bergold, A.F. and Carr, P.W., Anal. Chem., 59 (1987) 1286-1295.

Weliky, N. and Weetall, H.A., Immunochemistry, 2 (1965) 293-322.

Wingard, L.B., Biotechnol. Bioeng., S3 (1972) 3-13.

This Page Intentionally Left Blank

Chapter 2

The principle, history and use of bioaffinity chromatography

2.1. THE PRINCIPLE OF BIOAFFINITY CHROMATOGRAPHY AND NONCOVALENT INTERACTIONS IN COMPLEMENTARY BINDING SITES OF BIOSPECIFIC COMPLEXES

Bioaffinity chromatography is a type of adsorption chromatography which is based on the exceptional ability of biologically active substances to bind specifically and reversibly to complementary substances. These are generally called ligands or affinity ligands, or simply affinants for the purpose of bioaffinity chromatography. The complexes of active or regulatory sites of enzymes with their inhibitors, substrates, cofactors or effectors, antibodies with antigens or haptens, lectins with carbohydrates, complexes of nucleic acids and nucleotides, hormones and toxins with receptors, transport proteins with vitamins or sugars, etc., may be mentioned as examples.

Assuming that a single enzyme of the crude proteins has an affinity for the specific sorbent, the equilibrium between the attached affinity ligand L and the isolated enzyme E is given by the equation

$$E + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EL$$

Affinity constant (= equilibrium or association constant):

$$K_A = \frac{[EL]}{[E]\,[L]} \qquad \text{(e.g. } 10^8\ \text{M}^{-1}\text{)}$$

Dissociation constant:

$$K_A = K_L = \frac{[E]\,[L]}{[EL]} \qquad \text{(e.g. } 10^{-8}\ \text{M)}$$

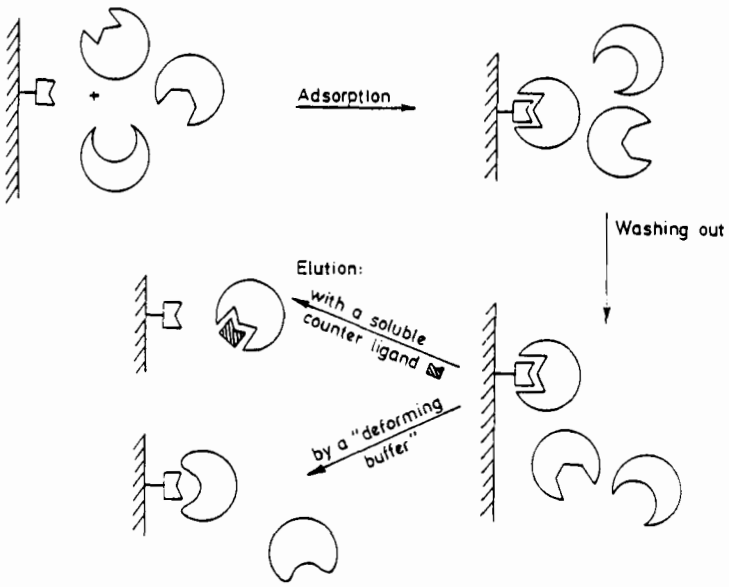
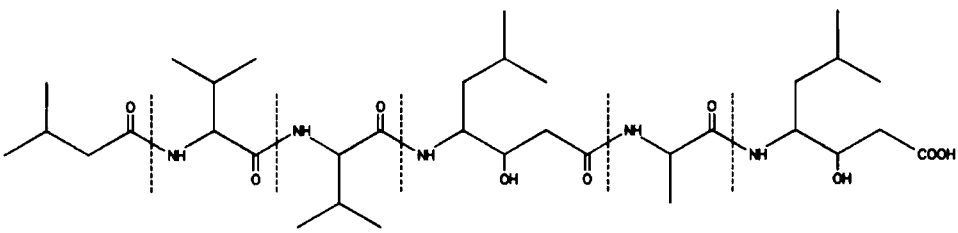


Fig. 2.1. Diagrammatic representation of the process of bioaffinity chromatography.

A solid support with a covalently bound affinity ligand is used as a stationary phase in a chromatographic column. A diagrammatic representation of the process of bioaffinity chromatography is given in Fig. 2.1. When a crude mixture containing the biologically active products to be isolated is passed through the column, then all the compounds which, under given experimental conditions, have no complementary binding site for the immobilized affinity ligand will pass through unretarded. By contrast, those products which do show affinity are adsorbed on the column. They can be released e.g. by a solution of a soluble affinity ligand or by a change of the solvent composition by so-called deforming buffers (e.g. by a change in pH, ionic strength or temperature; or alternatively by such dissociation agents as urea, guanidine, etc.). The formation of the biologically functioning complexes involves the participation of common molecular forces and interactions systematized under the terms of ionic bonds, hydrophobic bonds, London's dispersion forces, dipole-dipole interactions, charge-transfer interaction and so on. The simultaneous and concerted action of

several of these forces in the complementary binding site constitutes the basis of the high specificity and efficiency of the biospecific bond.

The number of noncovalent interactions in complementary binding sites of biospecific complex can be shown best by X-ray diffraction analysis. The affinity ligand most commonly used in the bioaffinity chromatography of aspartate proteinases is pepstatin, the naturally occurring peptide inhibitor:



Iva Val Val Sta Ala Sta

The mode of binding of the first part of the pepstatin molecule (the fragment isovaleryl-valyl-valyl-statine) to the binding site of penicillopepsin was elucidated by James and coworkers (1983).

Fig. 2.2 shows a three-dimensional representation of the binding of isovaleryl-valyl-valyl-statine ethyl ester to the active site of penicillopepsin an aspartate proteinase, as determined by X-ray analysis at 1.8 Å resolution. The hydrogen bonding interactions of the peptide with the enzyme are listed in Table 2.1. Table 2.2 lists the noncovalent interactions between the pepstatin fragment and penicillopepsin which occur at a distance of 4 Å or smaller. The total number of 87 includes a variety of interactions.

The high number of noncovalent interactions between the binding sites of microbial proteinases and pepstatin is the reason why the *Mucor miehei* proteinase cannot be eluted after adsorption to an isovaleryl-pepstatin-Sepharose column. After Kobayashi and coworkers (1982), however, had replaced immobilized *N*-isovalerylpepstatin by *N*-isobutyryl-, *N*-propionyl- or *N*-acetylpepstatin they were able to increase the yield of the enzyme eluted from 0 to 12, 46 and 90%, respectively. The affinity constants of these complexes were not reported.

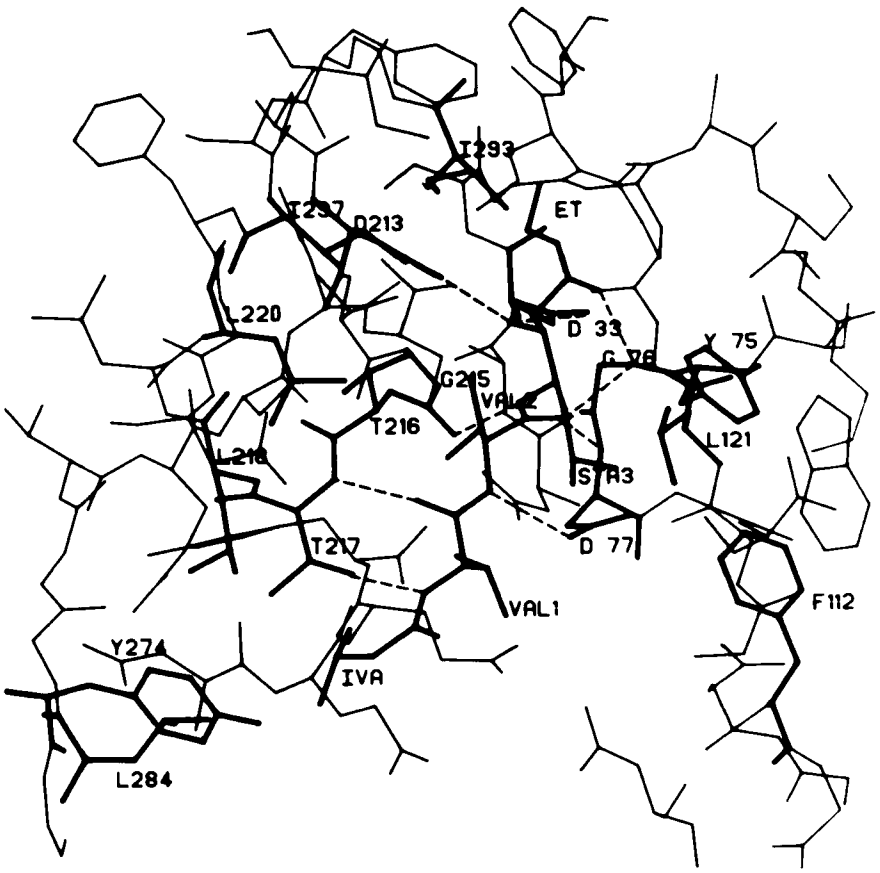


Fig. 2.2. Crystallographic analysis of binding interactions of Iva-Val-Val-StaOEt to the active site of penicillopepsin at 1.8 Å resolution. Hydrogen bonds between enzyme and inhibitor are shown as dashed lines; some of those residues of penicillopepsin implicated in binding are shown by thicker full lines. Reproduced with permission from M.N.G. James, A.R. Sielecki and J. Moult, Proceedings of the 8th American Peptide Symposium, V.J. Hruby and D.H. Rich, eds., Pierce Chem. Co., Rockford, Illinois, 1983, pp. 521-530.

The affinity constants of the biospecific complexes of modified pepstatin were determined by Kay and coworkers (1982). The value of the affinity constant K_i of the complex of cathepsin D with isovalerylpepstatin was equal to 10^{10} M^{-1} . After the isovaleryl group had been replaced by the lactoyl group the K_i of the complex of cathepsin D with lactoyl pepstatin dropped to 10^7 M^{-1} , i.e. a drop of three orders of magnitude.

Table 2.1.

Hydrogen-bonding involving $P_3P_2P_1$ and P'_1 residues

Pepstatin Fragment		Penicillopepsin	Dist(Å)
P_3	Val NH	Thr217 $O^{\gamma 1}$	2.83
	Val C=O	Thr217 NH	3.03
		Wat548 O	2.88
P_2	Val NH	Asp77 $O^{\delta 1}$	2.71
	Val C=O	Gly76 NH	3.21
		Asp77 NH	3.24

Pepstatin Fragment		Penicillopepsin	Dist(Å)
P_1	Sta NH	Gly215 C=O	3.07
	Sta-OH	Asp33 $O^{\delta 2}$	2.58
		Asp33 $O^{\delta 1}$	3.31
		Asp213 $O^{\delta 1}$	2.89
		Asp213 $O^{\delta 2}$	2.68
P_1'	Ester C=O	Gly76 NH	3.25
	Ester C=O	Wat606 O	2.86

Table 2.2.

Summary of non-bonding contacts (≤ 4.0 Å) between IvaValValStaOEt and penicillopepsin

PENICILLOPEPSIN / PEPSTATIN ANALOGUE	Glu 15	Asn 31	Asp 33	Gly 35	Tyr 75	Gly 76	Asp 77	Ser 79	Leu 121	Phe 190	Ile 211	Asp 213	Gly 215	Thr 216	Thr 217	Leu 218	Leu 220	Leu 284	Ile 297	Total
P_4Iva	1														3	1		1		6
P_3Val	1						3*						2	3	7(2)					16
P_2Val					1	4(1)	11(2)							3			1		1	21
P_1Sta		1	8(2)		5		1	1	2			4(2)	9(1)	2						33
P_1'ester				1	2	1(1)				2	2	2		1						11
Total	2	1	8	1	8	5	15	1	2	2	2	6	11	9	10	1	1	1	1	97

* Contacts within this boxed region are to atoms of residues that comprise the flap of penicillopepsin

This example shows that formation of complexes of lower affinity should by used for biospecific affinity chromatography. However, in connection with the use of biospecific complex formation in bioaffinity chromatography we must always take into consideration the fact that the affinity constant of the biospecific complex is not only influenced by the microenvironments of both solid and liquid phases, but also by steric

accessibility, the conformation and concentration of the affinity ligand, etc. Hence, the affinity constant of the complex formed between the compound to be isolated and the immobilized affinity ligand should lie in the range 10^4 - 10^8 M^{-1}. Complexes of higher affinity, characterized by constants higher than or equal to 10^9 M^{-1}, can be used to advantage for oriented immobilization by simple adsorption.

2.2 HISTORY AND USE OF BIOAFFINITY CHROMATOGRAPHY

In the history of bioaffinity chromatography the isolation of α-amylase by means of an insoluble substrate (starch) should be mentioned first; this was described in 1910 by Starkenstein, a professor at Prague University. The principle of bioaffinity chromatography, using affinity ligands covalently bound to a solid matrix, has been known for as long as 40 years. Campbell et al. were the first to use this principle in 1951, for the isolation of antibodies on a column of cellulose with covalently attached antigen. Bioaffinity chromatography was first used in the isolation of enzymes in 1953 by Lerman, who isolated tyrosinase on a column of cellulose with etherically bound resorcinol residues. In subsequent years bioaffinity chromatography was employed only rarely, the reason obviously being the character of the insoluble supports which did not offer sufficient possibilities for the formation of a complex between the product to be isolated and the attached affinant. Non-specific adsorption was often observed when supports having hydrophobic or ionogenic groups were used. A milestone in the development of the use of bioaffinity chromatography was the method of affinant attachment to agarose activated with cyanogen bromide, developed by Porath and coworkers (Porath et at., 1967; Axén et al., 1967; Axén and Ernback, 1971). Cuatrecasas and Anfinsen (1971) have shown that agarose (most often the commercial product Sepharose) possessed almost all the characteristics of an ideal support. In 1968, Cuatrecasas et al. successfully employed bioaffinity chromatography for the isolation of nuclease, chymotrypsin and carboxypeptidase A. They used the term affinity chromatography for the first time for this type of isolation.

This name, however, raised and still gives rise to many objections. Attempts have been made to replace it, for example, by the more accurate term "biospecific

adsorption" or "bioaffinity chromatography" (Porath, 1973, O'Carra et al., 1974), especially when it was found that a series of adsorbents, mainly those having a synthetic inhibitor bound by hydrophobic hydrocarbon chains, can sorb macromolecules on the basis of hydrophobic interactions (O'Carra, 1974). The exploitation of the formation of complexes of biological macromolecules on the basis of hydrophobic bonds gave rise to so-called hydrophobic chromatography (Shaltiel, 1974, 1984). However, distinguishing between a biospecific complex and a complex formed on the basis of nonspecific hydrophobic forces is no simple task, as has been observed in many instances. Often one substance bound to a carrier may form biospecific complexes with one group of macromolecules, while with another it may undergo complex formation exclusively on the basis of nonspecific hydrophobic interaction, and both types of bonding may take part in the formation of a bond with a third group of macromolecules. Hexamethylenediamine may be mentioned as an example. This compound, bound to Sepharose, was used by Jakubowski and Pawelkiewicz (1973) and Henderson et al. (1974) as a sorbent in the hydrophobic chromatography of amino acyl-transfer RNA synthetases or L-histidinol-phosphate aminotransferase; Toraya et al. (1976) indicate that hexamethylenediamine is a biospecific sorbent for amino oxidase from *Aspergillus niger*, while Vosbeck et al. (1973), when using the same sorbent for the isolation of aminopeptidases from *Streptomyces griseus*, reached no definite conclusion on which type of bond is operative during specific sorption. In consequence, bioaffinity chromatography was included among the affinity methods, along with affinity elution, covalent chromatography, hydrophobic chromatography, metal chelate and charge transfer chromatography, affinity density perturbation, affinity partitioning, affinity labelling, solid-phase radioimmunoassay and enzyme-linked immunoassay, affinity histochemistry, ultrafiltration affinity purification and affinity electrophoresis.

Progress in the development and use of affinity methods may be charted by consultation of: Proceedings of Symposium on Affinity Chromatography and Immobilized Biochemicals, Charleston, U.S.A. 1973 (Dunlop, 1974); International Symposium on Chromatographic Fractionation of Biopolymers, Birmingham, U.K. 1976 (Epton, 1978); 1st International Symposium on Affinity Chromatography, Vienna, Austria 1977

(Hoffmann-Ostenhof et al., 1978); International Symposium on Theory and Practice in Affinity Techniques, Gottingen, F.R.G. 1978 (Sundaram and Eckstein, 1978); 3rd International Symposium on Affinity Chromatography and Molecular Interaction, Strasbourg, France 1979 (Egly, 1979); 4th International Symposium on Affinity Chromatography and Related Techniques, Veldhoven, The Netherlands 1981 (Gribnau et al., 1982); 5th International Symposium on Affinity Chromatography and Biological Recognition, Annapolis, U.S.A. 1983 (Chaiken et al., 1983); 6th International Symposium on Bioaffinity Chromatography and Related Techniques, Prague, Czechoslovakia 1985 (Turková et al., 1986); 7th International Symposium on Affinity Chromatography and International Macromolecular Interactions, Oberammergau, F.R.G. 1987 (Jennissen and Müller, 1988); 1st International Conference on Dye-Protein Interaction, Compiegne, France 1988 (Vijayalakshmi and Bertrand, 1989); and the 8th International Symposium on Affinity Chromatography and Biological Recognition, Jerusalem, Israel 1989 (Wilchek 1990) and the 9th International Symposium on Affinity Chromatography and Biological Recognition, Yokohama, Japan 1991 (Ishii, 1992).

Such works regularly illustrate the development of novel bioaffinity supports and techniques, the conditions of bioaffinity chromatographies under low or high pressure and their analytical, clinical, industrial or biotechnological applications, the use of bioaffinity chromatography for the characterization of biomolecular interactions, etc. From the titles of several symposia it is evident that great interest is evoked by the theory of biological recognition.

The proceedings of the 6th International Symposium on Bioaffinity Chromatography and Related Techniques in Prague (Turková et al., 1986) contains the information about the International Interest Group in Biorecognition Technology which was affiliated to the International Union of Biochemistry. Recently the name of this organization has been changed to "Society for Molecular Recognition". The Society assists in the scheduling, support and promotion of scientific meetings including Symposia on affinity chromatography. Also, the Pierce Award in Affinity Chromatography was presented for the first time at the Symposium in Prague to Professor Klaus H. Mosbach from the University of Lund, Sweden. The recipient of the 1987 Pierce Prize was Professor Meir

Wilchek from the Weizmann Institute in Rehovot, Israel (Jennissen and Müller, 1988), in 1989 the Pierce Prize was awarded to Professor Christopher R. Lowe from the University of Cambridge, U.K. (Wilchek, 1990) and in 1991 to Dr. Irwin Chaiken from Biopharmaceuticals R&D, King of Prussia, USA (Ishii, 1992).

The books on affinity chromatography written by Lowe and Dean (1974) and edited by Jakoby and Wilchek (1974) which were mentioned in the first edition of the present work (Turková, 1978) have been followed by further books, such as "An Introduction to Affinity Chromatography" written by Lowe (1979) and "Affinity Chromatography: Bioselective Adsorption on Inert Matrices" written by Scouten (1981). The chapters about the theory, characteristics, principles and use of dyes in affinity chromatography were edited by Fichter (1982) and the book "Affinity Chromatography: Practical and Theoretical Aspects" was written by Mohr and Pommering (1985). In the book "Affinity Chromatography, a Practical Approach", edited by Dean et al. (1985), extensive chapters are presented on the use of affinity chromatography for the quantitative characterisation of molecular interactions and for cell separation. From many review papers I mention only those written by Wilchek et al. (1984), Turková (1984 a, b), Scopes (1987), Ostrove (1990) and Ostrove and Weiss (1990).

The ever-increasing use of genetic engineering has been reflected in the rapidly increasing number of publications devoted to the isolation of nucleic acids, genes, oligonucleotides and nucleic acid fragments, to the purification of special proteins and enzymes, as well as to investigations of protein-nucleic acid interactions. Since the template functions of nucleic acids, specifically the base-pairing mechanism, were used for chromatography in many papers, Schott reviewed them in his book "Affinity Chromatography-Template Chromatography of Nucleic Acids and Proteins".

The second most commonly used protein-affinity ligand interaction in bioaffinity chromatography is the dye-protein interaction, going under the term "dye-ligand chromatography" (Amicon Corporation, 1980). Synthetic textile dyes (Cibacron Blue F3GA, Procion Red HE3B, Procion Green H-E4BD, Yellow H-A etc.) have many advantages, particularly for large-scale applications. Compared to biological ligands, dye-ligands are inexpensive materials which are available in tonnage quantities

throughout the world. There is a wide range of chromophors available which are dye-based biologically, chemically, and photochemically stable adsorbents, potentially sterilisable *in situ* with no degradation of the ligand itself. Because they are reactive materials, they are very easily immobilized to hydroxyl-polymers, generally by a single-step process. Such adsorbents have a high capacity, a very broad binding capability in terms of the complementary proteins, and they are easily re-usable. Several thousand different types of proteins can interact with an immobilized textile dye. Examples are oxidoreductases, phosphokinases and nearly all coenzyme dependent enzymes, hydrolases, various transferases, a number of proteins which interact with mono- and polynucleotides, synthetases, hydroxylases, nearly all of the glycolytic enzymes, phosphatases, a whole variety of blood proteins and other non-enzyme proteins (Kopperschlager et al., 1982; Lowe and Pearson, 1984; Lowe et al., 1989; Stellwagen, 1990).

The interactions between the dyes and proteins are complex and not well understood. Scopes (1987) classified them for affinity purposes as "specific" and "nonspecific" according to whether the interaction is at the active site or not. All interactions are likely to involve practically all known types of forces between molecules. The dyes generally contain aromatic rings, sometimes heterocyclic, often fused, and by definition a long series of conjugated double bonds must be present to give a strong adsorption of light in the visible spectrum. Additionally, sulphonic acid groups are usually included to confer aqueous solubility; these groups are negatively charged at all pH values above zero. Some dyes contain carboxylates, amino, chloride, or metal complexing groups; most contain nitrogen both in and out of aromatic rings. Thus interactions with proteins are the result of hydrophobic, electrostatic and hydrogen bonding. Biellmann et al. (1979) used X-ray crystallography to study the binding of Cibacron Blue F3GA to liver alcohol dehydrogenase. They demonstrated similarities in the binding of this dye with the binding of the coenzyme NAD^+.

Clonis et al. (1986) described process scale high-performance liquid affinity chromatography (HPLAC) of lactate dehydrogenase using a 3.3 l column of glycol silica containing 2 mg of immobilized Procion blue MX-R/g silica. They isolated 100 mg of

the purified enzyme in a single step from a crude rabbit muscle preparation containing 1.8 g of protein. Vijayalakshmi, summarizing the First International Conference on Dye-Protein Interaction (Vijayalakshmi and Bertrand, 1989), stated that it is extremely stimulating and promising to see that the dye-ligand affinity systems have developed in different spheres and this domain is becoming one of the milestones in the progress of downstream processing in biotechnology. But she also said that only very little information is available on the toxicological aspects of the possible traces of dye molecules or their fragments which may be present in the final product. This situation is undoubtedly an obstacle to a genuine industrial exploitation of this rapidly developing field.

High-performance liquid bioaffinity chromatography (HPLBAC) permits the rapid isolation of the desired material and offers a rapid, microscale method for the thermodynamic characterization of molecular recognition (Fitzgerald and Swaisgood, 1989). The concept of HPLBAC was first introduced by Kato et al. (1977), who separated nucleic acid bases and nucleosides by the use of thymine attached to a methacrylate solid support, and by Ohlson et al. (1978), who demonstrated the ability of appropriately labelled bioaffinity matrices of microparticulate silica to serve as a means for the rapid purification of enzymes and antigens. The advantage of both supports is their high dimensional stability under high pressure conditions. Fig. 2.3 demonstrates the high performance liquid bioaffinity chromatography of crude porcine pepsin on hydroxyalkyl methacrylate support Separon H 1000 modified with 0.5 μmol of ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (B) in comparison with analogous chromatography on unmodified Separon (A) (Turková et al., 1981). The chromatography of pepsin on unmodified Separon (A) was carried out in order to check whether, under the given experimental conditions, elution of the material non-specifically sorbed to a solid support does not take place. In this instance all of the protein material with the whole proteolytic activity was washed in the front of the fraction obtained by elution with the equilibration buffer. By contrast, desorption at a high ionic strength carried out on a column of Separon modified with ε-amino-caproyl-L-Phe-D-Phe-OCH_3 yields a sharp peak of whole pepsin with a high activity. The baseline characteristics of the bioaffinity chromatographic system (C) were determined with the injection of sorption

buffer in the absence of protein. Reviews of high-performance liquid affinity chromatography have been published by Larsson (1984), Ernst-Cabrera and Wilchek (1988b) and Ohlson et al. (1989).

The success of bioaffinity chromatography has contributed to many of the advances in the biotechnological revolution (Scouten, 1983). The bioaffinity chromatographic steps in the production of urokinase *via* genetic engineering may be cited as an example. Ratzkin et al. (1981) isolated total mRNA from human fetal cells by the use of bioaffinity chromatography on poly(U) - Sephadex. The urokinase-specific messenger RNA was

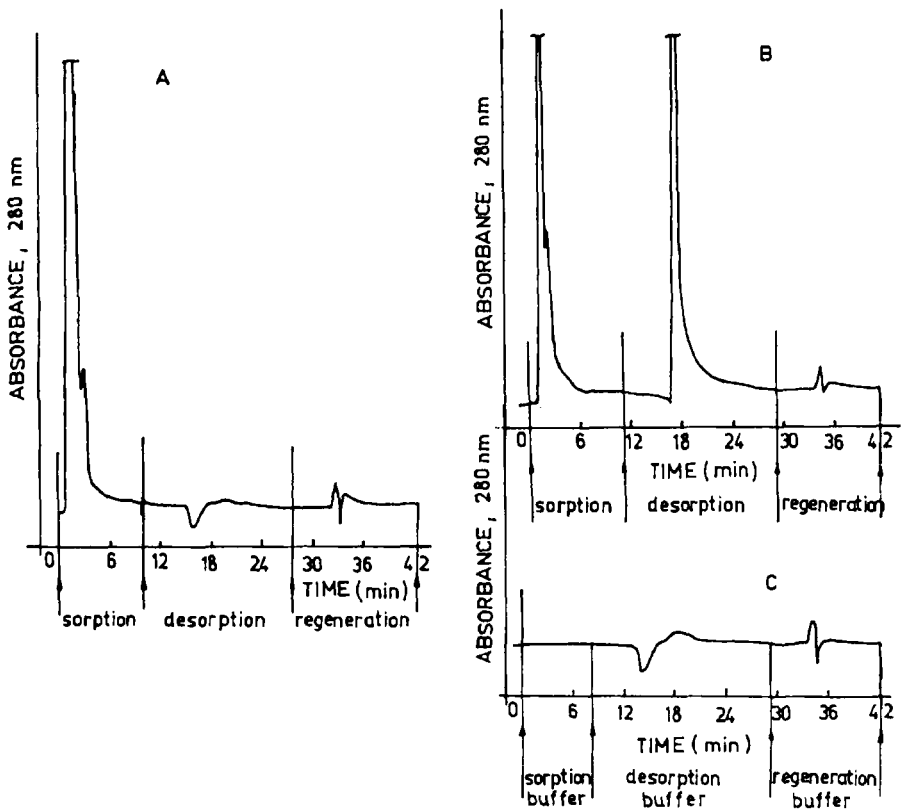


Fig. 2.3 High-performance bioaffinity chromatography of crude porcine pepsin on (A) Separon H 1000 (mean particle diameter 10 μm) and (B) Separon H 1000 modified with 0.5 μmol of ε-aminocaproyl-L-Phe-D-Phe-OCH_3 per gram of carrier. Column 100 x 4 mm I.D. Detector: UV (280 nm). Mobile phase: sorption buffer, 0.1 M sodium acetate (pH 4.5); desorption buffer, 0.1 M sodium acetate (pH 4.5) containing 1 M sodium chloride; regeneration buffer, 0.1 M sodium acetate (pH 4.5) containing 10 % 2-propanol. Injection: 10 μl of pepsin solution in sorption buffer (0.5 mg/μl). (C) baseline characteristics of bioaffinity chromatographic system (B). Injection: 10 μl of sorption buffer without protein. Data from J.Turková et.al., J.Chromatogr., 215 (1981) 165-179.

isolated from the total mRNA *via* classical ultracentrifugation methods and inserted into *Escherichia coli* plasmid pBR322. Reverse transcriptase and DNA polymerase (probably purified by bioaffinity chromatography) were used for the transformation of mRNA into cDNA and the copy of the cDNA. After the insertion into pBR322 and subsequent cloning colonies containing pBR322 - urokinase cDNA conjugate were prepared. Urokinase was isolated from the transformed *E. coli via* bioaffinity chromatography on a benzamidine - Sepharose column to obtain a urokinase enzyme molecule indistinguishable from the urokinase isolated from human tissue-culture preparations.

The branch of bioaffinity chromatography which employs immobilized antigens or antibodies for the purification of biologically active material is known by the term immunoaffinity chromatography. One of the reasons for the rapid growth of immunoaffinity chromatography has been necessity. The introduction of sensitive and specific methods in clinical chemistry increased the demand for a number of biologically active compounds. Thus, for instance, the use of immunoassay for the determination of steadily increasing numbers of various clinically significant compounds increased the demand for the preparation of various immunosorbents.

Fig. 2.4 (taken from Anderson et al., 1975) shows a scheme illustrating the use of the isolated pure antigen and pure antibody for the production of two compounds on a large scale. A rabbit was immunized with a small amount of antigen and the corresponding antibody was isolated from its serum. This antibody was coupled to a solid carrier and the prepared specific sorbent was then used for the purification of crude antigen in multiple chromatographic cycles. Fractions of non-adsorbed "U" and adsorbed "A" material were obtained by repeated chromatographic cycles. Combined fractions of the desorbed antigen were then used for both for the immunization of a larger number of rabbits and for the preparation of a column of the immobilized antigen. A monospecific antibody was then isolated on this column from the prepared serum. The isolated monospecific antibody in a subsequent step, was again coupled to the solid carrier, and a column was prepared for the sorption of the antigen on a large scale.

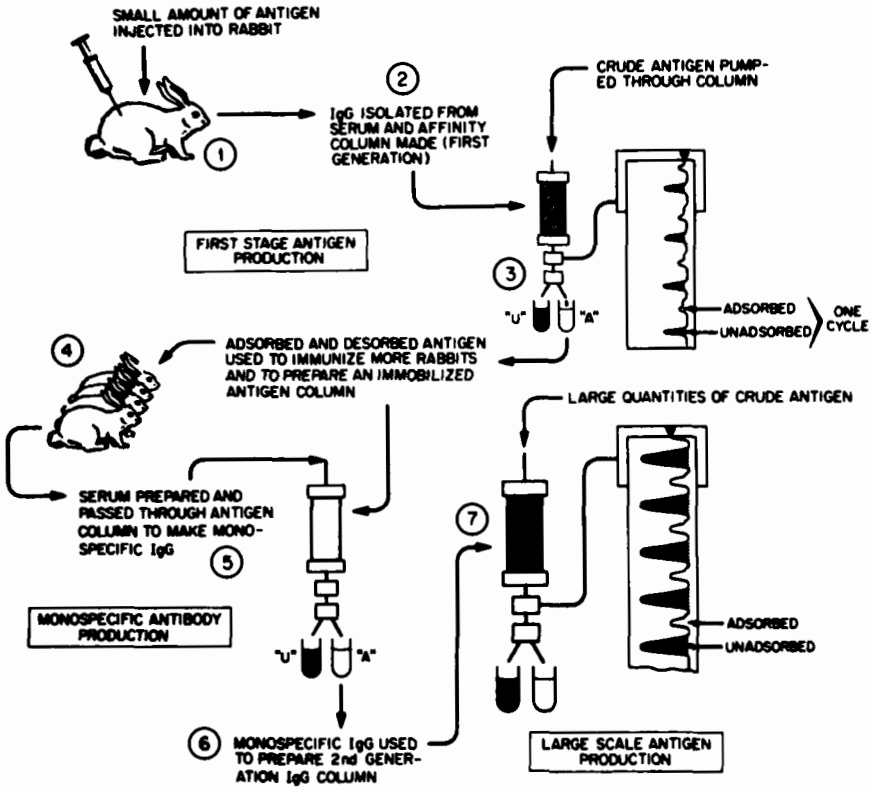


Fig. 2.4. Cyclic bioaffinity chromatography by bootstrapping technique. Reproduced with permission from N.G.Anderson et al., Anal. Biochem., 68 (1975) 371-393.

Fig. 2.5 shows a scheme for the fully automatic preparation of monospecific antibodies and antigens (Eveleigh, 1982). The method is based on a simple application of adsorption-desorption chromatography.

The sample is pumped by a peristaltic pump through a valve (VI) and flow detector (FD) to the bioaffinity column. The eluate from the column passes through a UV monitor and can be further transferred through a valve (V4) to the outlet, or through a valve (V5) collected as the non-adsorbed fraction "U" or *via* valve (V6) as the adsorbed fraction "A". The process is controlled by a microprocessor. In a fully automatic cycle the desorbed, specifically isolated, compound is pumped again through the flow detector (FD) to a Sephadex G-25 column, where it is desalted. The desalted fraction

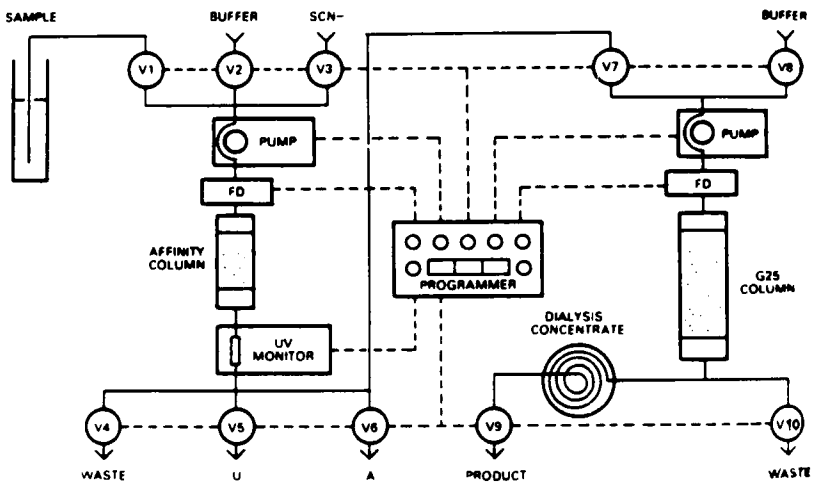


Fig. 2.5. Schematic diagram of an automatic immunoaffinity system. Reproduced with permission from J.W.Eveleigh, in T.C.J.Gribnau et al. (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam 1982, pp. 293-303.

is further concentrated and collected as a product. Flow detectors (FD) are used with the aim of preventing occasional losses of the sample or product in the case of a failure. Since a thiocyanate solution is suitable for the desorption and since this compound exhibits highly corrosive effects, all connection and valves must be made of inert plastic material and a peristaltic pump is used.

The discovery of the monoclonal antibody producing hybrid cell lines by Kohler and Milstein (1975) has resulted in the rapid growth of immunoaffinity chromatography. Multispecific responses of polyclonal antibodies to complex antigens are reduced to a series of monospecific responses by cloning. The exactly defined reactivity and the possibility of production in large quantities enable the use of immunoaffinity chromatography for attractive industrial scale applications in the isolation of the desired product in pure form from the crude fermentation broth. Proteins of potential therapeutic value e.g. interferons, interleukin 2, anti-clotting and clotting factors, insulin-like growth hormone and vaccines are already produced by bioengineering techniques and purified with immunoaffinity chromatography. This intensive commercial activity has even involved battles for patents, which can secure sales worth millions of dollars, as, for example, in the case of tissue plasminogen activator obtained by Johnston (1987) by

recombinant DNA technology (Ernst-Cabrera and Wilchek, 1988a). Chapters about immunosorbent separation have been written by Calton (1984) and Wilchek et al. (1984), a review of the use of immobilized antibodies for the purification of pharmaceutically active proteins was published by Ernst-Cabrera and Wilchek (1988a) and one on high performance immunoaffinity chromatography (HPIAC) by Phillips (1989).

The interaction between hormone, neurotransmitter or drug and the relevant receptor is selective and specific and belongs among the best known biological interactions. The receptor-bioaffinity purification process of recombinant human interleukin - 2 (rIL-2) was used by Weber and Bailon (1990) as a model system to demonstrate the utility of this approach for the purification of recombinant proteins. The receptor-affinity purified rIL-2 was shown to be biochemically and biologically more homogeneous than the immunoaffinity purified material. The efficiency of immunosorbents can be greatly increased by site oriented immobilization of immunoglobulins G through the Fc part of the molecule. The cross-linkage of a specific complex for oriented immobilization was first used in 1978 when Sepharose with covalently attached Protein A was employed for the oriented immobilization of IgG (Werner and Machleidt, 1978, Gersten and Marchalonis 1978). The bivalent binding potential of immobilized antibodies can also be achieved by immobilization *via* the carbohydrate moieties. The oligosaccharide moieties of polyclonal IgGs are primarily located on the Fc portion of the molecule in such a way that site-directed immobilization through the carbohydrate moieties should result in the antigen binding Fab regions being oriented away from the matrix, resulting in greater accessibility of antigen (O' Shannesy, 1990). Enzymatic oxidation of carbohydrate moieties of monoclonal antibodies by soluble and immobilized bifunctional enzyme complexes of neuraminidase and galactose oxidase and oriented immobilization on hydrazide or amino Eupergit C derivatives were used by Solomon et al. (1990) for the preparation of antibody matrix conjugates with high antigen-binding activities.

The exceptionally high affinity constant between avidin (or its bacterial counterpart streptavidin) and vitamin biotin has resulted in its widespread application in virtually all fields of biology and biotechnology (Wilchek and Bayer, 1990). The general idea of

the approach is that biotin, coupled to low or high molecular weight molecules, can still be recognized by avidin. A review of the application of avidin-biotin technology to affinity - based separations was published by Bayer and Wilchek (1990).

Oriented immobilizations of various affinity ligands on porous or nonporous solid supports can not only provide us with efficient biospecific adsorbents suitable for a variety of applications but also with models which may be used to advantage for studies of chemical processes in the living cell (Turková et al., 1988). The preparation of a model for the study of the formation of several biospecific complexes is illustrated in Fig. 2.6. In order to select an antibody fraction which interacts solely with the protein part of ovalbumin (OA) from the large population of immunoglobulins present in rabbit polyclonal antiserum against OA, this antiserum was applied to an immunosorbent represented by OA covalently bound by its single carbohydrate moiety after oxidation by $NaIO_4$ or by galactose oxidase to bead cellulose derivatized with adipic acid dihydrazide (Fig. 2.6A). The isolated immunoglobulin fraction was periodate oxidized and bound *via* its carbohydrate moiety to cellulose hydrazide (Fig. 2.6B). After the adsorption of OA it was determined that the molar ratio between immobilized IgG and adsorbed OA molecules was 1 : 1.7. Similar to the studies by Eveleigh and Levy (1977), the results of our experiments have shown that the immunological reactivity of an immunosorbent depends strongly on the surface density of the immobilized antibody.

The following step (Fig. 2.6B) served to verify the assumption that the bound OA was really oriented by its protein part towards the surface of the support and by its carbohydrate domain outwards, where it was freely accessible to interaction with concanavalin A (Con A), a substance which react specifically with carbohydrates. This assumption was fully confirmed. The specificity of the Con A - OA interaction was also verified. It was shown that Con A was not adsorbed on the original support with immobilized immunoglobulins only. Because the free IgG reacts with Con A in solution, this finding may be considered as evidence that IgG is immobilized *via* its Fc fragments, and that its carbohydrate part is not accessible for interaction with Con A. Con A and OA were repeatedly bound and eluted and thus the chosen IgG-OA-Con A model strongly suggests the applicability of the principle of oriented immobilization to the

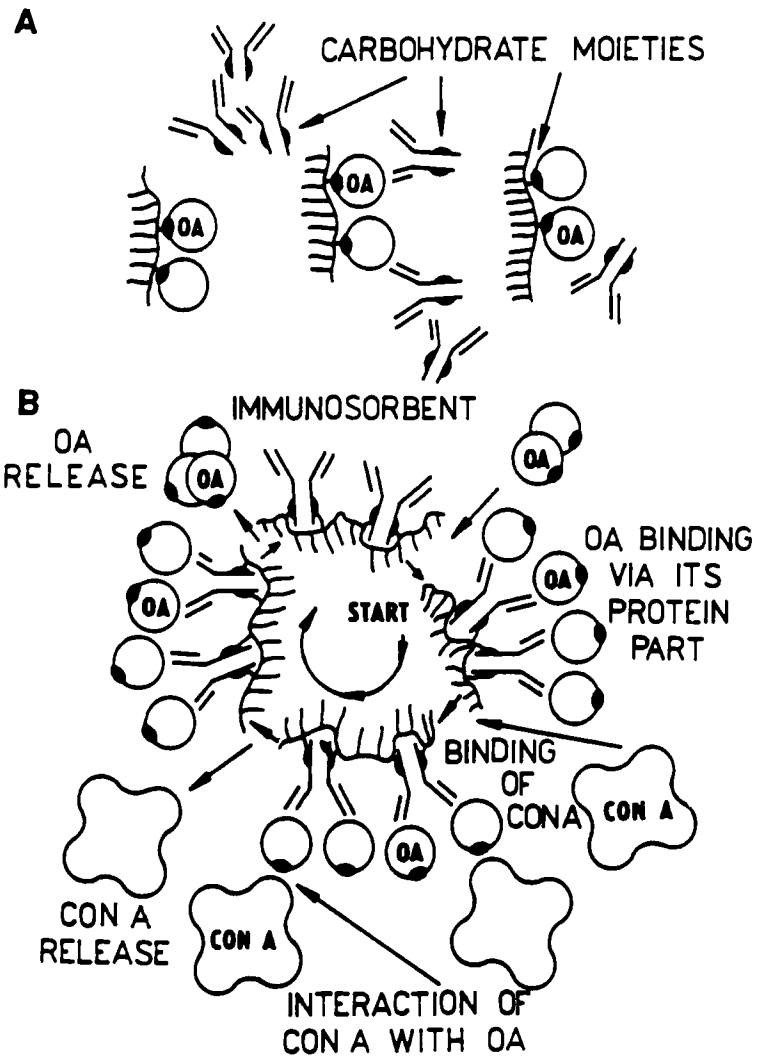


Fig. 2.6. Schematic drawing (A) of preparation of immunoglobulins (IgG) against ovalbumin (OA) by use of immunoaffinity chromatography on cellulose with OA attached through its carbohydrate moiety, and (B) of use of this oriented immobilized IgG (via carbohydrate moiety on hydrazide derivative of cellulose) after adsorption of OA for isolation of concanavalin A (Con A). Data from J.Turková et al., J.Chromatogr., 500 (1990) 585-593.

preparation of various sorbents suitable for the purification of different compounds in satisfactory yields and with good reproducibility. It may be expected that oriented immobilization will find a use not only in separation process, but also in analytical and research applications (Turková et al., 1990). Activation by enzymatic oxidation with

galactose oxidase in presence of catalase was used not only for the activation galactose-containing glycoproteins but also for the activation of galactose-containing solid supports Sepharose and O-α-D-galactosyl Separon (Petkov et al., 1990).

REFERENCES

Amicon Corporation, Dye-Ligand Chromatography, Amicon Corporation, Lexington, Massachussetts, USA, 1980, pp. C1-C83.

Anderson, N.G., Willis, D.D., Holladay, D.W., Caton, J.E., Holleman, J.W., Eveleigh, J.W., Atrill, J.E., Ball, F.L. and Anderson, N.L., Anal. Biochem., 68(1975) 371-393.

Axen, R. and Ernback, S., Eur. J. Biochem., 18 (1971) 351-360.

Axen, R., Porath, J. and Ernback, S., Nature, 214 (1967) 1302-1304.

Bayer, E.A. and Wilchek, M., J. Chromatogr., 510 (1990) 3-11.

Biellmann, J.-F., Samama, J.-P., Branden, C.I. and Eklund, H., Eur. J. Biochem., 102 (1979) 107-110.

Calton, G.J., Methods Enzymol., 104 (1984) 381-387.

Campbell, D.H., Luescher, E.L. and Lerman, L.S., Proc. Nat. Acad. Sci. U.S.A., 37 (1951) 575-578.

Chaiken, I.M., Wilchek, M. and Parikh, I. (Editors), Affinity Chromatography and Biological Recognition, Academic Press, Inc., Orlando, 1983, pp. 1-515.

Clonis, Y.D., Jones, K. and Lowe, C.R., J. Chromatogr., 363 (1986) 31-36.

Cuatrecasas, P. and Anfinsen, C.B., Methods Enzymol., 22 (1971) 345-378.

Cuatrecasas, P., Wilchek, M. and Anfinsen, C.B., Proc. Nat. Acad. Sci. U.S.A., 61 (1968) 636-643.

Dean, P.D.G., Johnson, W.S. and Middle, F.A. (Editors), Affinity Chromatography - a Practical Approach, IRL Press, Oxford 1985, pp.1-215.

Dunlap, R.B. (Editor), Advan. Exp. Med. Biol., 42 (1974) 1-377.

Egly, J.-M. (Editor), Affinity Chromatography and Molecular Interaction, INSERM, Vol. 86, Paris: INSERM Publications, 1979, pp. 1-542.

Epton, R. (Editor), Hydrophobic, Ion-exchange and Affinity Methods, Ellis Horwood, Chichester, 1978, pp. 1-353.

Ernst-Cabrera, K. and Wilchek, M., Med. Sci. Res., 16 (1988a) 305-310.

Ernst-Cabrera, K. and Wilchek, M., Trends Anal. Chem., 7 (1988b) 58-63.

Eveleigh, J.W., in T.C.J. Gribnau, J. Visser and R.J.F. Nivard (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982, pp. 293-303.

Eveleigh, J.W. and Levy, D.E., J. Solid-Phase Biochem., 2 (1977) 45-78.

Fiechter, A. (Editor), Advan. Biochem. Engineering, 25 (1982) 1-145.

Fitzgerald, R.J. and Swaisgood, H.E., Arch. Biochem. Biophys., 268 (1989) 239-248.

Gersten, D.M. and Marchalonis, J.J., J. Immunol. Methods, 24 (1978) 305-309.

Gribnau, T.C.J., Visser, J. and Nivard, R.J.F. (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982, pp. 1-584.

Henderson, G.B., Shaltiel, S. and Snell, E.E., Biochemistry, 13 (1974) 4335-4338.

Hoffmann-Ostenhof, O., Breitenbach, M., Koller, F., Kroft, D. and Scheinor, O. (Editors), Affinity Chromatography, Biospecific Sorption, Pergamon Press, Oxford, 1978, pp. 1-374.

Ishii, S. (Guest Editor), J.Chromatogr., 597 (1992) 1-452.

James, M.N.G., Sielecki, A.R. and Moult, J. in V.J. Hruby and D.H. Rich (Editors), Proceedings of the 8th American Peptide Symposium, Pierce Chem. Comp., Rockford, Illinois, 1983, pp. 521-530.

Jakoby, W.B. and Wilchek, M. (Editors), Methods Enzymol. 34 (1974) 1-810.

Jakubowski, H. and Pawelkiewicz, J., FEBS Lett., 34 (1973) 150-154.

Jennissen, H.P. and Müller.W. (Editors), Die Makromolekuläre Chemie, Macromolecular Symposia, 17 (1988) 1-497.

Johnston, K., Nature, 327 (1987) 450.

Kato, Y., Seita, T., Hashimoto, T. and Shimizu, A., J.Chromatogr., 134 (1977) 204-206.

Kay, J., Afting, E.G., Aoyagi, T. and Dunn, B.M., Biochem.J., 203 (1982) 795-797.

Kobayashi, H., Kusakabe, I. and Murakami, K., Anal. Biochem., 122 (1982) 308-312.

Kohler, G. and Milstein, C., Nature, 256 (1975) 495-497.

Kopperschläger, G., Bohme, H.J. and Hofman, E., Advan. Biochem. Eng., 25 (1982) 101-138.

Larsson, P.-O., Methods Enzymol., 104 (1984) 212-223.

Lerman, L.S., Proc. Nat. Acad. Sci. U.S., 39 (1953) 232-236.

Lowe, C.R., An Introduction to Affinity Chromatography, North Holland Publishing Comp., Amsterdam, 1979, pp. 269-522.

Lowe, C.R., Burton, N., Dilmaghanian, S., Mc Loughlin, S., Pearson, J., Stewart, D. and Clonis, Y.D. in M.A.Vijayalakshmi and O. Bertrand (Editors), Protein-Dye Interactions: Developments and Applications, Elsevier Applied Science, London and New York, 1989, pp. 11-20.

Lowe, C.R. and Dean, P.D.G., Affinity Chromatography, Wiley, London, 1974, pp. 1-272.

Lowe, C.R. and Pearson, J.C., Methods Enzymol., 104 (1984) 97-113.

Mohr, P. and Pommerening, K., Affinity Chromatography: Practical and Theoretical Aspects, Marcel Dekker Inc., New York, Basel, 1985, pp. 1-320.

O'Carra, P., Biochem. Soc. Trans., 2 (1974) 1289-1293.

O'Carra, P., Barry, S. and Griffin, T., Methods Enzymol., 34 (1974) 108-126.

Ohlson, S., Hansson, L., Glad, M., Mosbach, K. and Larsson, P.-O., Trends Biotechnol., 7 (1989) 179-186.

Ohlson, S., Hansson, L., Larsson, P.-O. and Mosbach, K., FEBS Lett., 93 (1978) 5-9.

O'Shannessy, D.J., J.Chromatogr., 510 (1990) 13-21.

Ostrove, S., Methods Enzymol., 182 (1990) 357-371.

Ostrove, S. and Weiss, S., Methods Enzymol., 182 (1990) 371-379.

Petkov, L., Sajdok, J., Rae, K., Šůchová, M., Káš, J. and Turková, J., Biotechnol.Techniques, 4 (1990) 25-30.

Phillips, T.M., Adv. Chromatogr., 29 (1989) 133-174.

Porath, J., Biochimie, 55 (1973) 943-951.

Porath, J., Axén, R. and Ernback, S., Nature, 215 (1967) 1491-1492.

Ratzkin, B., Lee, S.G., Schrenk, W.J., Roychoudhury, R., Chen, M., Hamilton, M.A. and Hung, P.P., Proc. Nat. Acad. Sci. U.S.A. 78 (1981) 3313-3317.

Schott, H., Affinity Chromatography - Template Chromatography of Nucleic Acids and Proteins, Marcel Dekker, New York, Basel, 1984, pp. 1-234.

Scopes, R.K. in R.K. Scopes, Protein Purification, Principles and Practice, Springer-Verlag, New York, 1987, pp. 126-172.

Scouten, W.H., Affinity Chromatography: Bioselective Adsorption on Inert Matrices, Wiley-Interscience, New York, 1981, pp. 1-348.

Scouten, W.H. in W.H. Scouten (Editor), Solid Phase Biochemistry, Wiley, New York, 1983, pp.1-15.

Shaltiel, S., Methods Enzymol., 34 (1974) 126-140.

Shaltiel, S., Methods Enzymol., 104 (1984) 69-96.

Solomon, B., Koppel, R., Schwartz, F. and Fleminger, G., J.Chromatogr., 510 (1990) 321-329.

Starkenstein, E., Biochem. Z., 24 (1910) 210-218.

Stellwagen, E., Methods Enzymol., 182 (1990) 343-357.

Sundaram, P.V. and Eckstein, F. (Editors), Theory and Practice in Affinity Techniques, Academic Press, London, 1978, pp. 1-246.

Toraya, T., Fujimura, M., Ikeda, S., Fukui, S., Yamada, H. and Kumagai, H., Biochim. Biophys. Acta, 420 (1976) 316-322.

Turková, J., Affinity Chromatography, Elsevier, Amsterdam, 1978, pp. 1-405.

Turková, J. in Z.Deyl (Editor), Separation Methods, Elsevier, Amsterdam, 1984a, pp. 321-361.

Turková, J. in V. Krumphanzl and Z. Řeháček (Editors), Modern Biotechnology, UNESCO, Paris, 1984b, pp. 437-524.

Turková, J., Bláha, K., Horáček, J., Vajčner, J., Frydrychová, A. and Čoupek., J., J. Chromatogr., 215 (1981) 165-179.

Turková, J., Chaiken, I.M. and Hearn, M.T.W. (Guest Editors), J. Chromatogr., 376 (1986) 1-451.

Turková, J., Fusek, M. and Šťovíčková, J. in A.Blažej and J.Zemek (Editors), Progress in Biotechnology, Vol.4, Elsevier, Amsterdam, 1988, pp. 245-260.

Turková, J., Petkov, L., Sajdok, J., Káš, J. and Beneš, M.J., J. Chromatogr., 500 (1990) 585-593.

Vijayalakshmi, M.A. and Bertrand, O. (Editors), Protein-DyeInteractions:Developments and Applications, Elsevier Applied Science, London and New York, 1989, pp. 1-342.

Vosbeck, K.D., Chow, K.F. and Awad, Jr., W.M., J. Biol. Chem. 248 (1973) 6029-6034.

Weber, D.V. and Bailon, P., J. Chromatogr. 510 (1990) 59-69.

Werner, S. and Machleidt, W., Eur. J. Biochem., 90 (1978) 99-105.

Wilchek, M. (Guest Editor), J. Chromatogr., 510 (1990) 1-375.

Wilchek, K. and Bayer, E.A., Avidin-Biotin Technology, Methods Enzymol., 184 (1990) 1-746.
Wilchek, M., Miron, T. and Kohn, J., Methods Enzymol., 104 (1984) 3-55.

Chapter 3

Choice of affinity ligands (affinants)

3.1 HIGHLY SPECIFIC AND GROUP-SPECIFIC MATRICES

All those compounds are suitable as affinity ligands for the isolation of biologically active products which bind such products specifically and reversibly. Hence, given the varied nature of biologically active materials, affinants represent a very wide range of chemical compounds. Their classification can therefore be based rather on biochemical function than on chemical structure.

Two main criteria determine the selection of an affinity ligand:

1) The affinant to be immobilized must possess a functional group that can be modified for attachment to the solid support without impairing or abolishing its recognition by the complementary molecule. Not all affinants that are suitable for a complementary binding of molecules also have suitable functional groups for their attachment to a solid support. These groups must first be introduced into the affinants, as well as suitably long spacing arms, which are usually indispensable in case of low-molecular weight affinity ligands, since such arms are necessary to enable a bonding interaction.

2) The affinity ligand should have an adequate affinity for the molecule to be purified. Satisfactory results have usually been obtained with specific complexes having an equilibrium constant, K_A, in the region of 10^4 - $10^8 M^{-1}$. However, it is important to mention here that the K_A values under consideration are those determined for the complex of the substance to be isolated with the immobilized affinity ligand and that therefore they need not correspond to the equilibrium constant values determined in solution. For the determination of K_A the most commonly used method is so-called quantitative affinity chromatography, which is based on the elution of biological macromolecular substances from an affinity matrix with soluble affinant solutions of various concentrations (this method will be described in Chapter 14).

Examples of affinants used for the isolation of enzymes, inhibitors, cofactors, antibodies, antigens, agglutinins, glycoproteins and glycopolysaccharides, nucleic acids,

nucleotides, transport and receptor proteins, hormones and their receptors, lipids, cells, viruses and other substances are shown in Tables 9.1,2 and 3. The practical utility of specific sorbents increases if, instead of the narrowly specific ligands, a so-called "general ligand" (Mosbach, 1974) is used for their preparation. As is implied by the name, a group-specific matrix prepared in this manner displays an affinity for a more-or-less large group of biological macromolecules. For example, the enzymes related to the metabolism of aspartic acid show group-specific adsorption affinity to N-(ω-aminohexyl)-L-aspartic acid-Sepharose. Asparaginase, aspartase, aspartate-β-decarboxylase and asparaginase modified with tetranitromethane (Tosa et al., 1974) could all be sorbed on this immobilized affinant.

If the immobilized affinity ligand displays an affinity for more than one complementary molecule, then the specific shape of the adsorption isotherm has important implications. Consider Fig. 3.1, for example, which shows adsorption isotherms for four enzymes, each of which displays different affinities for the immobilized affinant (Lowe and Dean, 1974). Enzyme 1 possesses a very high affinity for the specific sorbent with a dissociation constant of 10^{-7} - 10^{-8} M. Enzymes 2 and 3 have affinities for sorbents with dissociation constants of about 10^{-5} M, and enzyme 4 shows a very weak affinity with a dissociation constant of $> 10^{-3}$ M.

For the generalized Langmuir adsorption isotherm

$$q_i = \frac{k_1 k_2 C_i}{1 + k_1 C_i} \tag{3.1}$$

where q_i is the specific amount of the adsorbed substance i, C_i is concentration and k_1 and k_2 are constants. For low concentrations of C_i, Eq. 3.1 reduces to $q_i = k_1 k_2 C_i$, and for high concentrations of C_i to $q_i = k_2$. In general we can write

$$q_i = f(C_i)^n \tag{3.2}$$

where n = 0-1. It then follows that when the concentration of the ligand is sufficiently high, so that the adsorbent capacity is not a limiting factor, the specific amount of the

adsorbed substance i, q_i, is dependent on its concentration in the mobile phase, C_i, and not on its affinity toward the attached affinant. For a sample containing equimolar amounts of four enzymes, the amount of each of them adsorbed will be q_1, q_2, q_3 and q_4. In displacement elution, using concetration D of the displacer, enzymes 1, 2 and 3 of concentrations C_1, C_2 and C_3 will be eluted. Enzyme 4 will appear before the displacing solution because its adsorption isotherm is not intersected by the displacer line. An enzyme with a high affinity does not displace a less strongly bound enzyme even when, after the initial adsorption, a further amount of enzyme of high affinity is added. If the capacity of the adsorbent is exceeded, enzymes with both a high and a low affinity will appear in the retention volume of the eluate, i.e. not only those which are weakly adsorbed. This consequence is important in view of the differentiation of the enzymes which display an affinity towards general ligands.

Sometimes it becomes necessary to eliminate the contaminating proteins before adsorption on a specific adsorbent by the insertion of a preceding fractionating step. If

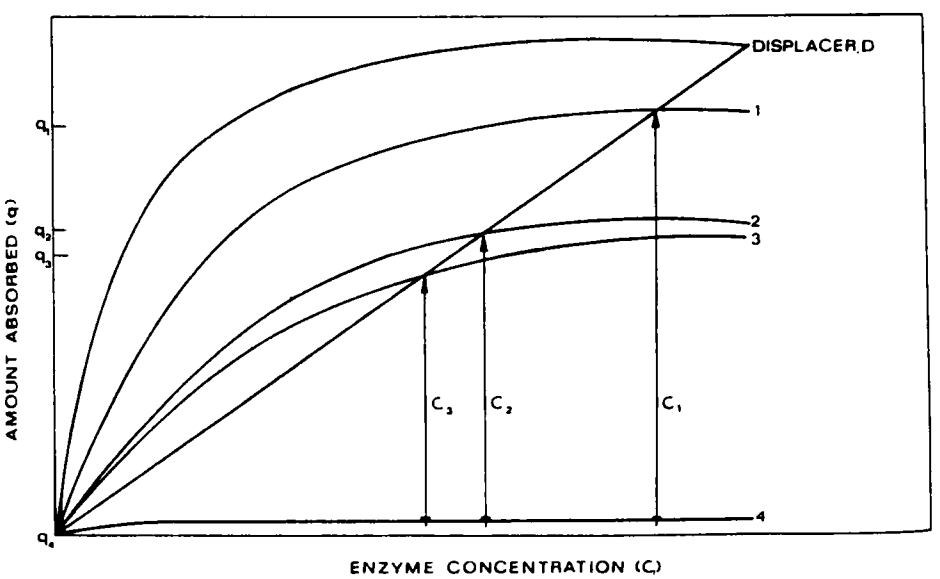


Fig. 3.1. Adsorption isotherms for four enzymes interacting with single immobilized affinity ligand. Reproduced with permission from C.R. Lowe and P.D.G. Dean, Affinity Chromatography, Willey, New York, London, 1974, p.91.

the conditions of adsorption, such as pH, ionic strength, temperature, flow rate or dielectric constant are changed, some enzymes can be excluded specifically. Furthermore, an inhibitor or other ligand may be added in order to prevent adsorption of some enzymes. The use of a solid support with small pores can exclude proteins of a high molecular weight. Increased selectivity can be further improved by using specific methods of elution. A knowledge of inhibitors or substrates of various enzymes can be utilized when planning the selective elution of individual enzymes. According to Ostrove (1990) a bioaffinity sorbent could also be chosen to bind the contaminating proteins, allowing the sample of interest to pass through the gel in the wash volume. This method of separation could result in a great saving of time and the avoidance of cleavage of the isolated molecule by degradative enzymes present in crude cellular extracts.

The selectivity of affinity ligands can also be affected by the nature of the solid support (Fritz et al., 1969). Proteolytic enzymes bound to the negatively charged copolymer of maleic acid with ethylene sorbed only those inhibitors having an isoelectric point below 4-5. If the strongly negative charges of the copolymer chain were neutralized by attachment of (for example) hexamethylenediamine and dimethylethylenediamine, the polyamphoteric derivative formed became suitable for the isolation of inhibitors with even lower isoelectric points.

As it will be discussed in detail in Section 3.3, antibodies show a high affinity for their corresponding antigens and *vice versa*. Difficulties with their liberation from complexes ensue from the strength of this interaction. The use of strongly chaotropic eluents in immunoaffinity chromatography can be circumvented by chemical modification of the immobilized affinity ligand (Murphy et al., 1976). For example, the elution of anti-glucagon-antibodies from a column of immobilized glucagon can be achieved under relatively mild conditions if the steric complementarity to the binding site of the antibody is partly perturbed by selective modification of the hormone, for example by reaction with 2-hydroxy-5-nitrobenzyl bromide, tetranitromethane, or hydrogen peroxide.

O'Carra (1974) recommends that we differentiate between affinity systems with small ligands and those with macroligands. Low molecular weight synthetic affinants are advantageous mainly as a result of their stability and better accessibility. The specific

sorbents prepared from them are usually better characterized, because they are attached *via* a pre-defined functional group. In order to increase their steric accessibility a spacer is inserted, in the majority of cases, between them and the surface of the solid support. High molecular weight affinants are predominantly proteins or nucleic acids. They often undergo denaturation, leading to an irreversible loss of activity. For such affinants the method of random attachment is usually not unambiguously defined. Attachment of glycoproteins through their carbohydrate moiety is suitable for the immobilization of antibodies and enzymes which have active sites in the protein parts of the molecules. Recent times have seen more use being made of oriented immobilization by the use adsorption of antibodies to insolubilized Protein A derived from *Staphylococcus aureus* or various biotinylated molecules to insolubilized avidin or streptavidin. Some examples are shown in Table 9.1,2 and 3.

3.2 ENZYMES AND THEIR EFFECTORS

The combination of several complementary binding sites on the surface of the enzyme molecule permits the formation of a relatively large number of biospecific complexes to be achieved; these complexes can be utilized for efficient isolation as well as for oriented immobilization.

Fig. 3.2 shows the surface of an enzyme molecule covered with several complementary binding sites. Such an enzyme could be, for instance, carboxypeptidase Y containing the complementary binding site for glycyl-glycyl-p-aminobenzylsuccinic acid, a specific inhibitor. The active site of the enzyme also contains a free SH- group. Carboxypeptidase Y is a glycoprotein whose carbohydrate moiety specifically interacts with concanavalin A, a lectin. The antigenic sites of the enzyme can be determined by investigation of the antigenic structures of the peptide chain in experiments with specific antibodies to this enzyme. Enzymes whose coenzymes are nucleotides can form biospecific complexes with nucleotides. Enzymes form complexes with substrates and their analogues, allosteric effectors, metal ions etc. The type of the complex formed determines the mode of their action in chemical processes which take place in the living cell.

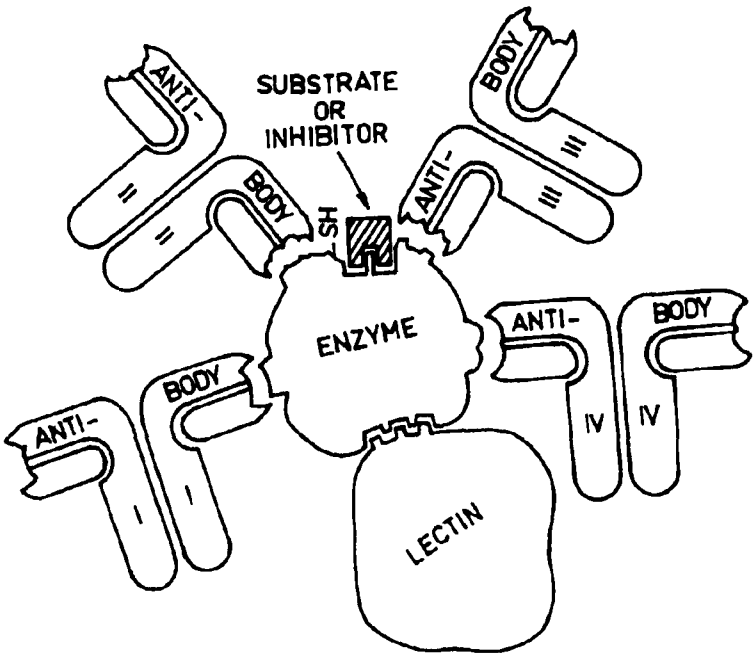


Fig. 3.2. Possible ways in which an enzyme may form biospecific complexes.

Fig. 3.3 shows the ways in which the enzyme can be isolated by the use of the formation of three specific complexes of carboxypeptidase Y. The conditions of formation of the biospecific complex can be determined from bioaffinity chromatography experiments with the enzyme. Absorbance at 570 nm shows peptidase activity, assayed with N-carboxybenzoxy-L-phenylalanine-L-alanine as substrate.

Mercury-Spheron was developed by the use of hydroxyalkyl methacrylate copolymers containing different amounts of mercury derivatives of p-acetaminophenoxyethyl methacrylates or methacrylanilides (Turková et al., 1975). The copolymer containing 15% of the mercury derivative of methacrylanilide was found to be the most convenient one for the isolation of SH-proteinases and was therefore used in this study.

Concanavalin A was bonded to Spheron with immobilized glucose (Filka et al., 1978) which had been activated by periodate oxidation (Vančurová et al., 1979). The bond between the lectin and the aldehyde group of the solid support was stabilized by reduction using sodium borohydride. Carboxypeptidase Y was liberated from the

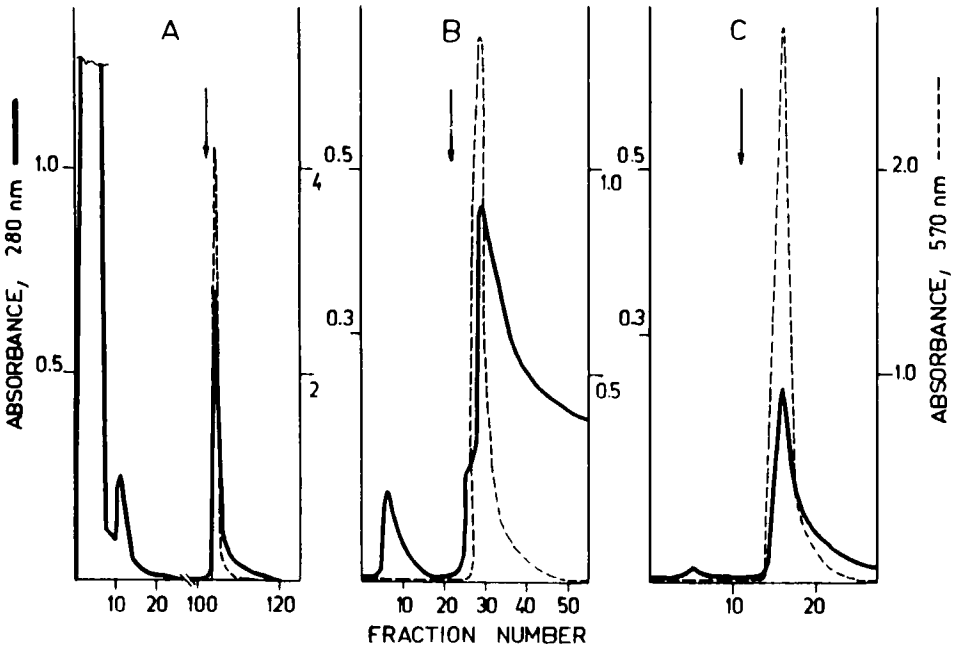


Fig. 3.3. (A) Gly-Gly-p-aminobenzylsuccinic acid-Spheron: the column (5 ml) was equilibrated with 0.01 M sodium acetate (pH 5.0). The sample of a yeast autolysate (50 ml) was added and then the column was washed with 1 M sodium chloride-0.01 M sodium acetate (1 l), pH 4.3. The elution (arrow) was performed with 0.01 M phosphate buffer (pH 7). (B) Mercury-Spheron: the column was equilibrated with 0.1 M sodium acetate (pH 6). After the application of sample, elution (arrow) was performed with 0.05 M mercaptoethanol - 0.1 M sodium acetate (pH 6.0). (C) Con A-Spheron: the column (10 ml) was washed with 0.1 M sodium acetate and 1 mM manganese chloride - calcium chloride - magnesium chloride and equilibrated with 0.4 M sodium chloride -0.1 M sodium acetate (pH 7). After the application of sample, elution (arrow) was performed with 0.1 M borate buffer (pH 6.5). Data from J. Turková et al., J. Chromatogr., 376 (1986) 315-321.

complex with immobilized concanavalin A by borate buffer. In Chapter 4 we shall discuss the advantages of oriented immobilization of glycoproteins through their carbohydrate moieties. Oriented immobilization of carboxypeptidase Y adsorbed to concanavalin A-Spheron was effected by covalent bonding according to the method of Hsio and Royer (1979) by use of crosslinking with glutaraldehyde (Turková et al., 1986). Using electron-cytochemical reaction and N-carbobenzoxy-(CBZ)-L-tyrosine-4-methoxy-2-naphthylamide as substrate Voříšek (1988) was able to show that *Saccha-*

romyces cerevisiae cells contain carboxypeptidase Y bound to vacuole membranes, probably through their carbohydrate moieties.

3.2.1 Naturally occurring high molecular weight and synthetic low molecular weight inhibitors

For the isolation of chymotrypsin and trypsin from a crude pancreatic extract the specific adsorbents were prepared by coupling of two naturally occurring proteinase inhibitors (ovomucoid for trypsin and polyvalent trypsin inhibitor, antilysine, for

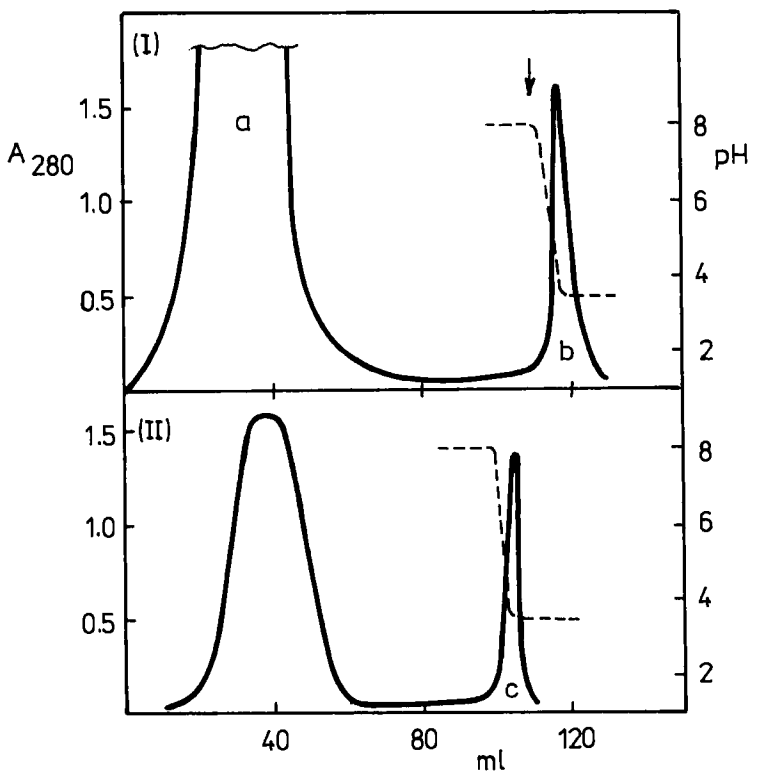


Fig. 3.4. Chromatography of crude pancreatic extract on ovomucoid-Spheron (I) and antilysine-Spheron (II). (I) A sample of active pancreatic extract (100 ml) was placed on a column of ovomucoid-Spheron (10 x 2 cm), which was subsequently eluted with an aqueous solution of ammonium formate (0.05 M formic acid adjusted to pH 8.0 with 5 % aqueous ammonia). Fractions (6 ml) were collected at 20-min intervals. The arrow designates the change in pH from 8.0 to 3.5 (0.1 M formic acid adjusted to pH 3.5 with ammonia). (II) The fraction (a) of material not adsorbed (180 ml) was placed directly on the antilysine-Spheron column (10 x 2 cm). The course of the chromatography was analogous to that described for (I). (a) Contaminants and chymotrypsin; (b) trypsin; (c) chymotrypsin; ---, absorbance at 280 nm; ---, pH. Data from J. Turková and A. Seifertová, J. Chromatogr., 148 (1978) 293-297.

chymotrypsin) and also synthetic low-molecular weight proteinase inhibitors (N-benzyloxycarbonylglycyl-D-phenylalanine for chymotrypsin and p-aminobenzamidine for trypsin) to a hydroxyalkyl methacrylate copolymer (Turková and Seifertová, 1978). In Figs 3.4 and 3.5 it is shown that identical results were obtained with specific adsorbents prepared both with high molecular weight and with low molecular weight inhibitors. Unlike the naturally occurring inhibitors, which could undergo denaturation because of their protein character, the synthetic low molecular weight inhibitors are completely stable. The capacity of specific adsorbents prepared with these inhibitors can be

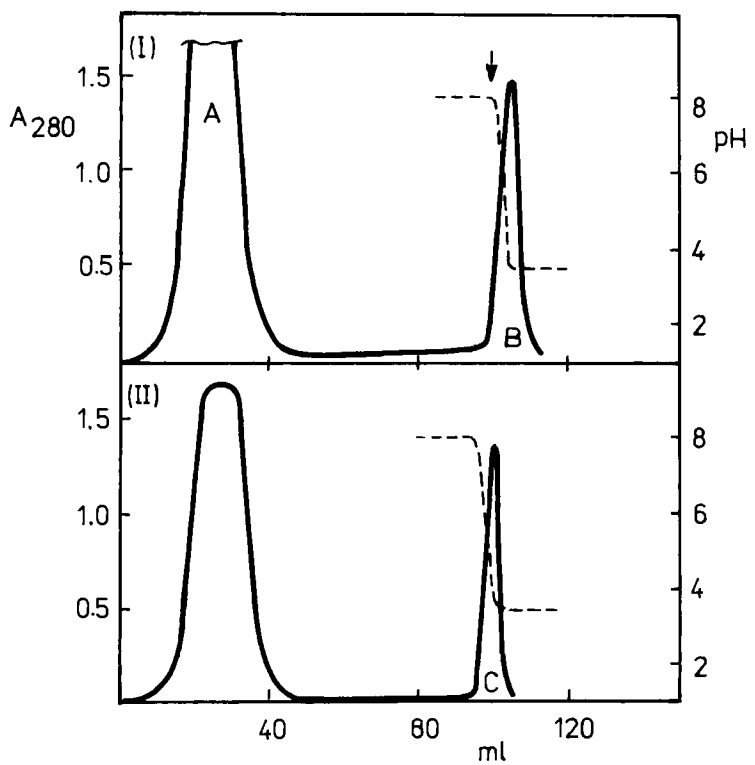


Fig. 3.5. Chromatography of crude pancreatic extract on N-benzyloxycarbonylglycyl-D-phenylalanine-NH_2-Spheron (I) and NH_2-benzamidine-NH_2-Spheron (II). (I) A sample of active pancreatic extract (100 ml) was applied to the column of N-benzyloxycarbonylglycyl-D-phenylalanine-NH_2-Spheron (6.0 x 1.5 cm). The course of the chromatography was identical with that shown in Fig. 3.4. (II) Fraction (A), filtered through a column of N-benzyloxycarbonylglycyl-D-phenylalanine, was placed directly on a column of NH_2-benzamidine-NH_2-Spheron (25 x 1 cm). The course of chromatography is identical with that shown in Fig. 3.4. (A) Contaminants and trypsin; (B) chymotrypsin; (C) trypsin; ---, absorbance at 280 nm; ---, pH. Data from J. Turková and A. Seifertová, J. Chromatogr., 148 (1978) 293-297.

regenerated virtually without limit, if a stable solid support and stable bonds between the support and the amino groups of peptide inhibitors are used.

3.2.2 Coenzymes

The catalytic activity of many enzymes depends on the presence of coenzymes or cofactors. Almost one third of the more than 2000 enzymes known so far require a nucleotide coenzyme (Mohr and Pommerening, 1985). Coenzymes have the function of co-substrates. Such enzymes will contain at least two specific binding sites, one of them for the coenzyme, which will be common to all of them, and one or more for the substrate. The latter site will then be dependent on the nature of the substrate and the catalysed reaction. The immobilized coenzymes will then selectively sorb those groups of enzymes which are utilized in bi- or multi-substrate reactions (Lowe and Dean, 1974).

The coenzymes nicotinamide adenine dinucleotide and other nucleotides of adenine, uridine, guanine and flavine, coenzymes of pyridoxal, folate and its analogues, biotin, lipoic acid, cobalamines and porphine derivatives are frequently employed as affinants. The function of immobilized cofactors as bioaffinity ligands depends on the spacer length and on the position of covalent binding. Fig. 3.6 demonstrates four positions on

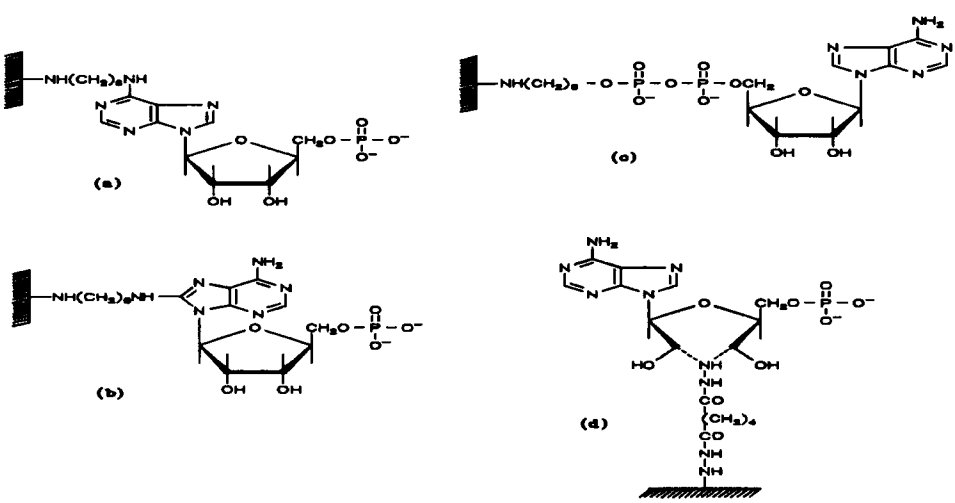


Fig. 3.6. The structures of several immobilized-AMP adsorbents. (a) N^6-(6-aminohexyl)-AMP-agarose. (b) 8-(6-aminohexyl)-AMP-agarose. (c) P^1-(6-aminohexyl)-P^2-(5'-adenosine)-pyrophosphate-agarose. (d) Ribosyl-linked AMP.

the adenine nucleotide which were derivatized before their attachment to CNBr-activated Sepharose 4B by Trayer and Trayer (1974).

When comparing the binding of various dehydrogenases and kinases on N^6-(6-aminohexyl)-5'-AMP-Sepharose and P^1-(6-aminohexyl)-P^2- (5'-adenosine)pyrophosphate-Sepharose, Harvey et al.(1974), expressed the strength of the interaction between the enzyme and the immobilized nucleotide by the so-called "binding" (β). This term represents the concentration of potassium chloride (mM) in the centre of the enzyme peak when the enzymes are eluted with a linear potassium chloride gradient (Fig. 3.7 and Table 3.1).

Immobilized inosine 5'-monophosphate was used for the large-scale purification of *E. coli* IMP dehydrogenase by Clonis and Lowe (1980). The use of a variety of immobilized nucleotides for binding NAD^+-dependent dehydrogenase was reviewed by Lowe et al. (1980).

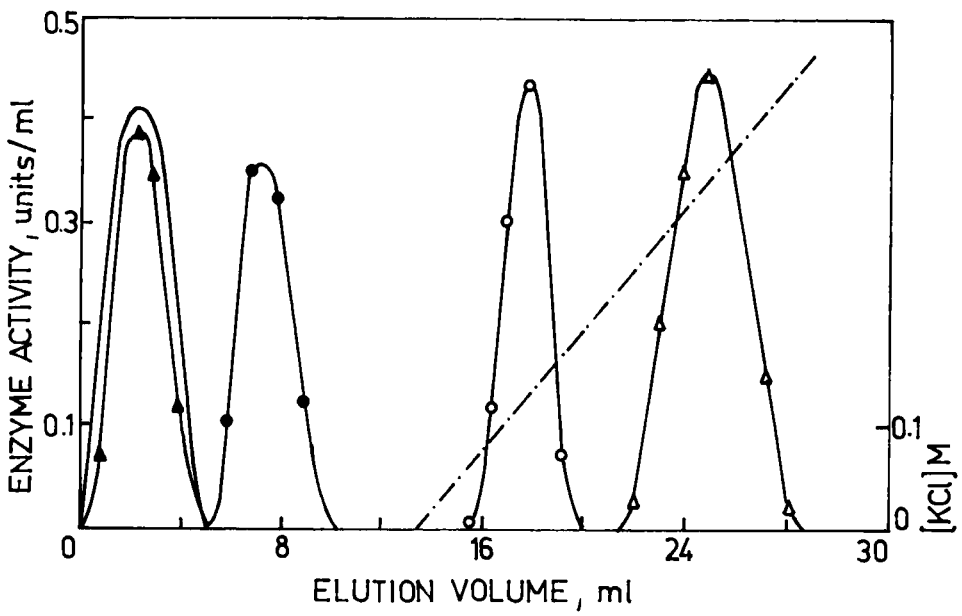
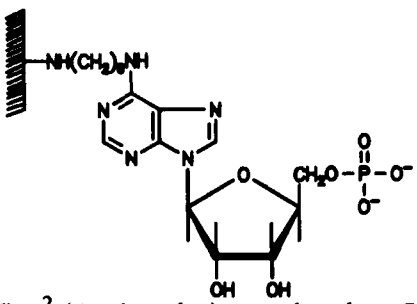


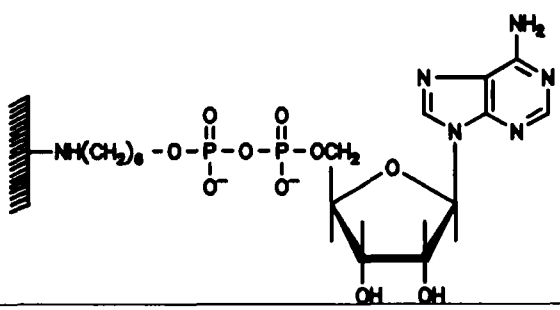
Fig. 3.7. Chromatography of a crude yeast extract on N^6-(6-aminohexyl)-5'-AMP-Sepharose. A sample (100 μl) of a crude yeast extract was applied to a column (50 x 5 mm) containing 0.5 g of N^6-(6-aminohexyl)-5'-AMP-Sepharose equilibrated with 10 mM KH_2PO_4-KOH buffer, pH 7.5. After washing through non-adsorbed proteins, enzymes were eluted with a linear salt gradient (0.1 M KCl; 20 ml total volume) at a flow-rate of 8 $ml.h^{-1}$. Inert protein (---), glucose 6-phosphate dehydrogenase (▲), glutathione reductase (●), malate dehydrogenase (o) and yeast alcohol dehydrogenase (Δ) were assayed in the effluent. Reproduced with permission from D.B. Crawen et al., Eur. J. Biochem., 41 (1974) 329-333.

Table 3.1.

Comparison of the binding of various enzymes to N^6-(6-aminohexyl-5'-AMP-Sepharose



and P^1-(6-aminohexyl)-P^2-(5'-adenosine)-pyrophosphate-Sepharose .



Enzyme		*Binding (β)**	
Code number	*Name*	*I*	*II*
E.C.1.1.1.27	Lactate dehydrogenase	>1000**	>1000**
E.C.1.1.1.49	Glucose G-phosphate dehydrogenase	0	170
E.C.1.1.1.37	Malate dehydrogenase	65	490
E.C.1.1.1.1	Alcohol dehydrogenase	400	0
E.C.1.2.1.12	p-Glyceraldehyde 3-phosphate dehydrogenase	0	>1000**
E.C.2.7.2.3	3-Phosphoglycerate kinase	70	260
E.C.2.7.1.40	Pyruvate kinase	100	110
E.C.2.7.1.1	Hexokinase	0	0
E.C.2.7.4.3	Myokinase	0	380
E.C.2.7.1.30	Glycerokinase	122	0

*Binding(β) is the KCl concentration (mM) at the centre of the enzyme peak when the enzyme is eluted with a linear gradient of KCl.

**Elution was effected by a 200-μl pulse of 5 mM NADH.

3.2.3 Dyes

A reactive textile dye in solution can function as a competitive inhibitor for the substrate, coenzyme, or effector of a variety of proteins, often with an affinity greater than that exhibited by the competitive molecule. This affinity probably results from the flexibility of the dye which can assume the polarity and geometry of the surface of a variety of competitive biomolecules. Immobilization of a single reactive dye generates a bioaffinity column that is useful in the purification of numerous proteins.

The history of dye-ligand chromatography is described in the chapter of the same name in the book by Scopes (1987a). Pharmacia Fine Chemicals attached a blue dye known as Cibacron Blue F3GA to a high molecular weight soluble dextran for use as a visible void volume marker for gel filtration. If they had selected a different dye, it is possible that the recent rapid expansion in the use of coloured adsorbents would not yet have happened. Workers were puzzled when yeast pyruvate kinase appeared in the void volume during gel filtration, indicating a much higher molecular weight than expected. But when the blue marker was omitted, the enzyme came out after the void volume.

The immobilization of Blue Dextran by entrapment in polyacrylamide gel, introduced by Kopperschläger et al. (1971) allowed the separation of phosphofructokinase in one step. The selectivity of the Cibacron Blue F3GA - enzyme interaction led Böhme et al. (1972) to the suggestion that the dye operates as an ATP analog. Stellwagen et al. (1975) expanded this concept by proposing that the dye would bind specifically to the dinucleotide binding fold of the respective protein. Thompson et al. (1975) published a Corey - Pauling - Koltin structural model of NAD and Cibacron Blue F3GA (Fig. 3.8). From X-ray studies of the binding of Cibacron Blue F3GA to liver alcohol dehydrogenase performed by Biellmann et al. (1979) it became evident that the dye binds at the nucleotide binding site of the enzyme with correspondences of the adenine and ribose rings, but not with the nicotinamide. However, this definitive work does not apply to all interactions involving immobilized Cibacron Blue F3GA, since many proteins that bind to this dye do not posses a nucleotide binding site, e.g. albumin and troponin (Hey and Dean, 1983).

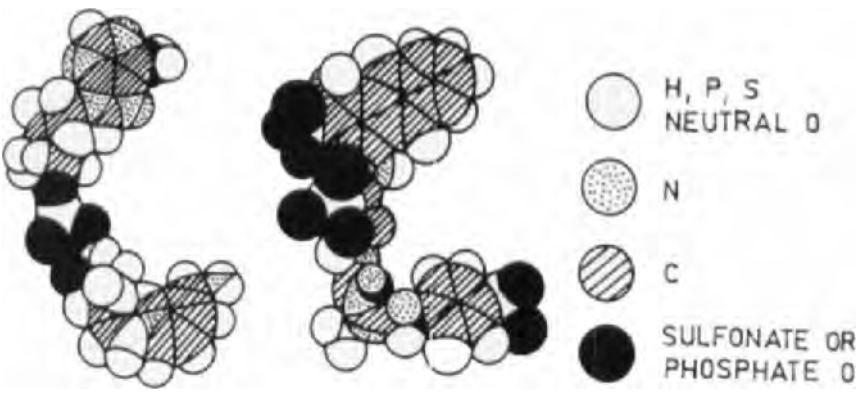


Fig. 3.8. Corey-Pauling-Koltin structural model of NAD and Cibacron Blue F3GA. NAD is shown on the left in the conformation in which it binds to dehydrogenases. The Blue A model is shown on the right in a conformation similar to the NAD. Note the close resemblance in size, orientation of π-electrons, and position of negatively charged sulphonate or phosphate groups. Reproduced with permission from S. T. Thompson et al., Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 669-672.

The chemical structure of Cibacron Blue F3GA and its reaction with hydroxyl group are presented in Fig. 3.9.

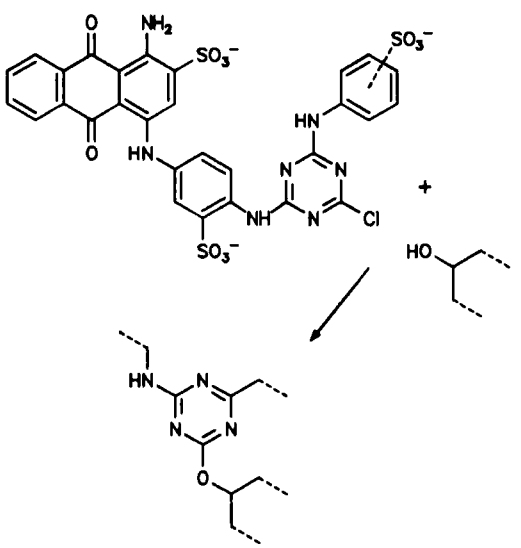


Fig. 3.9. The structure of Cibacron Blue F3GA and its reaction with hydroxyl groups.

The effect of increasing the chain length of the presaturating fatty acid on the interaction of human serum albumin with Cibacron Blue F3GA was studied by Metcalf et al. (1981), and the effect of albumin on binding and recovery of enzymes which interact with nucleotides in chromatography on Cibacron Blue-Sepharose was reported by Ramadoss et al. (1983). More than 250 papers on the molecular basis of the Cibacron Blue F3GA and related dye - protein interactions and their applications in protein purification have been reviewed by Kopperschläger et al. (1982).

In spite of the range of reactive dyes available, some proteins of interest are not retained by immobilized reactive dyes. The presence of a relatively low concentration of a metallic cation such as Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, or Al^{3+} in the chromatographic solvent has been found by Hughes et al. (1982) to facilitate the retention of a number of proteins. Such proteins can be selectively eluted by addition of a chelating agent to the chromatographic solvent in the absence of metal cations.

The grouping of dyes shown in Table 3.2 is taken from a paper by Scopes (1986) about strategies for enzyme isolation using dye-ligand and related adsorbents.

The ever-increasing use of dye-based affinity techniques in many fields of biomedical research and biotechnology is reflected in the 34 contributions from a world-wide gathering of experts at the First International Conference on "Modern Aspects of Protein-Dye Interaction: Role in Downstream Processing", which was held at the University of Compiegne, France, in July 1988 (Vijayalakshmi and Bertrand, 1989). Lowe (1989) in his report described many advantages of using dye ligands, particularly for large-scale applications. Compared to biological ligands, dye ligands are inexpensive materials available in tonnage quantities world-wide. There is a wide range of chromophors available which are dye-based biologically, chemically, and photochemically stable adsorbents, potentially sterilizable *in situ*, with no degradation of the ligand itself. Because they are reactive materials, they are very easily immobilized to hydroxyl polymers, generally by a single-step process. Such adsorbents have a high capacity, a very broad binding capability in terms of the complementary proteins, and they are easily re-usable. Several thousand different types of proteins would interact with an immobilized textile dye. Examples are oxidoreductases, phosphokinases and nearly all coenzy-

Table 3.2.

Grouping of Dyes

Group 1	*Group 2*	*Group 3*	*Group 4*	*Group 5*
P Blue MX-7RX	R Black GF	P Blue H-EG	P Black H-EXL	P Blue H-ERD
C Blue 2-RA	P Blue MX-R	P Blue H-EGN	P Blue H-GR	C Blue F-R
R Orange 3R	P Brown MX-GRN	P Blue H-4R	P Blue MX-G	P Brown H-5R
P Red MX-2B	C Brown 3-GRA	P Blue MX-3G	P Blue MX-4GD	P Green H-4G
P Rubine H-BN	P Navy H-4R	C Blue F3-GA	D Blue K-BL	P Green H-E4BD
P Turquoise H-A	P Orange MX-G	R Blue B	P Brown H-3R	P Navy H-ER
P Turquoise MX-G	R Orange FR	R Blue R	P Beown MX-5BR	P Red H-3B
C Turquoise 6-GE	P Red MX-5B	C Navy F-2R	P Orange H-ER	P Red H-8BN
R Violet R	P Scarlet MX-G	P Red H-E3B	P Orange MX-2R	P Red H-E7B
R Yellow GNL	P Scarlet MX-3G	P Rubine MX-B	P Red MX-7B	P Scarlet H-E3G
P Yellow H-A	C Turquoise GFP	P Scarlet H-2G	P Red MX-8B	P Yellow H-E3G
P Yellow MX-6G	C Yellow R-A	P Yellow H-E6R	C Red 3-BA	P Yellow H-E6G
P Yellow MX-8G	P Yellow MX-3G	P Yellow H-5G	P Violet H-3R	P Yellow H-E4R
	P Yellow MX-4R	P Yellow MX-R	P Yellow H-E6R	P Yellow MX-GR
		C Yellow 3-GP		

Group 1 dyes bind the least protein from crude extracts of tissues, and group 5 dyes the most. Actual groups may vary ±1 with different types of extract. P, I.C.I. Procion; C, Ciba-Geigy Cibacron; R, Hoechst Remazol; D, Sandoz Drimarene. Not all of these dyes are still commercially available.

me dependent enzymes, hydrolases, various transferases, a number of proteins which interact with mono- and polynucleotides, synthetases, hydroxylases, nearly all of the glycolytic enzymes, phosphatases, a whole variety of blood proteins and other non-enzyme proteins. A number of studies using classic enzyme kinetics, circular dichroism,

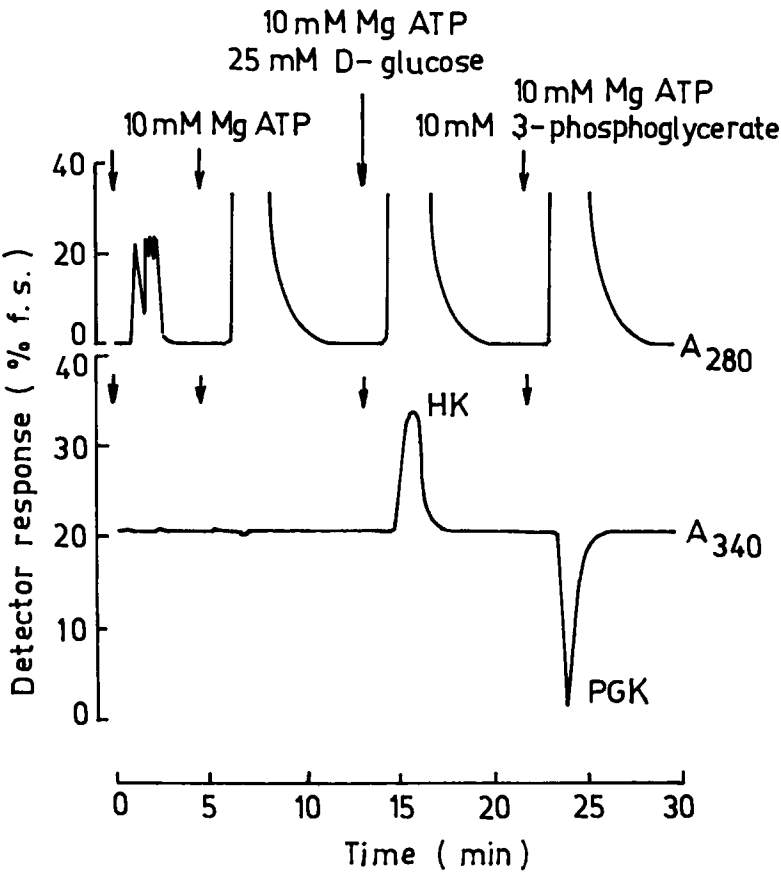


Fig. 3.10. Chromatography of a crude yeast extract on Cibacron Blue F3GA-silica with on-line monitoring of hexokinase (HK) and 3-phosphoglycerate kinase (PGK) activity. Sample applied at first arrow: crude yeast extract (2 μl); column irrigant, 0.1 M Tris-HCl buffer (pH 7.3) containing 0.5 mM EDTA, 5 mM $MgCl_2$ and 0.5 mM 2-mercaptoethanol; flow rates, 1 ml/min, both pumps; post-column reactor temperature, 40±0.1°C; eluents (400 μl), as indicated by the arrows. Reproduced with permission from C. R. Lowe et al., J. Chromatogr. 215 (1981) 303-316.

affinity labelling, X-ray diffraction, and other techniques have been utilized to demonstrate the specificity of dye binding to the active sites of proteins. A computer-aided study of the interaction between proteins and dye chromogens was presented by Stead (1989).

High performance liquid bioaffinity chromatography (HPLBAC) combines the inherent speed and resolving power of HPLC with the biological specificity of bioaffinity chromatography. Lowe et al. (1981) demonstrated the ability of Cibacron Blue F3GA

bound to silica in the selective and rapid resolution of complex mixtures of complementary proteins. The chromatography of a crude yeast extract on Cibacron Blue F3GA-silica with on-line monitoring of protein (280 nm), and of hexokinase (HK) and 3-phosphoglycerate kinase (PGK) activity (340 nm) shown in Fig. 3.10 may serve as an example. The experimental set-up for on-line monitoring of the elution profiles from the HPLBAC adsorbent is shown in Fig. 3.11.

Enzymes eluted from the HPLBAC column were detected with an on-line detector system comprising a reagent pump, post-column reactor equilibrated to 40 ± 0.1°C in a water bath, and a UV-visible monitor. The post-column detection of HK and PGK was accomplished with a "universal" assay medium comprising the reactions:

$$\text{D-glucose} + \text{ATP} \xrightarrow{HK} \text{D-glukose-6-phosphate} + \text{ADP}$$

$$\text{D-glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{G6PDH} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

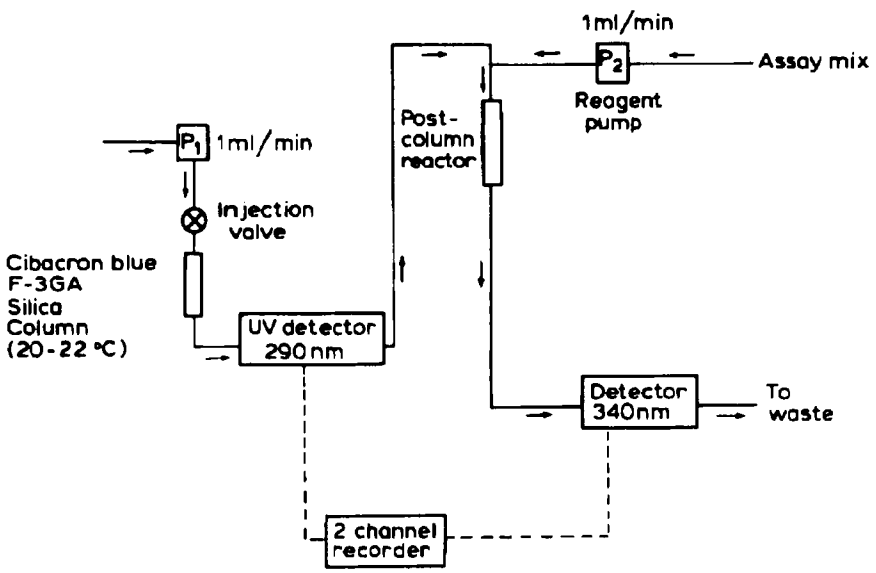


Fig. 3.11. The experimental set-up for on-line monitoring of elution profiles from HPLBAC adsorbents. Reproduced with permission from C. R. Lowe et al., J. Chromatogr. 215 (1981) 303-316.

for hexokinase activity and the reactions:

$$\text{3-phosphoglycerate} + \text{ATP} \xrightarrow{PGK} \text{glycerate-1,3,-bisphosphate} + \text{ADP}$$

$$\text{glycerate-1,3-bisphosphate} + \text{NADH} + \text{H}^+ \xrightarrow{G3PDH} \text{glyceraldehyde-3-phosphate} + \text{NAD}^+ + \text{phosphate}$$

As other examples we may mention the purification of anti-DNA antibodies from systemic lupus erythematosus serum (Pollard and Webb, 1982) or large-scale purification of staphylococcal enterotoxins (Brehm et al., 1990), both by dye - ligand chromatography. Hondmann and Visser (1990) described a method by means of which 96 different dye - adsorbents can be tested simultaneously for their ability to bind enzymes and to test their biospecific elution. Much useful information about chromatography on immobilized reactive dyes has been published by Stellwagen (1990), including representative purification procedures, regeneration and storage of immobilized dyes, preparation of immobilized reactive dye columns and newer technologies (Cibacron F with fluorine etc.)

In summary, many proteins have been purified to homogeneity in a single step using immobilized dye chromatography. Most aspects of immobilized reactive dye-protein interactions have been reviewed by Kopperschläger et al., (1982), Dean and Quadri (1983), Lowe and Pearson (1984), Scopes (1986, 1987 b). Immobilized dyes can function as an inexpensive stable reagent capable of impressive selectivity and capacity in the purification of a large number of quite different proteins (Stellwagen, 1990).

3.2.4 Substrates

Enzyme purification might be carried out by bioaffinity chromatography using real substrates as affinity ligands if the conversion to the corresponding products could readily be stopped or if it had only a small influence on the bioaffinity of the sorbents used. Gallop et al. (1957) used insoluble collagen for the bioaffinity chromatography of bacterial collagenase. Thermally modified casein was used for bioaffinity chro-

matography by Šafařík (1983). The purification of intracellular galactose oxidase from *Dactylium dendroides* by bioaffinity chromatography on Sepharose (a polymer of D-galactose and 3,6-anhydro L-galactose) was described by Mendonca and Zancan (1987). The active enzyme was only obtained when D-fucose was used as eluent. When galactose was used the enzyme lost its activity, probably due to hydrogen peroxide formation.

The biospecific sorption of cyclodextrin glucosyltransferase from *Bacillus macerans* on physically modified starch was described by Gottvaldová et al. (1988). The starch was heated in order to complete gelling, then the gel was cooled to room temperature and frozen to -20°C (12 h). The gel was subsequently thawed at room temperature and then frozen again (24 h). When thawed again, the gel was mixed in a laboratory mixer and freed from the excessively fine portion by decantation (Hanuš et al., 1975). The dependence of the sorption of glucosyltransferase on temperature was determined in the temperature range 5-40°C. The effect of temperature was very small in the range 5-15°C. At temperatures above 15°C the sorption efficiency decreased rapidly and at 25°C only 50% of the enzymatic activity was adsorbed. The capacity of modified starch for the sorption of cyclodextrin glucosyltransferase and the time course of this sorption were determined at 10°C. The sorption capacity also depended on the initial concentration of the enzyme in the cultivation broth. Column sorption was generally more effective than batch sorption.

The turnover of an enzyme-substrate system can be slowed down by using subzero temperatures in fluid media. Balny et al. (1979) described a chromatographic separation which was performed at a subzero temperature, where the turnover of the enzyme was very low or stopped. As a model system Sepharose-bound L-trialanine p-nitroanilide was used for the affinity binding of porcine pancreatic elastase, which was adsorbed to the column in a hypersaline medium at -14°C and eluted from the column at the same temperature using 50 % (v/v) ethylene glycol. The affinity adsorbent proved to be very specific since it did not retain trypsin, chymotrypsin and ovalbumin and retained only 20% of cytochrome c. The elution pattern of elastase from Sepharose-L-trialanine - p-nitroanilide is shown in Fig. 3.12.

This was the first report describing the use of a true substrate for the isolation of an enzyme by subzero temperature bioaffinity chromatography under conditions of very low enzyme turnover. Owing to the specificity of the adsorbent for elastase, this method can be probably used for the single-step isolation of this protease from crude extracts of pancreatic powder, leukocyte granular extracts or homogenates of macrophages. The last two extracts are worth mentioning because the method is particularly suitable for the purification of micro-amounts of elastase.

3.2.5 Other bioaffinity ligands

An example of the use of an allosteric effector for the preparation of a specific adsorbent is the use of the p-aminophenyl ester of dATP bound to Sepharose for the

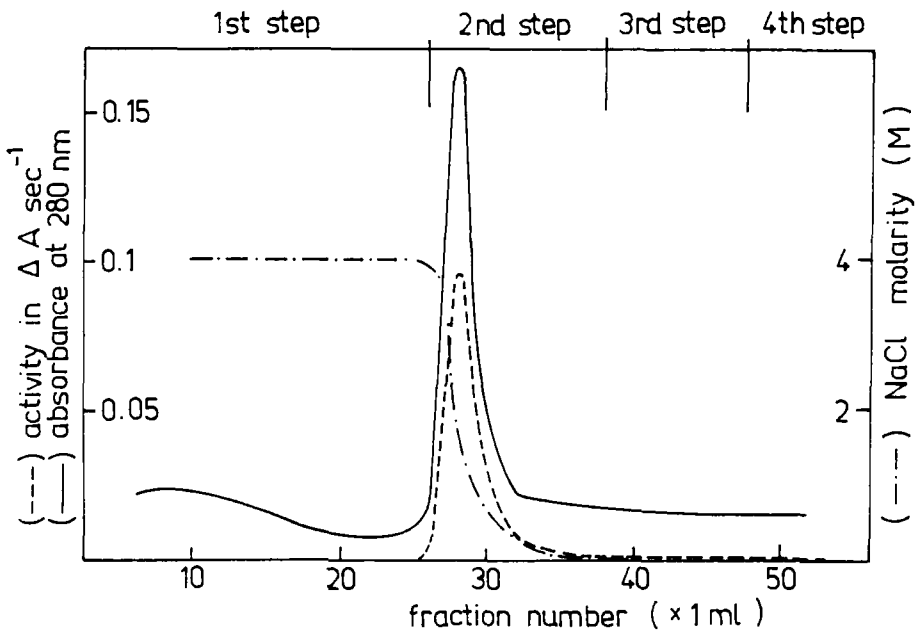


Fig. 3.12. Elution pattern of elastase (0.2 mg applied) from the bioaffinity column (2 x 1 cm). First step, 0.1 M Tris buffer containing 4 M NaCl, pH = 9.2, temperature -14°C; second step, 0.1 M Tris buffer containing 50 % (v/v) ethylene glycol, pa_H = 9.2, temperature -14°C; third buffer, same as the second but temperature = 0°C, pa_H = 8.6; fourth buffer, same as the second but temperature = 20°C, pa_H = 8.0. (---), Absorbance at 280nm; (- - -), activity in Δ A . sec^{-1} measured at 410 nm (20°C) using succinyl-L-trialanine p-nitroanilide as substrate; (-.-.-), molarity of the fractions. Reproduced with permission from C. Balny et al. (1979), J. Chromatogr. 168 (1979) 133-138.

isolation of T4 ribonucleotide reductase (Berlund and Eckstein, 1974). For the isolation of ADP glucosopyrophosphorylase from *Escherichia coli* Haugen et al. (1974) used bioaffinity chromatography on Sepharose with attached P^1-(6-phospho-1-hexyl)-P^2-(6-amino- 1-hexyl)pyrophosphate, which resembles the allosteric activator. Van Etten and Waheed (1980) used subunit bioaffinity chromatography for the isolation of aryl sulphatase; Chiancone and Gattoni (1986) used it as a tool for the purification of oligomeric and self-associating protein systems. Bacitracin is a family of branched antibiotic cyclopeptides exhibiting high affinity for proteases. Bacitracin-cellulose prepared with 2,4,6-trichlorotriazine was an efficient bioaffinity adsorbent for the preparation of highly active alkaline proteinase from the culture medium of *Bacillus subtilis* (Turková et al., 1987). Its flow properties, stability, and the possibility of repeated use make it a promising adsorbent for large-scale operations. Bacitracin coupled to epoxy silica Sorbsil C200 allowed a direct, one-step purification of homogeneous neutral proteinase from the culture supernatant of *Bacillus subtilis* containing the gene for neutral proteinase (Van Den Burg et al., 1989). High flow rates can be used, under conditions in which self-digestion is prevented. The bioaffinity sorbent has to be stable for repetitive use over long periods, without significant loss of capacity.

Benzeneboronic acid, a transition-state analogue for serine proteinases, binds to the catalytic center of subtilisin BPN' (Nakatani et al., 1978). Akparov and Stepanov (1978) attached p-(ω-amino-ethyl)phenylboronic acid to CH-Sepharose in the presence of water-soluble carbodiimide. This sorbent was shown to be suitable for the purification of subtilisin, α-chymotrypsin and trypsin.

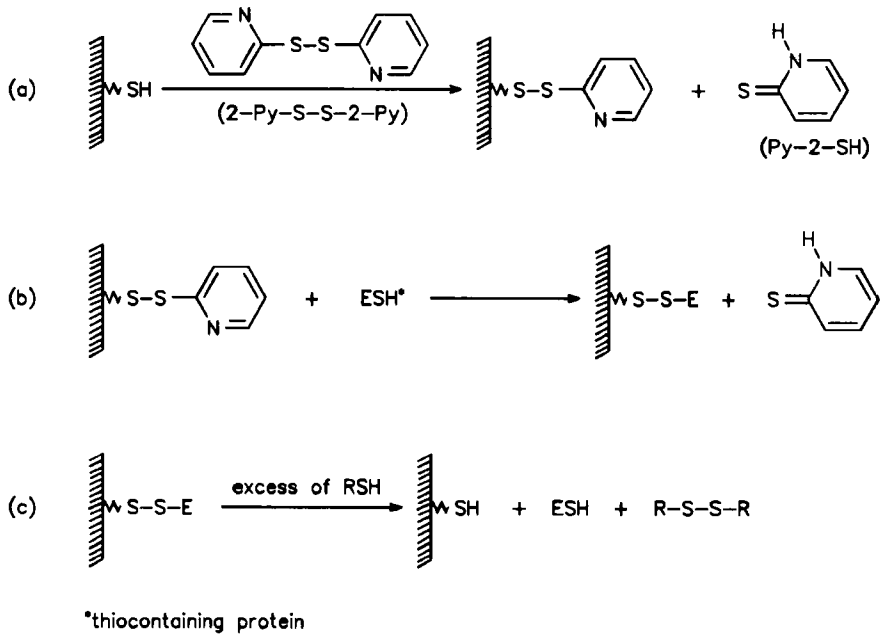
Polyacrylamide-boronate beads saturated with biomolecules were characterized by Maestas et al. (1980), as new general supports for the bioaffinity chromatography of enzymes. They coupled aminoethyl polyacrylamide beads to m-aminobenzeneboronic acid to obtain a product containing 1.3 mmoles of boronic acid per gram (dry weight) of gel. At pH 8.45 a number of enzyme substrates and cofactors including NAD^+, citric acid, pyridoxal, and epinephrine, were shown to bind to P-150-boronate beads packed in chromatography columns. At saturation, the P-150-boronate beads bound 2.5 to 80 μmoles of substrate or cofactor per 1 ml of wet packed gel. A P-150-boronate column

satured with uridine 5'-triphosphate was used to achieve a 1000-fold purification of the enzyme uridine 5'-diphosphate glucose pyrophosphorylase from the slime mold, *Physarum polycephalum*. Bouriotis et al. (1981) compared several different aminophenylboronic acid (APBA)-agarose matrices. Nucleotides are able to bind to these matrices in the presence of either NaCl or $MgCl_2$. NAD^+ and FAD are more tightly bound to these columns as compared to other nucleotides. The properties of NAD^+ and $NADP^+$ complexes with immobilized phenylboronic acids have been compared using pure 6-phosphogluconate and alcohol dehydrogenases. The $NADP^+$-dependent enzyme bound to the $NADP^+$ complex. Conversely the NAD^+-dependent enzyme was only retarded by the NAD^+-immobilized APBA complex. Purification factors of 14-fold were achieved for yeast glucose-6-phosphate dehydrogenase by choosing the appropriate concentration of presaturating $NADP^+$. The enzyme was selectively retarded and did not require specific elution. Bergold and Scouten (1983) reviewed the application of boronate affinity chromatography to the isolation and determination of biological substances in addition to other enzymes, peptides and glycoproteins.

The use of immobilized antibodies for the isolation of enzymes or their effectors is discussed in Section 3.3. The interaction of immobilized lectins with enzymes or their inhibitors containing carbohydrate moieties is the subject of Section 3.4. The description of possible advantages of the formation of a biospecific complex between avidin and biotin or other labelled compounds for the isolation or the determination of modified enzymes is given in Section 3.8.

For the isolation of proteins and peptides containing free SH groups it is advantageous to make use of the high affinity of mercapto compounds for heavy metal ions, mainly mercury. As an example one may mention the isolation of SH-protease from a crude extract of beans carried out on a column of hydroxyalkyl methacrylate gel containing a mercury derivative of methacrylanilide (Turková et al., 1975). SH-protease having an optimal proteolytic activity at pH 8 could be isolated in this way by a single chromatographic run from a mixture of proteolytic enzymes. For the isolation of papain, bromelain, chymopapain, ficin, propapain, creatine phosphokinase and phosphofructokinase, Brocklehurst et al. (1974) employed a polymer with 2,2'-dipyridyl disulphide.

The isolation of protein (ESH) containing a thiol group takes place according to the following scheme:



A covalent bond is formed between the isolated protein and the solid support, which is then split with an excess of low molecular weight thiol (RSH) after the unretained material has been washed out. In view of the formation of the covalent bond, this type of chromatography is called "covalent chromatography".

For purification of enzymes belonging to phosphoproteins and calcium-binding proteins the immobilized metal ion affinity (IMA) gels may be converted to biospecific adsorbents (Porath, 1988). Affinity ligands for the isolation of many enzymes are given in Table 9.1,2, and 3.

3.2.6 Use of immobilized enzymes

Bound enzymes are most commonly used as affinants for the isolation of inhibitors and cofactors. Fig. 3.13 shows the isolation of chymotryptic inhibitor from the crude extract of potatoes on Spheron with attached chymotrypsin (Fig. 3.13 B) in comparison

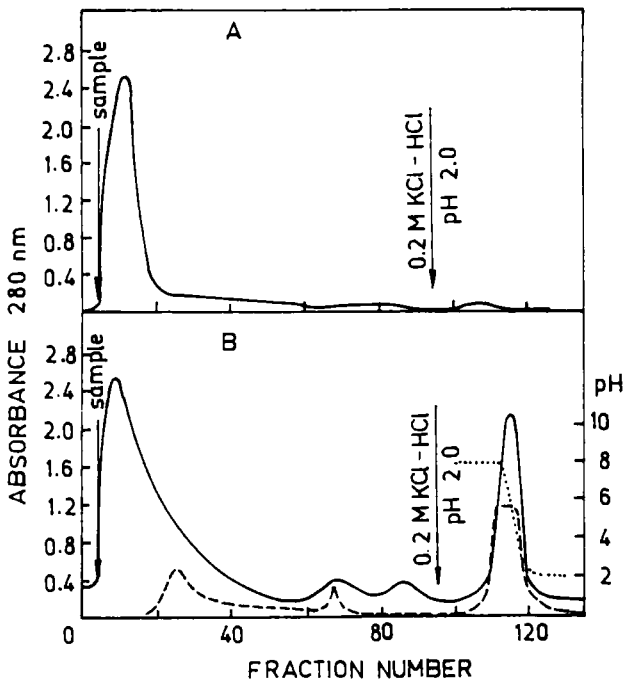


Fig. 3.13. Chromatography of a crude extract of potatoes on (A) Spheron 300 and (B) Spheron 300 chymotrypsin columns (20 x 18 mm). A 3-g amount of crude extract of potatoes was applied to the column and 10-ml fractions were collected at 1-h intervals. ---, Absorbance at 280 nm; - - -, inhibitor activity; ....., pH value. Vertical arrow, elution buffer changes from pH 8.0 (0.2 M Tris-HCl buffer) to pH 2.0 (0.2 M KCl-HCl). Data from J. Turková et al., Biochim. Biophys. Acta, 322 (1973) 1-9.

with analogous chromatography on unmodified Spheron (Fig. 3.13 A). The latter chromatography was carried out in order to check whether, under the given experimental conditions, elution of the material non-specifically sorbed to a solid support takes place or not(Turková et al., 1973).

Thelohan et al. (1989) demonstrated, that trypsin which has been immobilized on a silica support can be used as an HPLC chromatographic stationary phase for the stereochemical resolution of O- and N,O-derivatized amino acids. These chiral separations are possible due to the enzymatic activity of the support. This support offers a number of other possible applications based on the activity of the enzyme. These include the production of enantiomerically pure amino derivatives, as well as peptide mapping. Kito et al. (1990) developed a selective and sensitive assay of inosine, guanosine,

hypoxanthine, guanine and xanthine by high-performance liquid chromatography with immobilized enzyme reactors. Enzymes that catalysed the conversion of purine compounds were co-immobilized on aminopropyl controlled-pore glass packed in stainless steel tubing. The detection limits were 30-200 pg per injection.

3.3 ANTIBODIES AND ANTIGENS

A refinement of the technique has been the development of immunoaffinity chromatography, which adds the selectivity and specificity of immunological reactions to the separation process. Antigens or antibodies are increasingly used as tools to separate, determine or isolate complementary immunosubstances.

Antibodies induced by a certain antigen or haptens (chemically modified groups which are used as immunoagents after their attachment on proteins or synthetic polypeptides, for example) are characterized by a considerable degree of heterogeneity. When simple chemically defined haptens are used, the reasons for this heterogeneity may be the following (Lowe and Dean, 1974):

(1) The haptens may be attached to various parts of the carrier molecule, and consequently they are surrounded by a different micro-environment. This can be partly circumvented by using carrier proteins containing a single amino acid capable of a binding reaction, or by employing a synthetic polypeptide containing a single type of amino acid.

(2) The hapten can be oriented in various ways with respect to the surface of the antigen molecule.

(3) The antibodies may be directed against various parts of the hapten molecule. With antibodies against proteins the situation is much more complex because the protein contains various antigenic groups that are far less well-defined than in the case of simple haptens. Furthermore, serum contains several classes of proteins with antibody activity, such as IgG, IgM and IgA immunoglobulins.

The heterogeneity of the antibody binding sites results in a spectrum of dissociation constants for the antigen-antibody combinations (10^{-7} - 10^{-11} M; Bayer and Wilchek,

1990). By binding of antigen to a solid support, a specific immunoadsorbent is formed, which should possess the following properties:

(1) It should to be able to adsorb the complementary antibody from a mixture of components.

(2) The liberation of the adsorbed antibody from the specific adsorbent should be quantitative and should be carried out under conditions which are harmless for the specific antibody activity.

(3) It should possess a high capacity for the adsorption of the specific antibody.

(4) It should retain its biological activity after repeated use and storage.

(5) It should possess adequate mechanical properties, permitting centrifugation, filtration and use in a column.

The fulfilment of these requirements is not dependent on the quality and the amount of the bound antigen only, but also on the nature of the solid support and the nature of the bond.

Immobilized antibodies are used, by contrast, for the isolation of antigens. Antigens may belong to the most varied types of substances and therefore Tables 9. 1-3 present antibodies as affinants for the isolation of many different compounds.

Both polyclonal and monoclonal antibodies are currently prepared. The term "polyclonal antibody" is defined as the total population of antibodies present in an animal serum. Each antibody represents the secretory product from a single stimulated lymphocyte and its clonal progeny. A complex antigen such as a protein, glycolipid, etc., may contain many distinct antigenic determinants or epitopes, each of which is specifically recognized by antibodies from a single lymphocyte clone. These epitopes may be composed of amino acid sequences, conformational determinants, or molecular structures derived from post-translational modification (e.g. glycosylation, phosphorylation, or acetylation). The preparation of polyclonal antibodies, including the advantages and disadvantages of these antibodies, was described by Dunbar and Schwoebel (1990). The preparation of monoclonal antibodies, with the characterization of their advantages and disadvantages (e.g. procedure is expensive and time consuming), was described by Dunbar and Skinner (1990). The report by Kohler and Milstein

(1975) that a cell hybrid made by fusing normal spleen cells with malignantly transformed antibody-secreting cells (plasmacytoma or myeloma cell line) can provide a continuous source of antibody of predefined specificity has led to an explosion in the use of "monoclonal antibodies". Since a monoclonal antibody recognizes only one epitope on an antigen, the binding of an antigen to such a column should, in principle, be weaker than to one made with polyclonal antibodies, and the antigen should readily be eluted in the active form. Ackermans et al. (1985) presented a great advantage of monoclonal antibody immunoaffinity chromatography by a simple procedure for the purification of a saliva - interacting cell-wall protein from extracts of *Streptococcus mutans*, in which this receptor represents only a very small percentage of the total protein. The use of both monoclonal and polyclonal antibodies for oriented immobilization of enzymes is given in Chapter 10.

The principle of the antigen-antibody immunoglobulin G interaction is illustrated in Fig. 3.14. The dimeric antibody is divided into so-called Fc fragments or constant regions and the antigen binding F(ab) fragments termed "variable regions". The antigen or

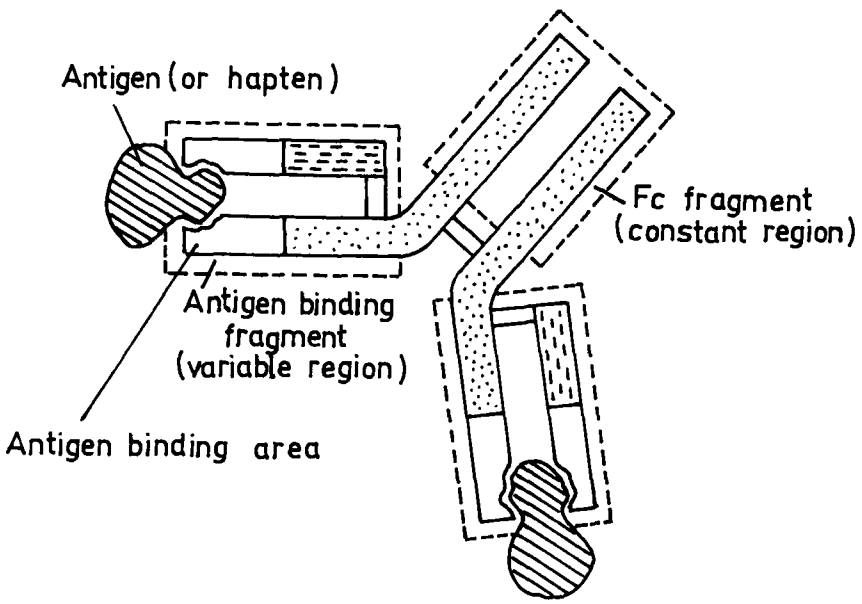


Fig. 3.14. Antigen-antibody interaction. Reproduced with permission from P. Mohr and K. Pommering. "Affinity Chromatography, Practical and Theoretical Aspects", Marcel Dekker, Inc., New York and Basel 1985, p. 132.

hapten conjugate binding areas are arranged on the top of the variable regions as has been shown by means of X-ray diffraction studies (Davies et al., 1975). It is an essential feature that these areas vary from antibody to antibody in their amino acid topography, and consequently only the complementary antigens can form real immunocomplexes.

The intermolecular forces that attract antibody and antigen to each other result in an attractive force that is capable of holding the complex together. These forces are van der Waals, Coulombic, hydrophobic and hydrogen bonding, all of which are relatively weak forces when acting by themselves. When two interacting molecules come into extremely close contact, the sum of their actions results in a relatively strong binding force. A diagrammatic presentation of these four forces is given in Fig. 3.15.

Protein A, which is a coat protein extracted from the bacterium *Staphylococcus aureus*, has the unique capacity to bind mammalian immunoglobulins, especially IgG. Table 3.3 published by Phillips (1989) describes the binding of different antibodies to Protein A. Protein A interacts with immunoglobulins *via* 2 non-immune mechanisms, the classical Fc-mediated binding and alternative F(ab)-mediated binding. Nilsson et al. (1983) showed that the mode of covalent linkage of Protein A to a solid matrix may influence the availability and nature of the binding sites expressing these two reactivities. Protein A-Sepharose CL4B showed higher reactivity with rat IgG1 and IgG2 than Staphylococci, whereas Protein A - Sepharose 6MB showed a lower uptake of these subclasses.

More than 20 papers about the isolation of IgG classes and subclasses using chromatography on Protein A - Sepharose were reviewed by Langone (1982). Bloom et al. (1989) characterized Protein A chromatography as an excellent technique for the purification of monoclonal antibodies to homogeneity due to its simplicity and high degree of antibody specificity. However, if the purified antibodies are manufactured for therapeutic use, a major safety concern is the possible presence of solubilized Protein A. Bloom et al. (1989) described the detection and reduction of Protein A contamination.

The cross-linkage of a specific complex for oriented immobilization was used first in 1978 when Sepharose with covalently attached Protein A was employed for oriented

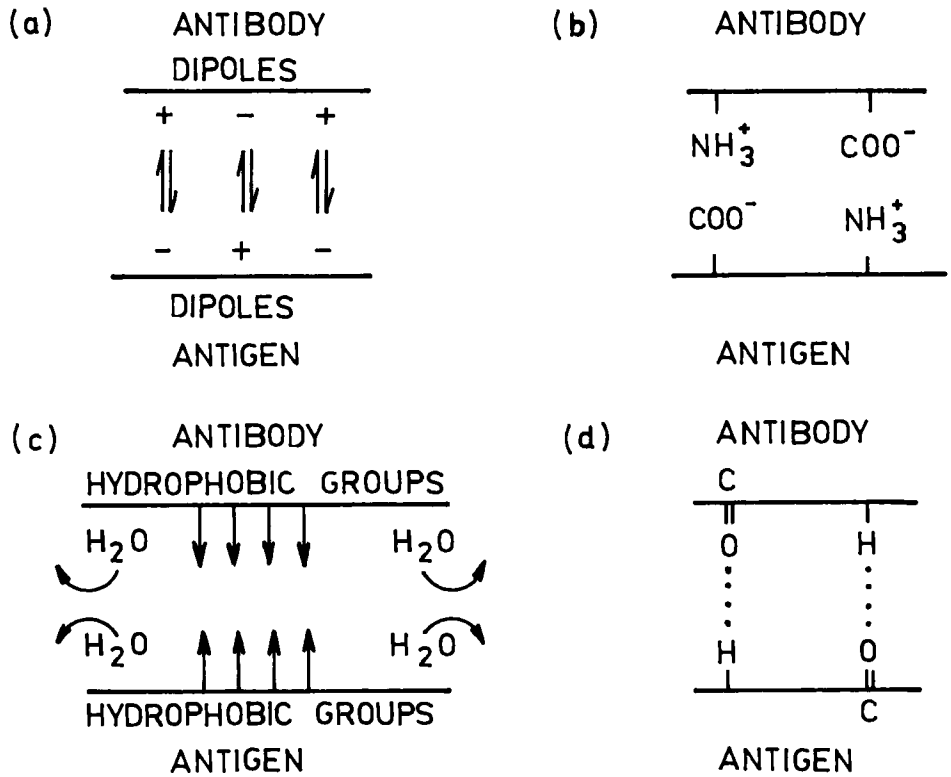


Fig. 3.15. The types of bonds involved in the formation of antibody/antigen complexes. a: Van der Waals forces. The attractive force is generated by the oscillating dipoles, which hold the two molecules together by charge attraction. b: The second strongest force is the Coulombic one, which generates attractive forces between ionized amino and one carboxyl groups of amino acid side chains. c: Hydrophobic bonding arises from the expulsion of water from between the two molecules, which brings hydrophobic groups into close proximity. d: Hydrogen bonding is the weakest of the attractive forces and is formed when two hydrophilic groups come into close contact. Reproduced with permission from T. M. Phillips, "The Use of HPLC in Receptor Biochemistry", Alan R. Liss, Inc., 1989, pp. 129-154.

immobilization of immunoglobulin G (cf. Fig. 3.16). Werner and Machleidt (1978) used N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride for the binding of IgG to immobilized Protein A. Gersten and Marchalonis (1978) employed dimethylsuberamide as a cross-linker for covalent binding of the same proteins. The binding of the IgG molecules through their Fc part was thus effected. This mode of binding makes the binding site of the antibody well accessible for interaction with the antigen. The affinity constant of the complex was high but the multiple use of immunoadsorbents under the

Table 3.3.

Binding of Different Antibodies to Protein A

Species	*Subclasess*	*Binding to Protein A*	*Species*	*Subclasess*	*Binding to Protein A*
Human	IgG1	Strong binding	Rat	IgG	Nonbinding
	IgG2	Strong binding		IgM	Nonbinding
	IgG3	Weak or nonbinding	Rabbit	IgG	Strong binding
	IgG4	Strong binding		IgM	Nonbinding
	IgA	Nonbinding	Sheep	IgG1	Nonbinding
	IgM	Nonbinding		IgG2	Strong binding
Mouse	IgG1	Weak or nonbinding		IgM	Nonbinding
	IgG2a	Strong binding	Goat	IgG1	Weak or nonbinding
	IgG2b	Strong binding		IgG2	Weak or nonbinding
	IgG3	Strong binding		Igm	Nonbinding
	IgM	Nonbinding			

conditions necessary for the elution of most antigens requires the cross-linkage of IgG with Protein A by a covalent bond.

When McLennan and Raney (1983) used dimethyl suberimidate as a crosslinker for anti-N^6-(Δ^2-isopentenyl) adenosine to Protein A-agarose, less than 30% of the protein was immobilized. A very effective crosslinking agent was iminothiolane, and immobilized antibody attached to agarose through Protein A had a much higher tRNA binding capacity than antibody attached directly to Sepharose. Crosslinking of the Protein A - antibody complex with glutaraldehyde, as used by Gyka et al. (1983), stabilized the antibody attachment.

The diagrammatic representation of the preparation of F(ab) and Fc fragments of IgG is shown in Fig. 3.17.

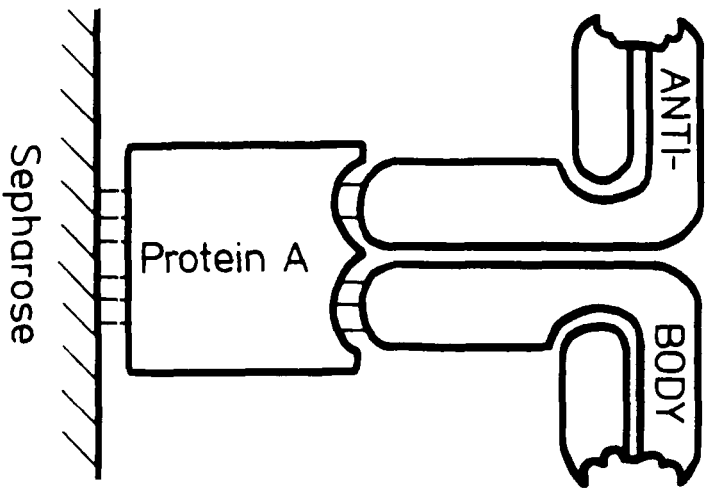


Fig. 3.16. Schematic drawing of oriented immobilization of immunoglobulin G by use of covalent crosslinking to staphylococcal Protein A attached to Sepharose. Covalent bonds are shown as full lines.

Two methods for synthesizing high-capacity immunoaffinity sorbents on Sepharose and Separon HEMA E-1000 were described by Prisyazhnoy et al. (1988). The first one was the oriented immobilization of monovalent immunoglobulin Fab fragments prepared by pepsin digestion *via* the formation of a covalent bond between the SH group of the Fab fragment at the C-terminus of the molecule and the maleimide covalently coupled to Sepharose. The second method was based on the oxidation of the immu-

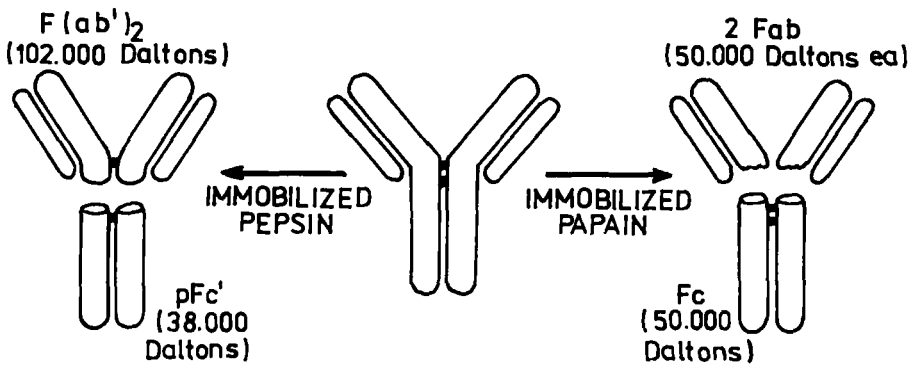


Fig. 3.17. Diagrammatic representations of the preparation of F(ab) and Fc fragments of IgG.

noglobulin carbohydrate component, located in the Fc fragment, by periodate with subsequent immobilization of the derivatives on hydrazide derivatives of Sepharose or Separon. Sorbents were obtained for the isolation of monoclonal antibodies from the culture supernatants and the elongation factor EF-G from a crude extract of *Escherichia coli* cells. These sorbents were characterized by a high capacity, minimal non-specific sorption and high stability. Site - specific immobilization of antibodies by their oligosaccharide moieties to new hydrazide derivatized solid supports was also described by O'Shannesy and Hoffman (1987) and Hoffman and O'Shannesy (1988).

In order to determine whether immobilization chemistry can be used to orient antibody on a support so that the bivalent binding potential can be fully utilized, Domen et al. (1990) developed three activated matrices that couple to different functional groups on the molecule. When AminoLink Gel was used to couple antibody randomly through primary amino groups, the molar ratio of immobilized antibody to recovered antigen averaged 1:1. Iodoacetyl groups on SulfoLink Gel couple through sulphydryls in the hinge region of the antibody molecule (after its cleavage with pepsin and treatment with 2-mercaptoethylamine) in theory leaving the antigen binding site still available. In comparison with the results of Prisyazhnoy et al. (1988) the antibody-to-antigen molar ratio was only slightly improved. But when antibody was attached by use of aldehyde groups generated by oxidation of its carbohydrate moieties to CarboLink Gel containing hydrazide groups, the molar ratio of immobilized antibody to purified antigen reached the optimum of 1:2. Parekh et al. (1985) published the refined structure at 2.8 Å of the rabbit Fc fragment of IgG based on crystal data. The illustration of the Fc fragment in their paper contained the two carbohydrate chains, each attached in conserved glycosylation sites at Asn 297. Part of their figure described the conformation and sequence of carbohydrate chains with N-acetylneuraminic acid and galactose as terminal sugars. Solomon et al. (1990) used concomitant treatment of the monoclonal antibodies against carboxypeptidase A or horseradish peroxidase with neuraminidase and galactose oxidase which generated aldehyde groups in the oligosaccharide moieties of immunoglobulins. Subsequent immobilization of neuraminidase and galactose oxidase on Eupergit C-adipic dihydrazide proved to be an efficient and selective system for the

enzymic oxidation of the monoclonal antibodies without any impairment of their immunological activity. Oriented immobilization of enzymically oxidized monoclonal antibodies on hydrazide or amino Eupergit C derivatives led to the formation of antibody matrix conjugates which possessed high antigen-binding activities. Oriented immobilization by the use of carbohydrate moieties is described in more details in Chapters 4 and 6.

Fig 3.18 shows the urokinase enzymatic activity of unbound and eluted fractions from bioaffinity colums prepared by Hérion and Bollen (1983) by the attachment of five different monoclonal antibodies against urokinase to activated agarose Affi-Gel 10.

All the active material present in the lyophilized crude preparation of urokinase derived from foamed urine was adsorbed to the column of immobilized AAU2 monoclonal antibody and was recovered at more than 100% after acid elution.

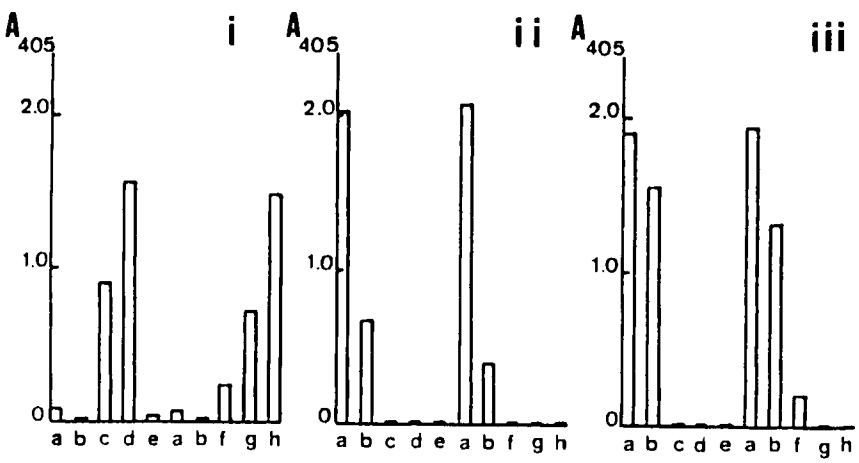


Fig. 3.18. Urokinase enzymatic activity of the unbound and eluted fractions from affinity columns prepared with five different monoclonal antibodies. The assays were performed with the chromogenic substrate S2444 (Kabi) and activities are expressed in A_{405} enzymatic units.
a, unbound fraction; b, wash with 0.5 M NaCl, 0.1% Tween 80; c, elution with acetate buffer pH 4; d, elution with acetate buffer pH 3; e, elution with acetate buffer pH 2.5; f, elution with 1 M KSCN; g, elution with 2 M KSCN; h, elution with 3 M KSCN. i, antibody AAU2; ii, antibodies AAU5 and AAU12; iii, antibodies AAU17 and AAU18. Reproduced with permission from P. Hérion and A. Bollen, Bioscience Reports, 3 (1983) 373-379.

Recently, high performance immunoaffinity chromatography (HPIAC) has been applied much more widely (Phillips 1989). Protein A-coated glass beads were developed as a universal support medium for HPIAC by Phillips et al. (1985). The preparation and mechanism of IgG antibody binding is demonstrated in Fig. 3.19. Protein A has the ability to bind to the Fc, or tail portion of IgG antibodies. Protein A is composed of five subunits, each with its own Fc receptor, but three of these receptors become inactive when the molecule is immobilized. If Protein A is applied as a coating to either solid or controlled pore glass beads, its Fc receptors become points of attachment on which the antibody can be immobilized. The bound antibodies are then covalently immobilized on the Protein A coat by crosslinking them with carbodiimide. In turn this attachment helps to orient the antigen receptors of the antibody toward the mobile phase of the column. In addition, the linear nature of the Protein A molecule becomes a spacer arm for the immobilized antibodies, thus preventing charges, which can rise from the bead surface,

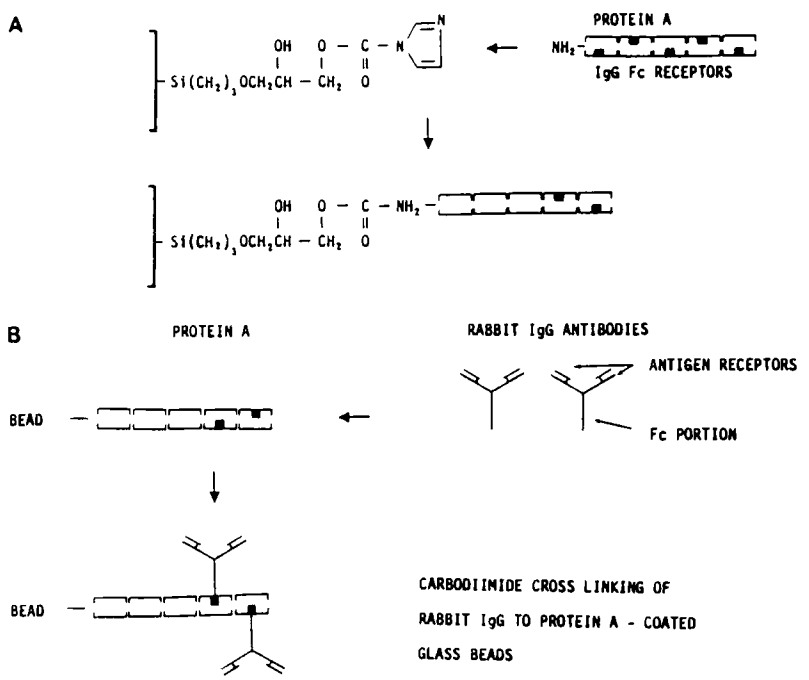


Fig. 3.19. Diagrammatic representation of the process for binding rabbit antibodies to Protein A-coated glass beads. (A) Illustration of the chemistry used to couple Protein A to the carbonyl diimidazole-derivatized glass beads. (B) Mechanism of IgG antibody binding to the Fc receptors on the Protein A-coated glass beads. Reproduced with permission from T. M. Phillips et al., J. Chromatogr., 327 (1985) 213-219.

inhibiting the formation of the antibody/antigen complex. However, disadvantages are that only certain classes of immunoglobulin are able to bind to Protein A. This problem was overcome by the development of avidin or streptavidin coated glass beads (Babashak and Phillips, 1988) and the use of an avidin-biotin system after biotinylation of carbohydrate moieties of antibodies with biotin hydrazine. Avidin-biotin system will be more discussed in Section 3.8.

Antibodies against carbohydrates were used by Dakour et al. (1986). They coupled anti-blood group A monoclonal antibody to beads of Sepharose or Concanavalin A - Sepharose for the separation and partial sequence analysis of blood group A-active oligosaccharides.

3.4 LECTINS, GLYCOPROTEINS AND SACCHARIDES

3.4.1 Importance of carbohydrate analysis

Many of the products prepared by biotechnological approaches, including recombinant genetic engineering, cell tissue culture, and monoclonal technologies, are glycoproteins. Recent large scale testing of glycoprotein-based pharmaceuticals has indicated that both the extent and type of glycosylation can play a central role in glycoprotein activity (Lee et al., 1990). The biosynthesis of glycoproteins occurs within the internal membrane systems of cells, the endoplasmic reticulum, and the Golgi apparatus. Glycoprotein oligosaccharide units are assembled and modified as the molecules move through succesive subcellular compartments en route to destinations outside the cell, as membrane components of the cell surface, or as components of membranes or contents of cellular organelles. The biological roles of carbohydrate units include facilitation of the secretion of certain proteins or their mobilization to the cell surface, induction and maintenance of the protein conformation in a biologically-active form, clearance of glycoproteins from plasma, their importance as antigenic determinants in differentiation and development, direction of the immune response and action as immune decoys and protection of peptide chains against proteolytic attack (Lee et al., 1990). Glycosylation also appears to affect the half-lives of certain proteins (Gerard, 1990). Multiple forms of carboxypeptidase Y isolated from various kinds of yeast

(Turková et al., 1988) which differed considerably in molecular weight (49 000 - 65 000) had identical amino acid composition of their acid hydrolysates. The differences in the carboxypeptidases Y isolated are due to differences in the content and the composition of their carbohydrate moieties. The stabilization of the structure and of the activity of carboxypeptidase Y by oligosaccharide chains has been determined by Chu and Maley (1982).

Enriched localization of glycoproteins and glycolipids in the surface membranes of cells has been demonstrated e.g. by reactions of cell surfaces with plant agglutinin or with anti-glycolipid antibodies. A significant role of complex carbohydrates on cell surfaces in controlling cell division and intercellular association has been predicted by Gahmberg and Hakamori (1973).

Glycoproteins are proteins to which carbohydrate moieties are covalently linked through glycosidic bonds. The linkages between the oligosaccharide and the protein are either N-glycosidic (carbohydrate linked to the amido nitrogen of asparagine) or O-glycosidic (carbohydrate linked to the hydroxyl oxygen of L-serine, L-threonine, and occasionally 5-hydroxy-L-lysine or 4-hydroxy-L-proline). Three major types of asparagine-linked oligosaccharides have been determined as high-mannose type or simple type, complex type, and hybrid type (Lee et al., 1990). Lectins are most important bioaffinity ligands for the specific and reversible interactions with carbohydrate groups or sequences. The dissociation constants of lectins with carbohydrate complexes lie in range 10^{-3} - 10^{-6} M (Bayer and Wilchek, 1990).

3.4.2 Lectins, isolation and application

Lectins are proteins or glycoproteins from plants, invertebrates, and bacteria. They form complexes with sugar residues of glucosides, oligo- and polysaccharides, glycoproteins, glycolipids, membrane proteins, enzyme-antibody and glycoprotein conjugates, as well as viruses, cell fragments and cells. Hydrogen bonding and electrostatic interactions are fundamentally involved in complex formation. Furthermore, the metal ions Ca^{2+} and Mn^{2+} are essential constituents of the sugar binding sites and are also essential for the tertiary structure.

Most known lectins are multimeric, consisting of noncovalently associated subunits, which are either identical (e.g. Concanavalin A) or different (e.g. *Ulex europeus* agglutinin). This multimeric structure is the reason for their ability to agglutinate cells or form precipitates with glycoconjugates (Mohr and Pommerening, 1985).

Bioaffinity chromatography for the purification of lectins was reviewed by Lis and Sharon (1981). They described three major types of biospecific adsorbents for the purification of lectins: (1) polysaccharides, either native or modified; (2) matrix- bound glycoproteins and glycopeptides; (3) matrix-bound monosaccharides and disaccharides. Fig. 3.20 shows four types of monosaccharide derivatives used for the synthesis of Sepharose-carbohydrate conjugates.

Fig. 3.21 presents the biospecific affinity chromatography of phytoagglutinins from the crude extract of sun hemp (*Crotolaria juncea*) seeds on ECD-Sepharose 3 h treated with 0.2 M hydrochloric acid at 50°C (Ersson et al., 1973). Galactan chains were hydrolysed without complete degradation of the gel matrix. This method increased the number of end galactosyl groups necessary for specific adsorption of hemagglutinins. The importance of this hydrolysis is most evident from a comparison with the chromatography of the same extract, under analogous conditions, on ECD-Sepharose which was not treated with acid (Fig. 3.21 A).

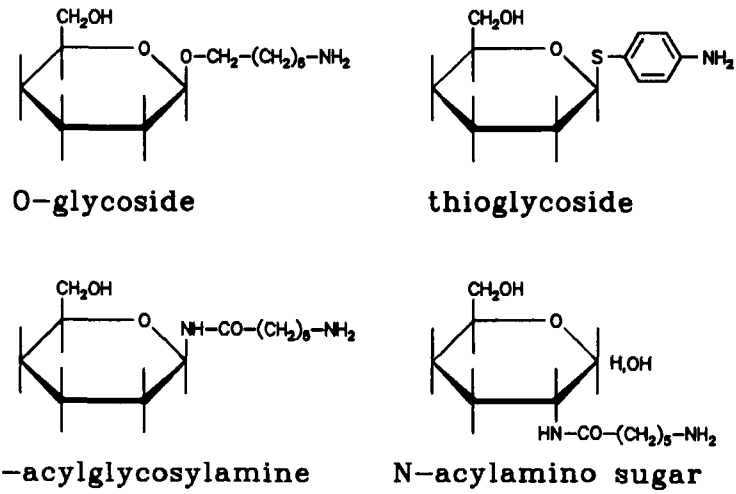


Fig. 3.20. Monosaccharide derivatives used for the synthesis of Sepharose-carbohydrate conjugates.

The isolation of wheat germ agglutinin (Lis et al., 1974) on Sepharose with covalently attached 2-acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine is an example of the use of a specific adsorbent prepared by covalent attachment of a monosaccharide on an insoluble carrier. Hořejší and Kocourek (1974) prepared a series of specific sorbents for the isolation of phytohemagglutinins from various sources by copolymerization of alkenyl-O-glycosides with acrylamide and N',N'-methylenebisacrylamide. The hydrophilic gels thus obtained contain sugars bound by O-glyco-

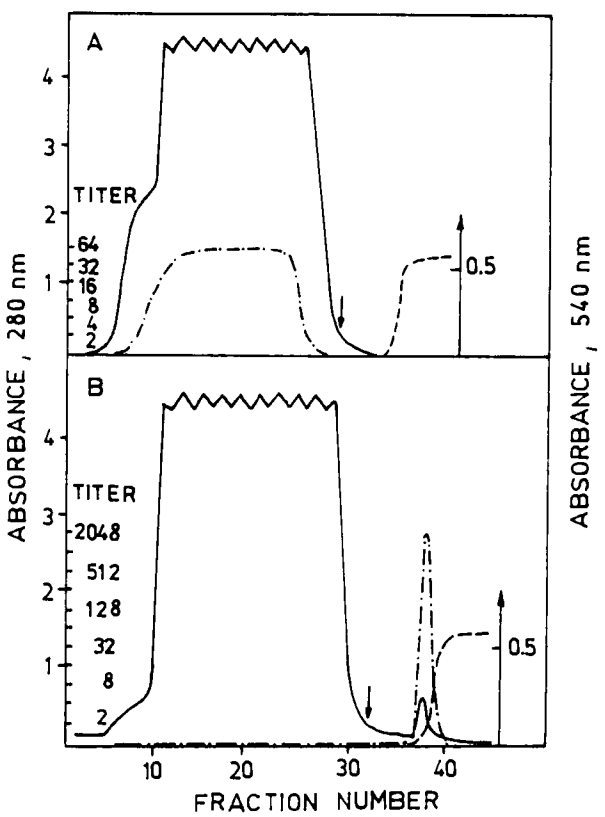


Fig. 3.21. Chromatography of clarified dialyzed crude extract on (A) untreated ECD-Sepharose 6B (prepared from Sepharose 6B by treatment with epichlorohydrin in alkaline medium, followed by alkaline hydrolysis), and (B) ECD-Sepharose 6B, treated with acid for 3 h (0.2 M HCl, 50°C). Columns, 100 x 19 mm; flow-rate, 15 ml/h; fractions, 2.5 ml. ---, A_{280nm}; -.-.-, haemagglutination titre; - - -, A_{540nm} (sugar concentration as determined by the orcinol method). Arrow indicates start of elution with buffer containing lactose. Reproduced with permission from B. Ersson et al., Biochim. Biophys. Acta, 310 (1973) 446-452.

sidic bonds to the alkyl side-chains of the matrix. Genaud et al. (1978) used yeast or stromas sediment polymerized with acrylamide for the bioaffinity chromatography of lectins from *Ricinus seeds*.

The isolation of glycoproteins by means of immobilized lectins makes use of their differing affinities for terminal carbohydrate residues characteristic of single glycoproteins. For the elaboration of a suitable procedure for the purification of the given glycoproteins or glycopeptides by means of lectins, Kristiansen (1974) recommended the following stages:

(1) identification of the terminal sugar or sugars in the carbohydrate part of the substance under consideration;

(2) selection of a lectin with a corresponding specificity;

(3) preparation of the selected lectin;

(4) immobilization of the lectin by a covalent bond to a solid support;

(5) choice of optimum conditions for the adsorption of the isolated substance on the immobilized lectin;

(6) choice of conditions for desorption.

It is possible to choose either non-specific elution, consisting mainly in a change in pH or salt concentration; or a specific method can be used, i.e. the displacement of the adsorbed glycoprotein by competing carbohydrates.

Assuming that the terminal sugar or sugars of glycoproteins have been determined, the choice of a suitable lectin can follow. In most instances lectins are not specific for one sugar only, although great differences exist in the degree of specificity. For example, lectin from the seeds of *Lotus tetragonolobus* has a narrow specificity for L-fucose, while concanavalin A (Con A) from *Canavalia ensiformis* has a broad specificity and binds most glycoproteins from human serum. In Table 3.4 the classification of lectins is given according to their main affinities. If not stated otherwise, plant seeds served as the source of lectin (Kristiansen, 1974).

The capability of mostly used lectins with reversible high-affinity binding to specific carbohydrate sequences published by Gerard (1990) is in Table 3.5.

Table 3.4.

Classification of lectins according to their main affinities

Group	*Specificity*	*Lectins*
I	L-Fucose	*Lotus tetragonolobus*, weakly inhibited by L-galactose *Ulex europaeus* (gorse); contains another lectin belonging to group VII *Ulex parviflorus*; weakly inhibited by other sugars
II	*N*-Acetyl-D-glucosamine	Group inhibited by *N*-acetylated chitodextrins *Triticum vulgare* (wheat germ); also inhibited by *N*-acetylneuraminic acid (NANA) *Solanum tuberosum* (potato tuber); also inhibited by muramic acid
III	*N*-Acetyl-D-galactosamine	*Dolichos biflorus* (horse gram) *Phaseolus lunatus* (lima bean; also called *P. limensis*) *Phaseolus vulgaris* (red kidney bean; black kidney bean, yellow wax bean; bean meal is source) *Vicia cracca*; also containing a non-specific lectin in group VIII *Euonymus europaeus* *Helix pomatia* (snail)
IV	D-Galactose	Group also inhibited by L-arabinose, D-fucose, lactose, raffinose, and melibiose *Crotalaria juncea* (sun hemp), β-specific *Ricinus communis* (castor bean) *Abrus precatorius* *Griffonia simplicifolia*
V	*N*-Acetyl-D-galactosamine and D-galactose	These lectins are inhibited almost equally by both sugars *Sophora japonica* (japanese pagoda tree) *Glycine max* (soybean), α-specific *Caragana arborescens* *Bandaeirea simplicifolia*, α-specific *Bauhinia variegata*, var. *candida* *Momordia charantia* *Erythrina subrosa* *Coronilla varia*, α-specific *Crotalaria zanzibarica* *Arachis hypogea*, β-specific

Table 3.4 (*continued*)

Group	*Specificity*	*Lectins*
VI	D-Glucose	*Sesamum indicum* *Pisum sativum* (garden pea); inhibited about four times better by D-mannose
VII	β-Glycosides and β-*N*-acetylglucosaminides	Group inhibited most trongly by *N,N'*-diacetylchitobiose, but also by salicin [2-(hydroxymethyl)phenyl-β-D-glucopyranoside], phenyl-β-D-glucopyranoside and cellobiose *Ulex europaeus* (gorse); contains another lectin belonging to group I *Ulex galli* *Ulex nanus* *Cytisus sessilifolius;* inhibited also by lactose *Laburnum alpinum*; inhibited also by lactose *Clerodendrum viscosum*; pulp is source
VIII	Methyl-α-D-mannoside, D-mannose, D-glucose, *N*-acetyl-D-glucosamine, L-sorbose	Sugars listed in decreasing order of inhibition *Pisum sativum* (garden pea); also inhibited by D-glucose, but only about one quarter as efficiently *Lens culinaris* (common lentil) *Canavalia ensiformis* (jack bean; gives Con A); bean meal is source. *Vicia crassa*; also contains a lectin in group III *Lathyrus sativus* L.
IX	*N*-acetylneuraminic acid	*Limulus polyphemus* (haemolymph of horseshoe crab) *Triticum vulgaris* (wheat germ); also in group II

Separations of glycopeptides and oligosaccharides by the use of lectin bioaffinity chromatography were reviewed by Merkle and Cummings (1987). A rapid, sensitive and specific technique for fractionating cell-derived asparagine-linked oligosaccharides by serial chromatography on ConA-Sepharose, pea lectin-Sepharose and leukoagglutinating phytohemagglutinin-agarose was described by Cummings and Kornfeld (1982).

Codogno et al. (1985) determined, using ConA-Sepharose, WGA-Sepharose and agaroses with three other lectins, that N-linked oligosaccharides derived from cell surface glycoproteins of 8-day chick embryo fibroblasts differ from those of 16-day chick embryo fibroblasts. Yamashita et al. (1986) used five lectin-Sepharose columns (contai-

Table 3.5.

Lectins commonly used for glycoprotein purification

Lectin	Specificity	Comments
Concanavalin A (ConA)	Structures containing α-linked mannose such as the N-acetylglucosaminyl core disaccharide in Asn-linked glycoproteins	Broad use due to ubiquitous α-linked mannose containing structures. pH optimum 5.6. As with other mannose-binding lectins, Ca^{2+} and Mn^{2+} are part of the binding site. Buffers may be Ca^{2+}/Mn^{2+} free. Avoid chelators
Lentil lectin (LcH)	Similar to ConA, narrower specificity in that substitution to the core saccharide may enhance or diminish binding	Sometimes employed sequentially with ConA.
Peanut agglutinin (PA)	Recognizes Gal(β1-3)GalNAc	Common core structure to many O-linked membrane glycoproteins; however, often requires sialidase treatment to expose binding disaccharide
Jacalin	Recognizes Gal(β1-3)GalNAc	Unlike peanut lectin, this lectin will recognize substituted disaccharide, eliminating the need for sialidase treatment. Specific for O-linked oligosaccharides
Wheat germ agglutinin (WGA)	Recognizes N-acetylglucosamine dimer or trimer structures/chitobiose. Also lower affinity interactions with sialic acid, mannose-containing structures	Widely used for membranes glycoprotein purification, which frequently contain Asn-linked oligosaccharides
Ricinus communis agglutinin I (RCA I)	Reacts with terminal (nonreducing) Gal, less strongly with GalNAc.	May be useful with both N- and O-linked structures. Since these residues are often not terminal, but penultimate, treatment with exoglycosidases (neuraminidase, fucosidase) may uncover binding properties
Soybean agglutinin	Recognizes terminal α- or β-GalNAc	Complementary to RCA I lectin

ning ConA, WGA, RCA, LcH and AAL = *Aleuria aurantia* lectin) for the adsorption of ribonucleases from various human organs and body fluids (Table 3.6). The behaviour of ribonucleases extracted from human viscera showed that almost all contain carbohydrates and that there are organ-specific differences in the structure of these carbohydrates.

The large variation in retentivity of Con A and WGA toward the glycoproteins peroxidase, fetuin and asialofetuin, determined by Rassi et al. (1988) by use of HPLBAC, is shown in Fig. 3.22.

3.4.3 Antibodies against carbohydrate antigens

The increased availability of well characterized monoclonal antibodies prepared against specific oligosaccharide structures should make it possible to determine the structure of carbohydrate chains of unknow glycoproteins (Lee et al., 1990). The content of Feizi's review article (1985) dealt with the demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental

Table 3.6

Binding of ribonucleases from various human organs and body fluids to lectin-Sepharose columns

Source of RNases	*Immobilized lectin*				
	Con A	*AAL*	*WGA*	*RCA*	*LcH*
	(% of bound RNases)				
U_S-urine	4	82	42	0	7
U_L-urine	72	87	0	100	49
Pancreas	50	51	48	35	28
Lung	27	67	82	7	39
Leukocyte	0	88	100	11	4
Kidney	15	89	45	0	24
Liver	37	80	42	5	20
Spleen	24	84	78	0	38
Stomach	45	22	79	65	0
Semen	70	78	0	40	42

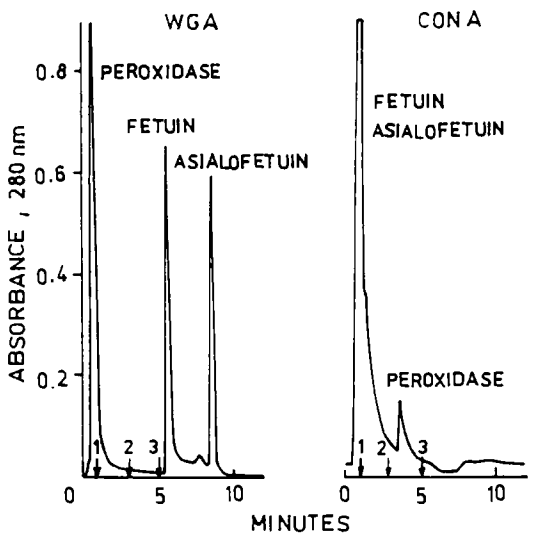


Fig. 3.22. Chromatogram of glycoproteins. Columns, 100 x 4.6 mm, WGA-silica (left panel) and Con A-silica (right panel); flow rate 1.5 ml/min; temp. 22°C. Stepwise elution: 1 min with 0.2 M NaCl in 25 mM Tris/HCl, pH 7.0; (1), 2 min with 0.5 M methyl-α-D-glucopyranoside in above eluent; (2), 2 min with 0.4 M N-acetyl-α-D-glucosamine in the first eluent; (3), 10 min with 0.1 M glycine/HCl, pH 3.0. Reproduced with permission from Z. E. Rassi et al., Makromol. Chem., Macromol. Symp., 17 (1988) 305-319.

antigens. The association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation patern of total serum IgG was described by Parekh et al. (1985). Immobilized antibodies against carbohydrate antigens used as biospecific adsorbents could become a fundamental analytical tool for future studies of the glyco-conjugates of whole cells and their receptors.

3.4.4 Boronic acid ligands

The interaction between borate and sugars has been known since the year 1842 (Bergold and Scouten, 1983). Boronates react readily and under mild conditions with a wide range of polar functional groups that are oriented in the proper geometry. Although complexes of 1,2-diols, 1,3-diols, and catechol-type compounds are the most used, a variety of other functional groups, such as 1,2-hydroxy acids and 1,2-hydro-xylamines, could also react with boronates to form complexes.

The specific interaction of boronic acid ligands with 1,2-cis-diol compounds resulted in bioaffinity chromatographic techniques for the separation of some glycoproteins. Abraham et al. (1983) investigated the use of m-aminophenylboronic acid immobilized on agarose as the affinity matrix for the separation and quantitation of the glycosylated hemoglobin (glyco Hb) in blood samples from newborn infants, normal and diabetic adults, and adults having certain hemoglobinopathies. Fig. 3.23 shows the separation of the glycosylated and nonglycosylated hemoglobins in the red cell hemolysates of a normal adult and a patient with insulin-dependent diabetes mellitus. Since the buffers used in the affinity methods were patented products, attempts were made to replace them with the following buffers prepared in our laboratory: 0.05M HEPES (N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid) buffer, adjusted to pH 8.5 and containing 10 mM $MgCl_2$ and 0.02 % sodium azide for the First Fraction Elution Buffer, 100 mM sorbitol for the Second Fraction Elution Buffer, and 0.1 M Tris HCl, pH 8.5 containing 0.5M NaCl, for the Regeneration Buffer. No significant differences in the results were obtained by replacing the commercial buffers with the home-made buffers.

3.5 NUCLEOTIDES AND NUCLEIC ACIDS

The ever-increasing use of genetic engineering is reflected in the rapidly increasing number of publications devoted to the isolation of nucleic acids, genes, oligonucleotides and nucleic acid fragments, to the purification of special proteins and enzymes, as well as to investigations of protein-nucleic acid interactions. Schott (1984) summarized this interesting field in his book "Affinity chromatography - Template chromatography of nucleic acids and proteins".

3.5.1 Oligonucleotides and polynucleotides

When oligonucleotides of a defined sequence are immobilized on cellulose, base pairing can take place with complementary oligonucleotides having either an homologous sequence or an alternating one according to the Watson and Crick theory. Those nucleotides undergoing such a base-pairing are adsorbed onto the cellulose and can thus be separated from any non-complementary partners present in the mixture (Schott,

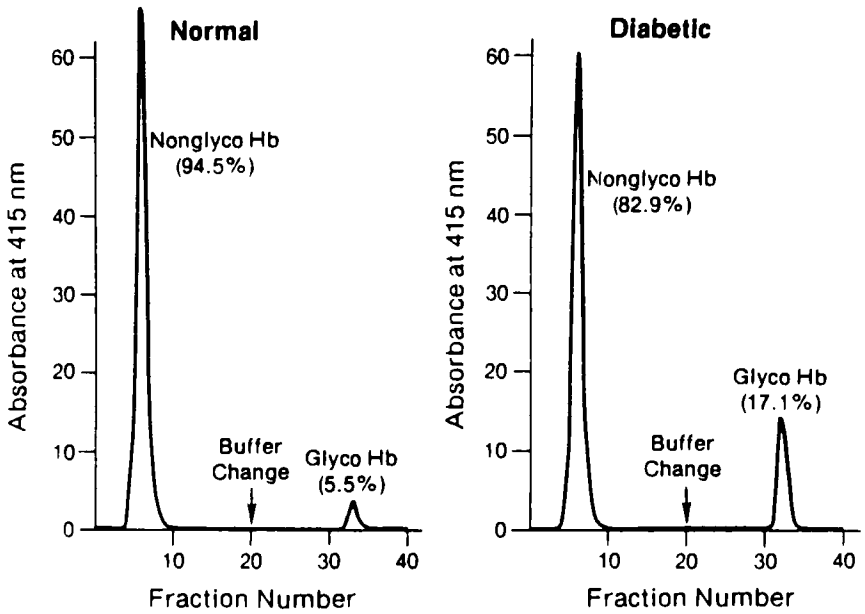


Fig. 3. 23. Bioaffinity chromatographic separation of glycosylated and nonglycosylated hemoglobins present in red cell hemolysates of a normal adult and a diabetic patient. Twenty-five milligrams of Hb were applied to a macrocolumn (1 by 10 cm) of phenylboronate-agarose and developed first with the First Fraction Elution Agent and then with the Second Fraction Elution Buffer and fractions of 1.5 ml were collected. Reproduced with permission from E. C. Abraham et al., J. Lab. Clin. Med., 102 (1983) 187-197.

1974). Schott et al. (1974) described selective adsorptions of complementary oligonucleotides in the mobile phase on the immobilized template if chromatography takes place under the conditions necessary for base pairing. Desorption was then carried out with a temperature gradient. In this paper the application of the principles of molecular biology for the selective separations of nucleotides and peptides was, for the first time, called template chromatography.

The chromatography of peptides on poly(vinyl alcohol) substituted with oligodeoxythymidylic acid and bound irreversibly on DEAE-cellulose by ionic bonds has been employed for the study of the interactions of peptides with nucleotides (Schott et al., 1975). The quantitative measure of the peptide-nucleotide interaction is the increase in the retention of a peptide on oligonucleotide-DEAE-cellulose in comparison with that on unmodified DEAE-cellulose. In order to eliminate possible effects of various column parameters, Schott et al. (1975) expressed all elution volumes relative to the

elution volume of alanine, which displays no measurable retention on these columns. The relative elution ratio (V_r) is thus obtained as the ratio of the elution volume found for the investigated peptide to that for alanine ($V_r = V_{obs}/V_{Ala}$). The peptide-oligonucleotide interaction is then evaluated on the basis of the difference in the relative elution volumes obtained by chromatography on both columns. Fig. 3.24 shows the separation of nucleic acid bases and nucleosides by high-performance bioaffinity chro-

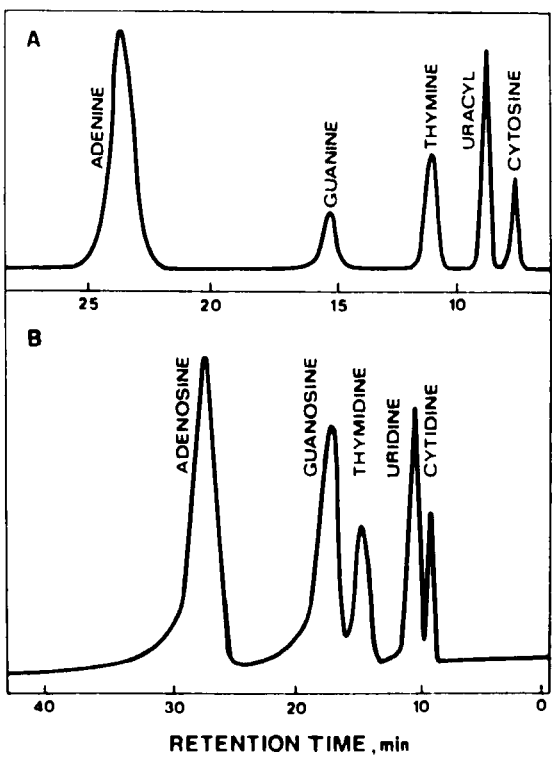


Fig. 3.24. (A) Chromatogram of a mixture of five nucleic acid bases obtained by using resins coupled with 8.0 % thymine (the porous spherical resins were synthesized by suspension polymerization of glycidyl methacrylate in the presence of diluent. The attachment of thymine to the glycidyl metacrylate in the resin so formed was conducted in dimethylformamide at 65°C in the presence of potassium carbonate). The chromatographic measurement was carried out in a stainless-steel column (610 x 7.6 mm) at 25°C on a high-performance liquid chromatograph using distilled water as solvent. The eluate from the column was monitored with a UV detector at 254 nm. The flow rate was 3.5 ml/min and the pressure drop was 45 kg/cm^2. (B) Chromatogram of a mixture of five nucleosides obtained by using resins coupled with 16.4 % thymine. Resin particle size, 15-20 μm; flow rate, 3.1 ml/min; temperature, 30°C. Reproduced with permission from Y. Kato et al., J. Chromatogr., 134 (1977) 204-206.

matography employing a column packed with porous spherical resins of diameter 12-15μm, coupled with thymine (Kato et al., 1977).

Many animal and viral messenger ribonucleic acids (mRNAs) are rich in polyadenylic acid. This was used in a number of instances (see Table 9.1) for isolations carried out by chromatography on supports containing oligothymidylic acid. In Fig. 3.25 the chromatography of RNA from duck reticulocytes on oligodeoxythymidylic acid-cellulose (Pemberton et al., 1975) is illustrated as an example. About 94-96 % of the RNA was eluted in peak 1 and contained ribosomal and transfer RNA. Peak 2 contained, in addition to ribosomal RNA, 10S globin messenger RNA. A very effective separation was demonstrated on the basis of the analysis of single peaks by centrifugation in sugar

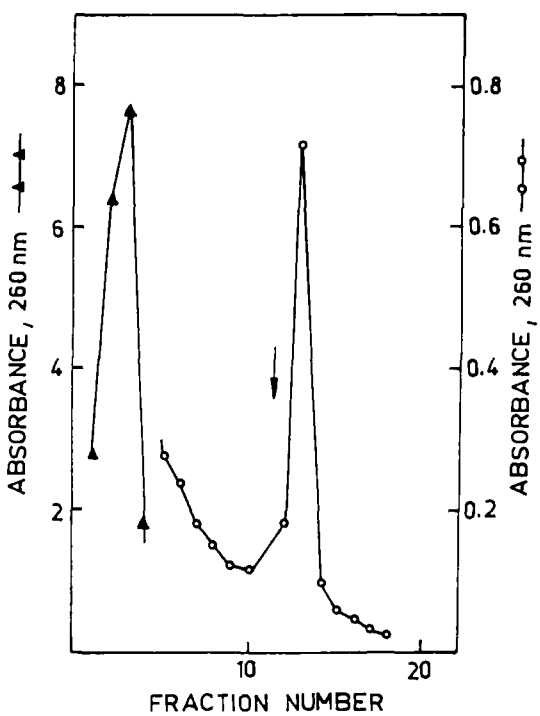


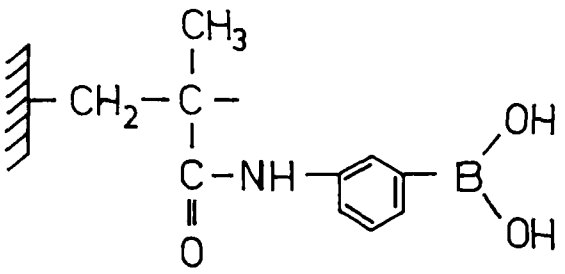
Fig. 3. 25. Chromatography of duck reticulocyte RNA on oligodeoxythymidylic acid-cellulose. The column (40 x 5 mm) was equilibrated with 0.5 M NaCl-0.5 % sodium dodecyl sulphate-0.01 M Tris buffer, pH 7.5 (starting buffer). The samples applied to the column were dissolved in water and then diluted with an equal volume of doubly-concentrated starting buffer. The column was washed with approximately 30 ml of starting buffer and then eluted with 0.5 % sodium dodecyl sulphate-0.01 M Tris buffer, pH 7.5. All of the operations, after the addition of starting buffer to the samples, were carried out at room temperature. RNA was precipitated with two volumes of ethanol at 20°C after adjusting the NaCl concetration to 0.1 M. Reproduced with permission from R. E. Pemberton et al., Anal. Biochem., 66 (1975) 18-28.

gradients. De Larco and Guroff (1973) tested a series of celluloses in comparison with their derivatives for their ability to bind homonucleotide oligomers. They determined that celluloses display considerable sorption, the degree of which varies with various celluloses. As the treatment of cellulose with sodium hydrogen sulphite caused a reduction in the amount of sorbed polyadenylic acid, De Larco and Guroff concluded that the binding was caused by lignin-like contaminants. Oligodeoxythymidylic acid and also deoxyribonucleic acid (DNA) complementary to ovalbumin mRNA (cDNA) coupled to cellulose were used by Rhoads and Hellmann (1978) for the isolation of ovalbumin mRNA from hen oviduct polysomes. Wetekam et al. (1975) used Sepharose with covalently bound polyuridylic acid for the isolation of RNA from animal cell polysomes. For the purification of DNA, Edelman (1974) also used an affinity sorbent prepared from Sepharose. However, he did not use a material with affinity for DNA in this instance, but immobilized lectins for the elimination of polysaccharidic contaminants, which can only be eliminated from DNA with great difficulty. Most often concanavalin A-Sepharose is used because the most commonly occurring contaminating polysaccharides are glycogen fractions or starch-like substances. If polysaccharides are present which, in addition to glucose, fructose or mannose, also contain other terminal groups, then yet other immobilized lectins must be used.

3.5.2 Affinants of nonbiological origin

Nucleic acid dye chromatography is described in the book of Mohr and Pommerening (1985). The use of dyes for the preparation of biospecific adsorbents for the separation of several DNA species has been developed by Bünemann and Müller (1978). By copolymerization on polyacrylamide bases certain base-specific dyes, such as the guanine-cytosine-specific phenyl neutral red, and adenine-thymine-specific malachite green are immobilized as ligands. It is assumed that the malachite green ligand contacts one of the two grooves of the DNA helix. For this reason the malachite green adsorbent can be applied for the separation of double-stranded nucleic acids from single-stranded or partially single-stranded nucleic acids. For example, Bünemann and Müller (1978) demostrated the elution of three different bacterial double-stranded DNA adsorbed by

a malachite green-substituted column. For the chromatography of mononucleotides, oligonucleotides and transfer RNA molecules, Schott et al. (1973) used sorbents containing a dihydroxyboryl derivative:



3.5.3 Antibodies against polynucleotides

Antibodies specific to double-stranded RNAs were purified from complexes of polyadenylate.polyuridylate (poly(A).poly(U)) and anti-polyinosylate.polycytidylate antibodies (antipoly(I).poly(C) antibodies) by a method of dissociation with 5 % dimethylsulphoxide - alkali solution and DEAE-cellulose column chromatography. Kitagawa and Okuhara (1981) coupled the purified antibodies to CNBr-activated cellulose. In Fig. 3.26 it is shown that synthetic and naturally occuring double-stranded RNAs were bound specifically by the antibody-linked cellulose and were eluted with alkali solution (pH 10.5) or 10 % dimethylsulphoxide.

3.5.4 Protein-nucleotide or nucleic acid interactions

The possibilities of the use of immobilized nucleotide coenzymes for the isolation of about 30 % of the approximately 2000 enzymes found in the cell has already been described in Section 3.2.2. DNA, RNA, tRNA, and oligonucleotides immobilized to various polymer supports can be used for the isolation of many proteins; e.g. several DNA polymerases, the RNA polymerases, DNA regulatory proteins, RNA-dependent DNA polymerase, repressor proteins, ribosomal proteins, tRNA synthetases, and viral DNA- and RNA-binding proteins (Schott, 1984).

The matrix which is commonly applied in template chromatography is oligo-deoxythymidylate (oligo(dT)) with strands 12-18 nucleotides long covalently attached

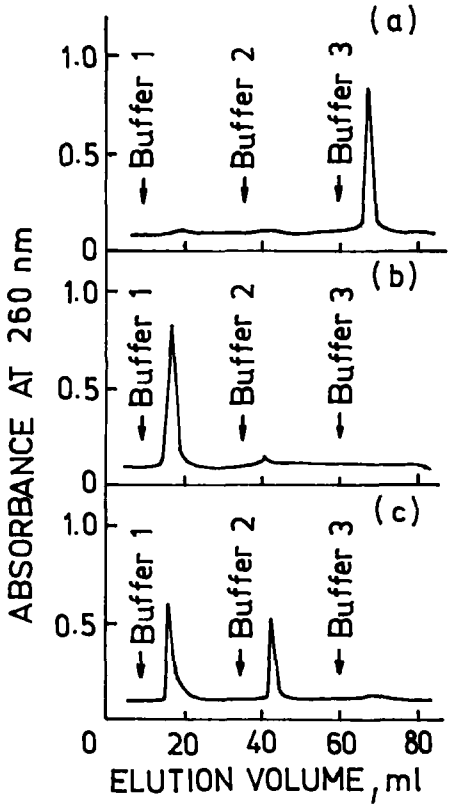


Fig. 3.26. Chromatography of nucleic acids on antipoly(I) . poly(C) antibody-linked cellulose. The column (1.0 x 3.2 cm), which contained 2.8 mg of antibodies coupled to 0.6 g of cellulose, was packed and equilibrated with 0.01 M Tris-HCl (pH 7.6) containing 0.15 M NaCl (buffer 1). Various nucleic acids (100 μg) were eluted stepwise from the column at a flow rate of 40 ml/hr by 0.1 M Tris-HCl (pH 7.6)/0.15 M NaCl (buffer 1); 0.01 M Tris-HCl (pH 7.6) containing 1 M NaCl (buffer 2); and 0.02 M sodium bicarbonate (pH 10.5) (buffer 3). (a) Poly(I).poly(C); (b) baker's yeast tRNA; (c) E. coli rRNA. Reproduced with permission from Y. Kitagawa and E. Okuhara, Anal. Biochem., 115 (1981) 102-108.

to microgranular cellulose. As an example the use of this biospecific sorbent for the purification of terminal deoxynucleotidyltransferase from calf thymus tissue by Okamura et al. (1978) is shown in Fig. 3.27.

The preparation of fairly stable DNA-cellulose by the very simple attachment of single- and double-stranded DNA to cellulose by drying and subsequent lyophilization of a DNA-cellulose paste was described by Alberts and Herrick (1971). This DNA-cellulose has been used most often up to present day.

The feasibility of using site-specific DNA affinity for the purification of specific DNA-binding proteins was tested by Herrick (1980) who demonstrated that lac repressor bound more tightly to plasmid DNA containing the lac operator than to a DNA lacking the operator when the DNA was coupled to either a cellulose or a Sepharose matrix. As a result of the tight binding, elution of the protein from the site-specific DNA-cellulose column required buffers with high ionic strength. Consequently, fractionation through the site-specific DNA-cellulose matrix resulted in considerable purification, efficiently separating the lac repressor from other DNA-binding

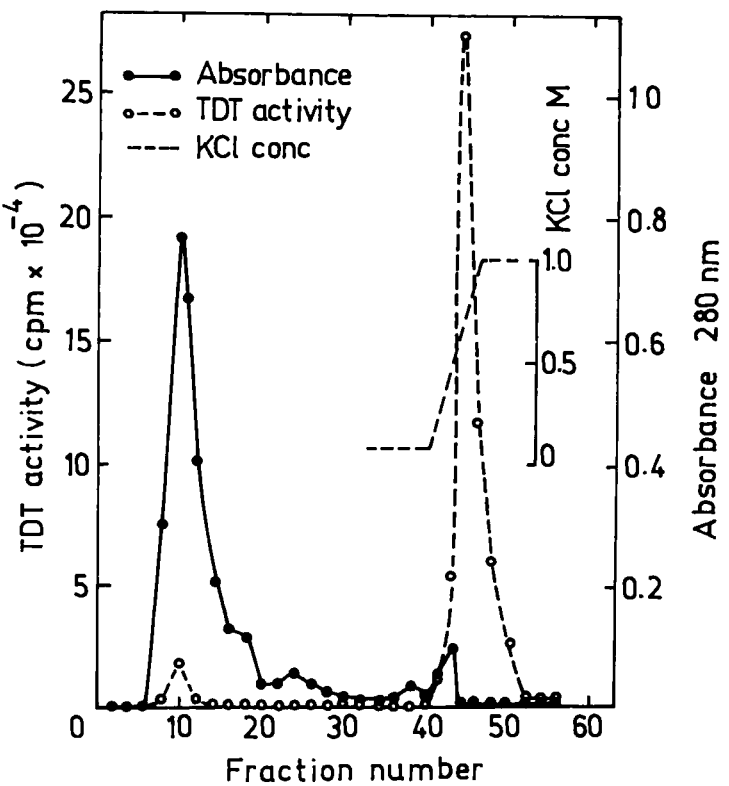


Fig. 3.27. Purification of terminal deoxynucleotidyltransferase (TDT) by an oligo(dT)$_{12-18}$-cellulose column. The column was washed with 35 ml of buffer containing 40 mM KCl, 1 mM dithiothreitol, 10 mM K_2PO_4 (pH 7.1), and 20 % glycerol. The enzyme activity was eluted with 1 M KCl in the same buffer. o-o, TDT activity in 25 μl; .--., absorbance at 280 nm. Reproduced with permission from S. Okamura et al., J. Biol. Chem., 253 (1978) 3765-3767.

proteins. Further work showed that an even higher ionic strength was required when a specific DNA was present in multiple copies (Oren et al., 1980).

The procedure of Rosenfeld and Kelly (1986) and of O'Neil and Kelly (1988) entailed the construction of a plasmid containing the recognition sequence in multiple tandem copies arranged in a head-to-tail direct orientation in order to maximize the stability of the insert. After plasmid amplification and purification, the DNA is coupled to cellulose to yield the DNA affinity resin. To separate the protein of interest from proteins that bind tightly and nonspecifically to DNA, a chromatography step involving a nonspecific DNA-cellulose column can be introduced as a prelude to the fractionation through the site-specific DNA resin.

Recently Eisenberg et al. (1990) have published a review on the purification of DNA-binding proteins by site-specific DNA affinity chromatography. They described the numerous advantages which they experienced with the purification of DNA-binding protein (OBF1) by use of DNA-cellulose prepared by the method of Alberts and Herrick (1971) as follows. (1) The DNA affinity matrix has a high protein capacity, which is determined by the number of copies of the protein recognition sequence inserted into the plasmid. It is possible to construct a plasmid in which the protein-binding sequences comprise at least 50% of the total DNA content. Once inserted into the plasmid, followed by DNA amplification in *E. coli*, very large quantities of these sequences could be obtained. Since about 1 mg of DNA can be coupled to 1 g of cellulose powder, it is relatively easy to prepare sufficient quantities of the DNA affinity matrix for a large-scale purification. (2) The protein appears to bind more tightly to the DNA on the column than in solution. They found that in solution the OBF1-DNA complex could not be formed in the presence of NaCl concentrations above 0.2 M, while the binding of OBF1 to the multimeric site-specific DNA-cellulose matrix occured at 0.4 M NaCl. The tight binding to the site-specific DNA-cellulose column ensures effective separation of OBF1 from other DNA-binding proteins by simple salt elutions and, when combined with standard ion-exchange chromatography, a highly purified protein may be obtained.

3.6 RECEPTORS, BINDING AND TRANSPORT PROTEINS, HORMONES, VITAMINS, TOXINS, GROWTH FACTORS, LIPIDS AND OTHER SUBSTANCES

3.6.1 Receptor substrates

The primary effect of some hormones is aimed at the plasma membrane of the target cells. The term "receptor" is usually understood to embrace those components of plasma membranes which are involved in the effect of the particular hormone. It seems that they are localized exclusively on the surface of the membrane cells. In order to elucidate the effect of hormones on a molecular basis, it is necessary to purify and identify these specific membrane receptor structures. The amount of these structures in the tissues is very small in comparison with other material present. For example, the concentration of glucagon receptor on liver cells membranes is very low: 2.6 pmole/mg of protein (Krug et al., 1971). The interaction of such a small amount with the immobilized hormones must be very effective in order to permit a strong binding of large membrane fragments. The interaction of hormones with their complementary receptors is specific and of high affinity. The dissociation constants of receptor complexes with hormones, toxins etc. lie in the range 10^{-9} - 10^{-12} M (Bayer and Wilchek, 1990). It is very difficult to isolate such small amounts by conventional methods. The use of biospecific chromatography on highly effective immobilized hormones or receptors permits such amounts to be concentrated selectively and to be isolated in a relatively high yield.

Although technical difficulties in the development of suitable methods for the determination of receptor activities, as well as difficulties with solubilization, for a long time prevented the development of the use of bioaffinity chromatography for the purification of receptors, a series of membrane fragments with specialized receptor activity has been isolated. The receptors isolated and the affinity ligands used are given in Tables 9-1,2,3. Bioaffinity chromatography of an insulin receptor solubilized with a detergent by use of diaminodipropylaminosuccinyl-N-phenylalanyl-insulin-agarose is shown in Fig. 3.28 (Cuatrecasas, 1972).

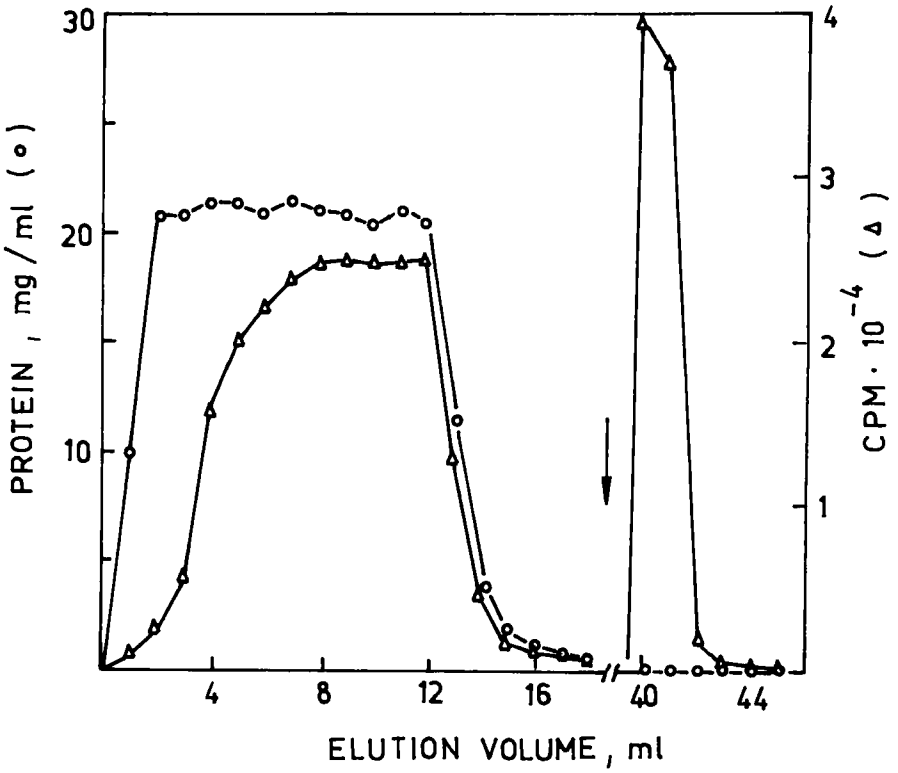


Fig. 3.28. Bioaffinity chromatography of detergent-solubilized insulin receptors of liver-cell membranes on affinity columns containing diaminodipropylaminosuccinyl-N-phenylalanyl-insulin-agarose. Liver-cell membranes were homogenized, extracted with 2 % (v/v) Triton X-100 by shaking at 24°C for 40 min, and centrifuged. The supernatant was dialyzed for 16 h at 4°C against Krebs-Ringer hydrogen carbonate buffer, pH 7.4, containing 0.1 % (v/v) of Triton X-100. Then 12 ml of the supernatant were slowly chromatographed at 24°C on an affinity column (V_t = 1.3 ml, in a Pasteur pipette) that had been washed for 20 h with 0.1 M $NaHCO_3$ buffer, pH 8.4, followed by equilibration (2 h) with Krebs-Ringer hydrogen carbonate buffer containing 0.1 % (v/v) of Triton X-100. The column was washed thoroughly (note break in abscissa) before elution (arrow) with 0.05 M sodium acetate buffer, pH 6.0, containing 4.5 M urea and 0.1 % (v/v) of Triton X-100. After application of this buffer to the column, the flow was stopped for 15 min before resumption of chromatography. Fractions of 1 ml were collected for determinations of protein, and the specific binding of [^{125}I] insulin was determined with the polyethylene glycol assay. Reproduced with permission from P. Cuatrecasas, Proc. Nat. Acad. Sci. U.S., 69 (1972) 1277-1281.

3.6.2 Antibodies against receptors

The introduction of monoclonal antibodies against receptors and their subunits as affinity ligands rather than receptor substrates greatly advanced the application of immunology to membrane biochemistry. A discussion of monoclonal antibodies against

acetylcholine, insulin, adrenergic, transferrin, thyrotropin, neurological, viral and complement receptors was presented by Phillips (1989).

Autoantibodies are naturally occuring antibodies that are produced against components of the host's own body. They are produced against several different receptors and usually arise in specific, clinically defined diseases. They are a useful source of reagent for use in the biospecific isolation of receptors and other clinically relevant proteins.

Anti-idiotypic antibodies can mimic receptor substrates, bind to membrane receptors and therefore can be used to isolate active receptors from detergent-solubilized membranes. Fig. 3.29 shows high performance immunoaffinity chromatography (HPIAC) of specific phosphorylcholine receptors (Phillips, 1989) by use of biotinylated mouse monoclonal anti-idiotypic antibody adsorbed to streptavidin-coated glass beads, as elaborated by Babashak and Phillips (1988). HPIAC analysis was performed on isolated membranes from primed T cells obtained from mice immunized with phosphorylcholine (PC). These membranes were prepared by hypotonic lysis of the cells, followed by detergent NP40 solubilization of the membrane-rich fraction obtained by ultracentrifugation. The preparation was passed through a Sephadex G25 column to remove excess detergent prior to injecting it into the HPIAC column. Using the monoclonal anti-idiotype as the immobilized ligand, a single peak was eluted by sodium thiocyanate gradient at 24 min (Fig. 3.29, peak B). This peak was shown to bind the antigen actively, but also demonstrated some cross-reactivity to other related antigens.

3.6.3 Receptor-affinity chromatography

Receptor affinity chromatography described by Kohanski and Lane (1985) contained the isolation of insulin receptor by use of biotinylated insulin in conjunction with avidin-Sepharose CL-4B. Receptor-affinity chromatography based upon the specificity and reversibility of the receptor-ligand interaction for the purification of biomolecules by use of matrix-bound receptor was described by Weber and Bailon (1990). As a model system they described the purification of recombinant human interleukin-2 from microbial and mammalian sources using the soluble subunit of the human interleukin-2 receptor (recombinantly produced by the gene-linked co-aplication technology elabo-

rated by Weber et al., 1988) attached to silica-based NuGel P-AF polyaldehyde poly-N-hydroxysuccinimide (NuGel P-AF PNHS). A comparison of receptor and immunoaffinity purification methods showed, that the binding capacity of the immobilized receptor is higher than that of the immunosorbent and the receptor-affinity purified interleukin-2 was more homogeneous than the immunoaffinity purified material.

Cholera toxin (the exotoxin from *Vibrio cholerae*), is the protein responsible for gastrointestinal manifestations of clinical cholera. In the medium of a *Vibrio cholerae* culture, the toxin occurs in a very low concentration of up to about 1 mg per litre of medium. The toxin is bound very strongly and specifically on certain brain gangliosides. It seems that monosialogangliosides localized in the membrane are natural receptors that interact specifically with cholera toxin, which results in a stimulation of the activity of adenylate cyclase in the tissue. Gangliosides bound to solid supports are affinity

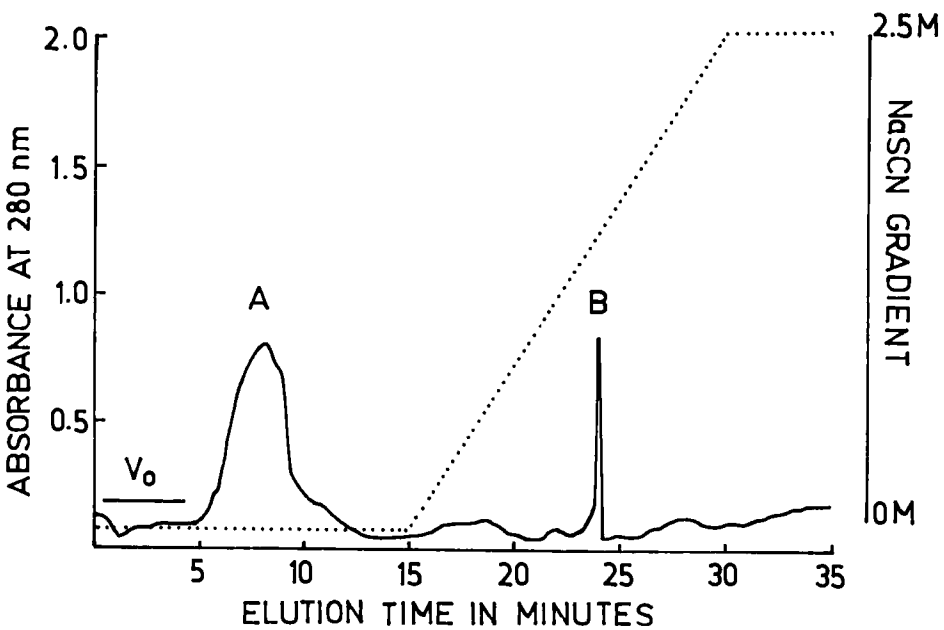


Fig. 3.29. Isolation of specific T-cell PC receptors using a streptavidin-immobilized mouse monoclonal anti-idiotypic antibody HPIAC column. The column was 150 x 4.6 mm, run at 1 ml/min at 4°C. The receptors were recovered at 24 min using a sodium thiocyanate concentration gradient. V_o represents the void volume of the column. Detection was at 280 nm. The initial running buffer was 0.01 M phosphate, pH 7.0. Reproduced with permission from T. M. Phillips, in A. R. Kerlavage (Editor), The Use of HPLC in Receptor Biochemistry, Alan R. Liss, New York 1989, p. 148.

sorbents for cholera toxin. This toxin also binds certain glycoproteins, such as fetuin and thyroglobulin. The affinity chromatography of ^{125}I-labelled toxin from *Vibrio cholerae* on agarose with attached fetuin or on ganglioside-diaminodipropylamino-agarose was achieved by Parikh and Cuatrecasas (1974).

3.6.4 Vitamins, lipids, hormones and other substances

The restricted supply of vitamins and hormones in animals led to the development of mechanisms for adsorption, transport and conservation of these trace substances. Specific transport or binding proteins play an important role in such processes, preventing the rapid urinary loss which would occur if the vitamins or the hormones were not bound in plasma in corresponding complexes. Binding proteins are present in very low concentrations. Proteins firmly binding vitamin B_{12} (transcobalamin I and II) are present, for example, in concentrations of 80 and 20 mg per 1000 l of human plasma, respectively. However, they usually have a high affinity for complementary vitamins or hormones. The dissociation constants of these complexes range from 10^{-7} to 10^{-16} M (Lowe and Dean, 1974). In view of their low concentration, they could not be obtained by classical purification procedures, and large volumes of the starting material combined with a specific interaction of high affinity led to the use of bioaffinity chromatography. As in the antibody-antigen interaction, the subsequent dissociation of the protein from the bioaffinity adsorbent becomes the crux of the isolation. For example, in order to free avidin from biocytin-Sepharose, 6 M guanidine-hydrochloric acid solution of pH 1.5 had to be used (Cuatrecasas ans Wilchek, 1968). In Table 9 further examples are given of the isolations of binding and transport proteins with the affinity ligands used.

Dodecylamine is a useful affinity ligand for the isolation of lipids (Deutsch et al., 1973). For the isolation of hormones, corresponding antibodies, transport proteins or lectins serve as bioaffinity sorbents (see Table 9). An example is the affinity chromatography of sheep luteinizing hormone on Sepharose with covalently bound anti-luteinizing hormone immunoglobulin fraction (Gospodarowicz, 1972). Nakano et al. (1978) used

immobilized bovine serum albumin and salicylic acid for the study of drug-protein binding.

3.7. SPECIFIC PEPTIDES

When determining amino acids present at the active site or on the surface of the protein molecule, chemical modification of proteins with subsequent isolation of the labelled peptides is used with success. The isolation of a peptide containing a modified residue is not easy, however, mainly because the modifying reagent often reacts with different residues to give products in various yields. For this reason the protein hydrolysate contains several modified peptides, each of which is present in amounts less than 1 μM. The conventional methods for the isolation of peptides require tedious and time-consuming procedures in which each step usually considerably decreases the final yield of the peptide. The use of bioaffinity chromatography on a sorbent specific for a modifying reagent permits a one-step isolation of the modified peptide. Wilchek (1974) distinguished three categories of protein modifications:

(1) site-directed modification or affinity labelling of the residues at the active site of the protein;

(2) selective modification of one or several residues due to their hyper-reactivity or the localization on the protein surface;

(3) general modification of all side-chains of certain amino acids with a group-specific reagent, aimed at sequencing.

To the first group belong reactions of analogues of substrates with the amino acids of the enzyme active site or the reaction of the hapten in the active sites of antibodies. In these instances the specific and reversible binding of analogues at the binding site of the protein is followed by the formation of a covalent bond at or near to the site of the labelling. A general scheme for the isolation of labelled peptides from an affinity-labelled protein has been described by Wilchek (1974). The native protein (enzyme or antibody) bound covalently to a solid support sorbs only the affinity-labelled peptide from the hydrolysate of the same affinity-labelled protein. After washing the column, the specifically sorbed labelled peptide can be eluted under conditions such that

dissociation takes place. Wilchek (1970) made use of this method for the isolation of affinity-labelled peptides from staphylococcal nuclease after reaction with the bromoacetyl derivate of deoxythymidine-3'-p-aminophenylphosphate-5'-phosphate and with bromoacetyl-p-aminophenylphosphate. Fig. 3.30 shows the affinity chromatography of labelled peptides from tryptic hydrolysate of modified nuclease on nuclease-Sepharose. Table 9.1 gives further examples of isolations of affinity-labelled peptides.

Specific antibodies with a high affinity can be induced against almost all small molecules, such as haptens, attached to suitable macromolecules. The specific antibodies induced in this manner, when bound covalently to a solid support, represent suitable specific sorbents for peptides that contain the corresponding small molecules. An example is the isolation of nitrotyrosyl peptides from a tryptic digest of nitrotyrosyl lysozyme by Helman and Givol (1971), using anti-nitrotyrosyl antibodies attached to Sepharose as the affinant. If an enzymatic digest of the nitrated protein is passed through a column containing the attached nitrotyrosine antibody, all peptides (except those which contain nitrotyrosine) emerge as the first peak. The nitrotyrosine peptides are then eluted with 1 M ammonia. The procedure described can be used in topographical studies aimed at the determination of tyrosine residues located on the surface of the molecule.

After the introduction of the sequenator in the determination of amino acid sequences of proteins, the purification of peptides became the rate-determining step. The development of sensitive and efficient methods for the selective isolation of modified peptides makes it possible to obtain even very small amounts of peptides from proteins that are not accessible in larger amounts (receptors, transport proteins, etc.). The use of anti-dinitrophenyl antibodies for the isolation of peptides containing cysteine, methionine, tryptophan, histidine and lysine residues was given as an example by Wilchek (1974).

Peptides containing free SH groups and all other mercapto compounds have a high affinity for heavy metal ions, particularly mercury. The mercurial derivative of hydroxyalkyl methacrylate gel used for the isolation of SH-proteinase from beans (mentioned in Section 3.2.5) was also used for the isolation of peptide with a free SH group from

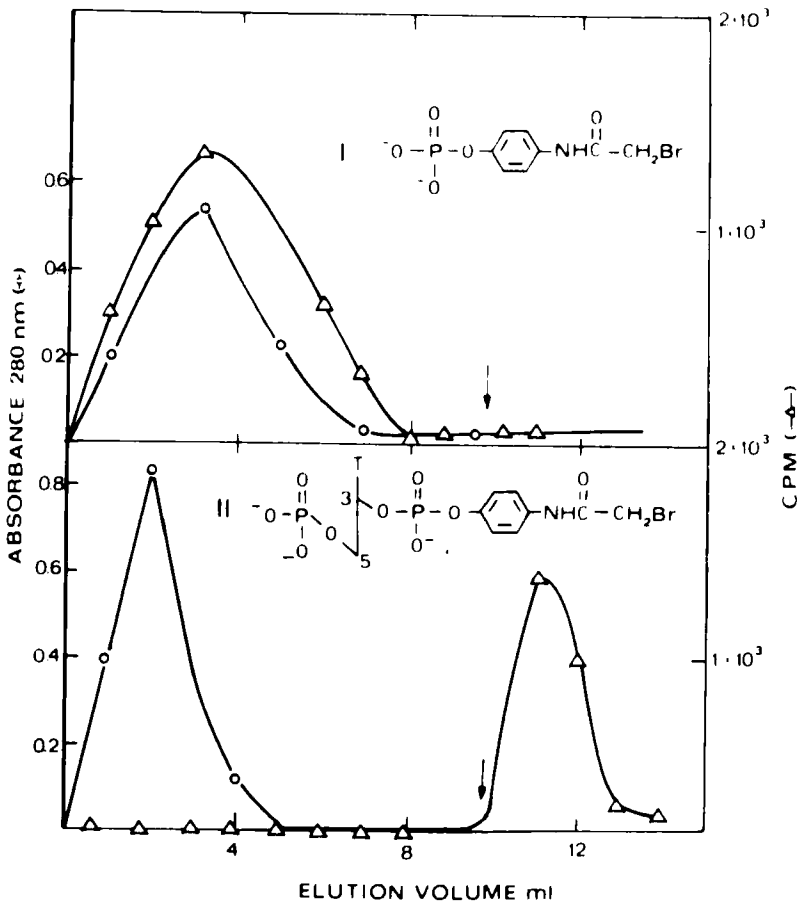


Fig. 3.30. Affinity chromatography on a nuclease-Sepharose column (20 x 5 mm) of affinity-labelled peptides with reagents I and II. The columns were equilibrated with 0.05 M borate buffer, pH 8.0, containing 10 mM $CaCl_2$. Tryptic digests of modified nuclease (1.7 mg) were applied in 0.5 ml of the same buffer. After 10 ml of buffer had passed through, the bound peptides were eluted with ammonia solution, pH 11.0 (arrow). Reproduced with permission from M. Wilchek, FEBS Lett., 7 (1970) 161-163.

chymotryptic hydrolysate of serum albumin (Turková et al., 1975). Thiol-disulphide interchange on agarose-(glutathione-2-pyridyl disulphide) conjugate, called "covalent chromatography" by Brocklehurst et al. (1974), was used by Egorov et al. (1975) as a rapid and specific method for the isolation of thiol-containing peptides from large proteins. In Table 9.1 examples are given of the isolation of specific peptides and of both peptides and proteins containing free SH groups.

3.8 BIOTIN AND AVIDIN OR STREPTAVIDIN

The binding of water-soluble vitamin biotin to the egg white protein, avidin, or to its bacterial counterpart, streptavidin, is paralleled by one of the biggest drops in free energy ever observed for noncovalent interactions. The change of enthalphy, Δ H, for biotin bound to avidin and streptavidin has been determined by Green (1966) as being -86 and -98.9 kJ/mol biotin, respectively. Avidin or streptavidin can bind four molecules of biotin. The Δ H value for the binding of four biotin molecules was determined by Green to be -334 kJ/mol avidin. The change in entropy for this reaction was found to be zero. The affinity constant for biotin-avidin determined by Green is of the order of 10^{15} M^{-1}. The extraordinarily high affinity constant of the biotin-avidin complex makes this system an efficient tool for study and application in various fields of biology, protein chemistry and biotechnology.

In the past decade the interaction between biotin and avidin or streptavidin has provided the basis for establishing a new avidin-biotin technology (Wilchek and Bayer, 1990). The general idea of the approach is that biotin, coupled to low or high molecular weight molecules, can be recognized by avidin. The biotinylation of membranes, nucleic acids, antibodies and other proteins has been developed in many laboratories. Fig. 3.31 shows biotin and various derivatives, suitable for various types of bonds. Fig. 3.32 shows that a spacer arm should be used when protein macromolecules are modified by biotin. Such a modification can be used for the preparation of complexes with immobilized avidin or streptavidin.

Literature survey of applications of avidin-biotin technology was published by Wilchek and Bayer (1990a) containing references of papers about the use of avidin columns in the isolation of biologically active materials, direct labelling, immunocytochemical and nonimmunocytochemical localization of biologically active materials, direct and indirect labelling of protein blots, immunoassays mediated by avidin-biotin technology and the use of biotinylated gene probes.

Information on biotin-binding proteins, the preparation of biotin, avidin and streptavidin derivatives, assays for avidin and biotin, and applications, classified in a similar

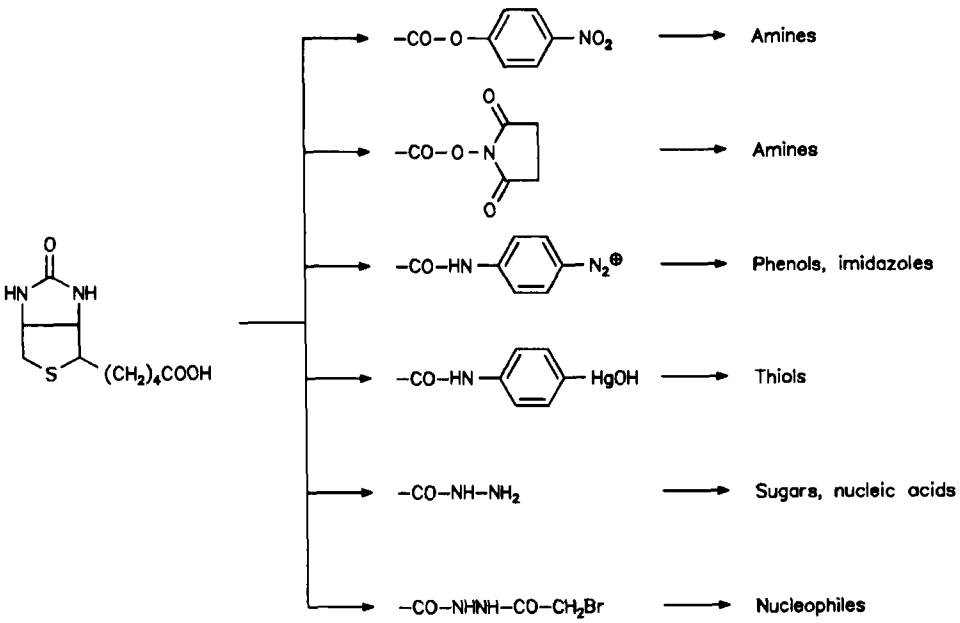


Fig. 3.31. Biotin and derivatives.

way as a literature survey, is given in Volume 184 of "Methods in Enzymology" under the title "Avidin-Biotin Technology" edited by Wilchek and Bayer (1990).

The first example of the use of an avidin-biotin complex in bioaffinity chromatography was the isolation of avidin from egg white on a biocytin-Sepharose column (Cuatrecasas and Wilchek, 1968). The conditions required for its dissociation were extremely drastic. Avidin was eluted by 6M guanidinium hydrochloride, pH 1.5. In order to decrease the strong affinity of streptavidin to biotin Bayer et al. (1986) developed an improved method for the preparation of stable iminobiotin-containing Sepharose and used it for the single-step purification of streptavidin from culture broth of *Streptomyces avidinii*.

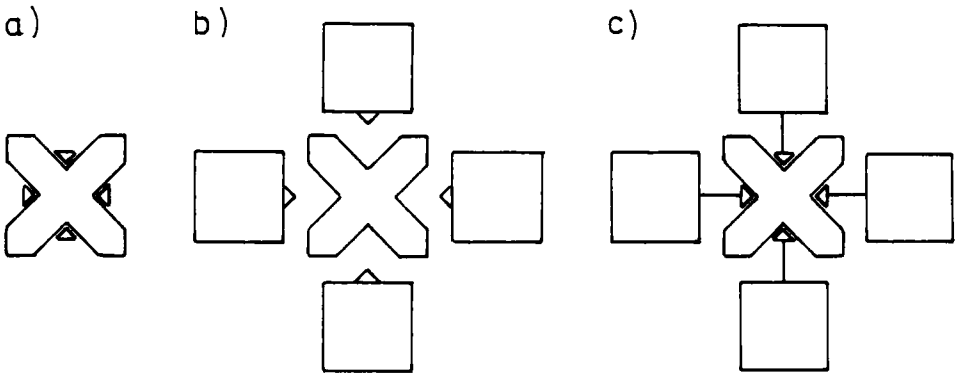


Fig. 3.32. Schematic drawing of (a) normal binding of biotin to avidin or streptavidin; (b) steric hindrance caused by macromolecule; (c) unhindered binding restored by use of spacer arm.

Native biotin-containing proteins and peptides isolated directly on columns of immobilized avidin or streptavidin are given in Table 9.1-3. To show the difficulty encountered in eluting the adsorbed material from avidin columns Wilchek and Bayer (1990a) listed in a Table the isolation of biotin-containing systems together with the elution conditions. For example acetyl-coenzymeA carboxylase aposubunits were eluted by Guchhait et al. (1974) with 6M guanidine-HCl, pH 2, acetyl-coenzymeA carboxylase by Beaty and Lane (1982) with 0.1 mM biotin. Henrikson et al. (1979) developed an effective way of releasing the biotin-containing enzymes (propionyl-coenzymeA carboxylase and methylmalonyl-coenzymeA pyruvate transcarboxylase) from avidin columns by the use of immobilized avidin monomers which takes advantage of the lower affinity constants (Green and Toms, 1973) and permits the release of biotin-containing material under mild conditions.

The isolation of peptide or protein receptors posed a number of difficult problems owing to their scarcity, their location in a complex, lipid-rich environment, and the difficulty with which even a starting plasma membrane is prepared. The coupling of peptide hormones to activated Sepharose results in nonspecific attachment which can generate a number of different species with varying affinities for the desired receptor. For these reasons Finn et al. (1984) have devoted their efforts to developing biospecific

sorbent based on avidin-biotin technology where the hormone can be attached in a targetted fashion. Fig. 3.33 shows the application of the avidin-biotin systems to the isolation of hormone receptors (Finn and Hofmann, 1990).

Soluble receptor (R) is percolated through a bioaffinity column to form the complex shown in the center of Fig. 3.33. The column is then exhaustively washed to remove contaminating materials. Alternatively, the biotinylated hormone ligand (B-H) can be added to a solution of solubilized receptor to form the soluble complex BHR. Percolating a solution containing this complex through a column of immobilized succinylated avidin (SA), as in Fig. 3.33 B will result in the formation of the same complex obtained by method A. Both these schemes have been used for the isolation of insulin receptors from human placenta. Removal of functional insulin receptor can be achieved by eluting with acetate buffer, pH 5 (Finn et al., 1984) or with buffer containing 1M NaCl and biotin (Kohanski and Lane, 1985).

Some important characteristics and structural studies of avidin and streptavidin have been published by Bayer and Wilchek (1990). Both proteins are tetramers with molecular weight 67 000. The genes for both proteins have been cloned and expressed in *Escherichia coli*. The primary sequences of both proteins are known. Avidin and the truncated form of streptavidin show an overall homology of about 40%. Chemical modification studies have shown that the single tyrosine (Tyr-33) and its homologue in streptavidin (Tyr-43) play a role in biotin binding. This was confirmed by X-ray crystallographic studies of streptavidin. The elucidation of the three-dimensional structure of streptavidin showed that the streptavidin subunit consists essentially of an extremely stable β-barrel consisting of a series of eight juxtaposed β-structures connected by turns. The biotin site is inside the barrel and, in binding biotin, some of the turns fold over to stabilize the complex.

Despite the fact that streptavidin is currently about 100 times more expensive than avidin, the replacement is sometimes justified since non-specific binding can be averted. Avidin is highly positively charged at neutral pH, with an isoelectric point, pI, higher than 10. Consequently, it binds in a non-specific manner negatively charged molecules such as nucleic acids, acid proteins or phospholipids. This can result in non-specific

staining of, for example, the nucleus and cell membranes. Avidin is also a glycoprotein and therefore interacts with other biological molecules such as lectins or other sugar-binding materials *via* the carbohydrate moiety. The advantage of streptavidin lies in the fact that it is a neutral, non-glycosylated protein (pI lower than 7). In terms of correcting the positive charge, the lysines of avidin can be easily derivatized by succinylation, acetylation, etc. A variety of avidin derivatives with average pI values 7 or lower are now commercially available. The removal of the carbohydrate residue from avidin is much more difficult.

Haueple et al. (1983) have demonstrated that a far more effective approach in the purification of lactogenic and somatogenic receptors from rabbit liver or mammary membranes by use of biotinylated growth hormone is to replace the immobilized avidin with the immobilized streptavidin. They compared adsorptions of biotinylated and non-biotinylated growth hormone-binder complexes to both bioaffinity columns. Non-specific adsorption of non-biotinylated hormone-binder complexes was found only on

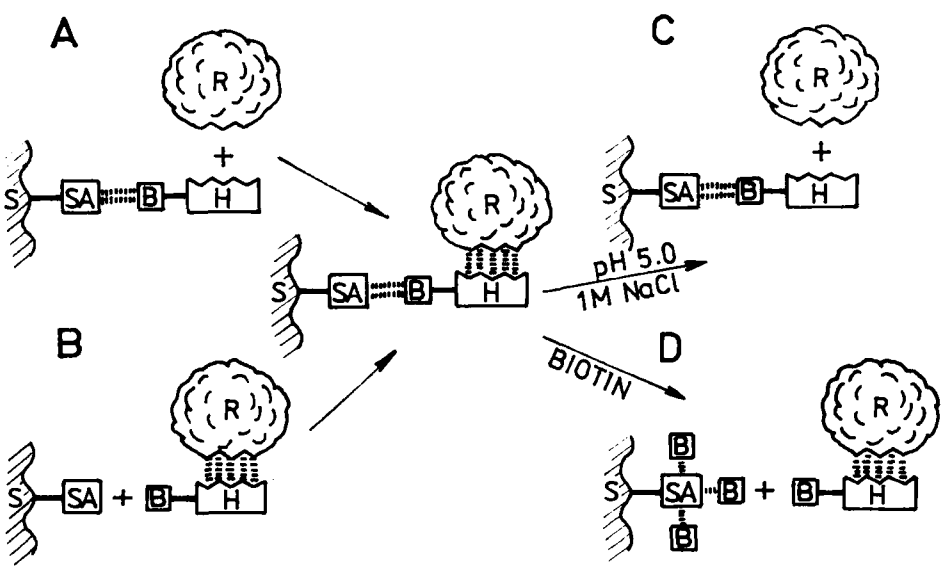


Fig. 3.33. Application of the avidin-biotin system to the isolation of hormone receptors. Dashed lines represent noncovalent bonds. Reproduced with permission from F. M. Finn and K. Hofmann, Methods Enzymol., 184 (1990) 244-274.

avidin-agarose. The purification of membrane proteins utilizing the biotin-streptavidin affinity has been reviewed by Kraehenbuhl and Bonnard (1990).

The separation of complementary strands of plasmid DNA using the biotin-avidin system was described by Delius et al. (1985). Biotin-labeled strands were eluted from avidin agarose with 50% guanidine isothiocyanate/formamide. Rapid and efficient recovery of the DNA-protein complexes was achieved by Herman et al. (1986), when they used DNA labeled with the chemically cleavable biotinylated nucleotide Bio-19-SS-dUTP and streptavidin-Sepharose. The elution conditions in this isolation are SS-reduction of cleavable biotin analogue. The advantage of the use of streptavidin column was demonstrated by the fact that, in low ionic strength buffers, nonbiotinylated DNA bound to avidin but not to streptavidin. Therefore, the biotinylated DNA-protein complexes can be selectively bound to a streptavidin column under conditions that should stabilize labile DNA-protein complexes. A method that can be generalized to link virtually any DNA substrate to a chromatography matrix at its ends *via* streptavidin-biotin linkage has been described by Fishel et al. (1990). Biotin-streptavidin affinity selection as a valuable tool permitting the analysis of the RNA components of splicing complexes assembled on a wide variety of pre-mRNA substrates has been reviewed by Grabowski (1990). The review of the isolation of cell surface glycoproteins by the use of biotinylated lectin written by Cook and Buckie (1990) has also demonstrated the advantage of immobilized streptavidin to obviate dissociation of complexes formed between biotinylated concanavalin A and membrane glycoproteins.

The technique for the isolation of detergent-solubilized membrane antigens described by Updyke and Nicolson (1984) is based on an immunoaffinity adsorption which allows the binding of biotinylated monoclonal antibodies with antigens in solution, followed by the adsorption of the antibody-antigen complexes to immobilized streptavidin. Antibodies were biotinylated using D-biotin-N-hydroxysuccinimide ester. The advantage of biotinylation of carbohydrate moieties of antibodies using biotin hydrazine for the same type of immunoaffinity chromatography has already been described in Section 3.3 (Phillips and Frank, 1988). Tables 9.1-3 present the examples of the application of avidin-biotin technology for the isolation, determination or removal of various materials.

3.9 CELLS AND VIRUSES

The bioaffinity chromatography of cells, cell organelles and membranes, phages and viruses was reviewed by Sharma and Mahendroo (1980) under the title "cellular affinity chromatography". Affinity ligands, in their review, are divided into two types: lectins and non-lectin ligands, which contain hormones, neurotransmitters and related ligands with affinity for cell surface receptors, and further inhibitors of membrane-bound enzymes.

3.9.1 Lectins and glycoconjugates

A vital role in many fundamental cellular processes - such as cell-cell recognition (binding of bacteria, aggregation of slime mold and sponge cells, sperm-egg binding, interactions between cells in the immune system etc.), transmission of extracellular stimuli, and regulation of cell movement, growth, division, and differentiation - is played by cell membrane glycoconjugates: glycoproteins and glycolipids. They are inserted or intercalated to varying degrees into the bilayer, disrupting its continuity and forming mosaic-like arrangements. The saccharides usually found in glycoproteins or glycolipids of cell membrane glycoconjugates include N-acetylneuraminic acid, galactose, mannose, fucose, N-acetylglucosamine, and N-acetylgalactosamine (Mohr and Pommerening, 1985). A review on the use of immobilized glycoconjugates for cell recognition studies was published by Schnaar (1984). In the review cited (Schnaar, 1984) the difference in carbohydrate recognition by chicken and rat hepatocytes was demonstrated. Hepatocytes dissociated from chicken livers bound readily to immobilized N-acetylglucosamine, but not to other carbohydrate derivatized surfaces, while rat hepatocytes bound to galactose derivatized surfaces.

Helix pomatia lectin- Sepharose 6 MB is recommended for blood typing, cell surface studies and lymphocyte fractionation in the booklet published by Pharmacia Fine Chemicals (1978). Thiolated concanavalin A immobilized through cleavable mercury-sulphur bonds to mersalyl -Trisacryl GF-05 beads adsorbed mouse thymocytes which were recovered by dithiothreitol treatment (Bonnafous et al., 1983).

Affinity ligands can also be coupled with the fibres through special linkers, permitting the liberation of the cells by a specific chemical or enzymatic cleavage. The binding of erythrocytes and thymocytes on various fibres as a function of the number of concanavalin A molecules bound per centimetre of the nylon fibre was described by Edelman and Rutishauser (1974). The basic principle of their fibre fractionation of cells is shown in Fig. 3.34. Suitable molecules or macromolecules are coupled in a suitable chemical form with nylon fibres strung on a frame. The dissociated cells are then shaken with the fibre in a suitable medium and the non-sorbed cells are washed out. The coupled cells can then be transferred into another medium and there further characterized, or they can be released into the medium by plucking them from the taut fibre with a needle,

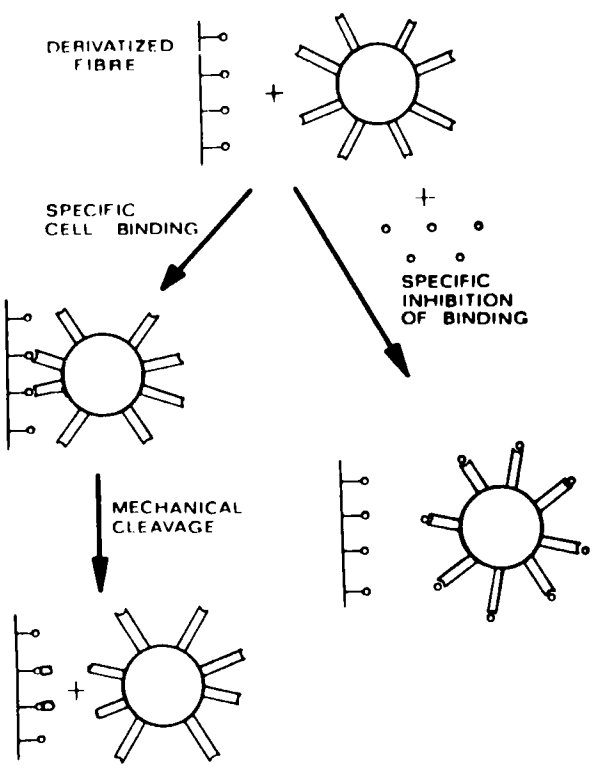


Fig. 3.34. General scheme for fibre fractionation. Reproduced with permission from G. M. Edelman and U. Rutishauser, Methods Enzymol., 34 (1974) 195-225.

which serves to split off the cells from their points of attachment. Fractionation can be achieved on the basis of specific bonding to a single component on the cell surface, or on the basis of the differences in binding affinity, or on the number and distribution of cell surface receptors of equal specificity.

The rapid isolation of human erythrocyte plasma membrane was achieved by Kaplan et al. (1984) on an affinity matrix consisting of wheat germ agglutinin covalently bound to Sepharose 6MB. After binding the washed cells to the bioaffinity matrix, they were washed extensively and lysed. The resulting ghosts were washed and then eluted from the matrix with N-acetyl-β-D-glucosamine.

Homogeneous concanavalin A labelled with ^{125}I was used as an affinity ligand by Wallach et al. (1972) in a bioaffinity approach called affinity density perturbation. Its principle is illustrated in Fig. 3.35. Concanavalin A was converted into a density perturbant by means of glutaraldehyde by covalent bonding to coliphage K29, a stable icosahedron of diameter 450 Å and density 1.495 g/ml. The membrane fragments were prepared from hog lymphocytoplasmatic membranes, and contained a large amount of receptor for concanavalin A. The interaction of membrane fragments carrying the receptor with the perturbant reversibly increased the buoyant density in a caesium chloride gradient from about 1.18 for untreated membranes to a broad zone with maximum density at 1.30-1.40. This relatively broad density distribution of the membrane-concanavalin A-K29 complex reflects the microheterogeneity in the distribution of the receptor sites. An addition of excess α-α trehalose was used for the dissociation of the complex of concanavalin A with its receptor.

In general, the above method can be applied not only for the isolation of receptor sites for hormones, transmitters, drugs, lectins and specific antigens and antibodies, but also for the mapping of the topology of the membrane and cell.

3.9.2 Antibodies and antigens

Manderino et al. (1978) successfully prepared T and B memory cells from lymph nodes of rabbits preimmunized with keyhole limpet hemocyanin by use of columns with goat anti-rabbit Fab fragments of immunoglobulin G (IgG) attached to CNBr-activated

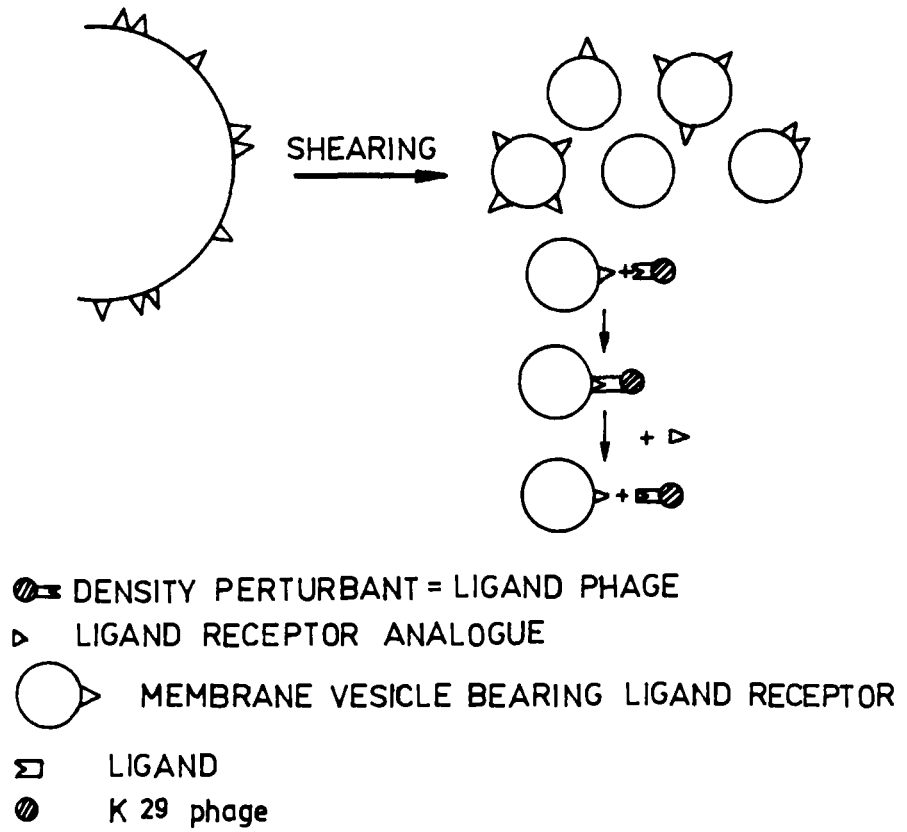


Fig. 3.35. The principle of affinity density perturbation. A plasma membrane bearing multiple receptors (Δ) is sheared into membrane fragments carrying different numbers of receptors in varying distributions. These are reacted with the ligand coupled to the density perturbant, i.e. K29 phage, producing a membrane-receptor-ligand-phage complex with a higher density than that of the membrane itself and a lower density than that of the density perturbant. Addition of a low molecular weight dissociating agent (Δ) returns the membrane and density perturbant to their original densities. Reproduced with permission from D. F. H. Wallach et al., FEBS Lett., 21 (1972) 29-33.

Sepharose 6MB macrobeads. The various positive immunoselection techniques available were reviewed by Basch et al. (1983). The same autors also describe liquid-phase positive immunoselection performed using either a fluorescence-activated cell sorter or by using "cellular engineering" to protect a cell from an otherwise noxious environment. The enzyme catalase coupled to specific antibody has been used for this purpose and renders cells resistent to hydrogen peroxide. Cells having IgG on their

surface or cells coated with IgG antibody specific for particular membrane antigen were separated by Ghetie and Sjöquist (1984) by the use of Protein A-Sepharose 6MB. Bioaffinity chromatographic separation of T cells by the use of monoclonal antibodies was discussed by Braun and Kümel (1986) as a valuable tool in studies of the function of T cells and their subsets. According to these authors a broad array of procedures has been developed, of which an indirect use of matrix-bound second antibody appears to be the most practical and advantageous with respect to purity, functional activity, and viability of separated cells. Widespread use of indirect immunoadsorbents is discussed in a review on the immunoaffinity purification of subcellular particles and organelles by Richardson and Luzio (1986).

Thomas and Lansdorp (1988) used tetramolecular monoclonal antibody complexes to selectively crosslink a subset of human peripheral blood T cells (CD8 positive) to glass beads coated with fluorescein isothiocyanate-conjugated bovine serum albumin (BSA-FITC). Immunoadsorption of T cells into glass beads using tetramolecular complexes of monoclonal antibodies is shown in Fig. 3.36.

Several examples of the isolations of viruses are also presented in Table 9.1. One of the most frequently employed affinants is bound antibodies. An example is the use of Sepharose with coupled IgG from chronically infected mink for the isolation of Aleutian disease virus (Yoon et al., 1973).

3.9.3 Other affinity ligands

In some cases rather than immobilized lectins, antibodies or Protein A, other specific and reversible cell surface receptor-ligand interactions can be successfuly utilized. As an example one may mention the isolation of acetylcholine receptor-bearing neuronal cells from chick embryo sympathetic ganglia by the use of snake venom α-bungarotoxin immobilized on Sepharose 6MB macrobeads. Dvorak et al. (1978) prepared a biospecific sorbent from diazotised p-aminobenzoylhexylamino-Sepharose 6MB, which resulted in the formation of an azo linkage with one or other of the two histidine residues. In the conformation of α-bungarotoxin determined by X-ray crystallography the two histidines are well removed from the active site and their use for immobilization is

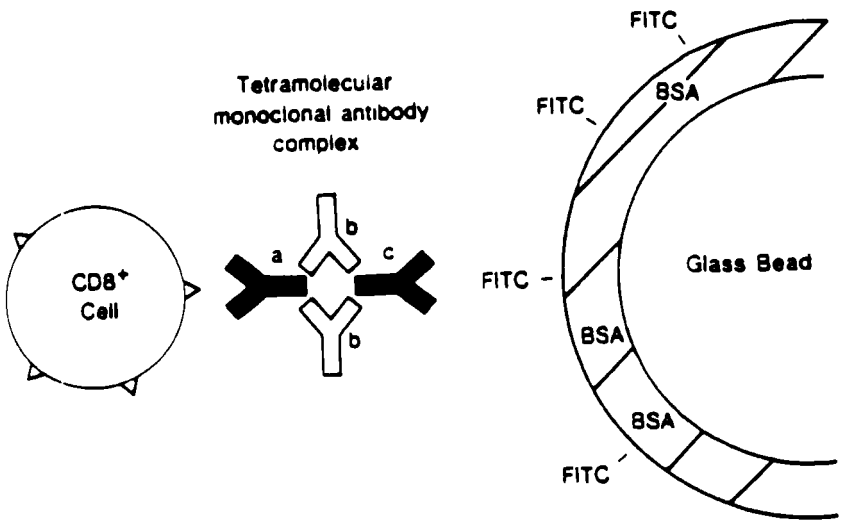


Fig. 3.36. Schematic representation of the crosslinking of $CD8^+$ T lymphocytes to glass beads coated with fluorescein isothiocyanate-conjugated bovine serum albumin (BSA-FITC). The crosslinking reagent is a tetramolecular antibody complex comprised of an anti-CD8 mouse IgG1 monoclonal antibody molecule (a), two rat anti-mouse IgG1 monoclonal antibody molecules (b), and an anti-FITC mouse IgG1 monoclonal antibody molecule (c). Reproduced with permission from T. E. Thomas and P. M. Lansdorp, J. Immunol. Methods, 112 (1988) 219-226.

unlikely to affect the biological activity of the attached affinant. Sympathetic ganglion cells were prepared from 19-d chick embryos. After trypsinisation (0.025 %) the cells were dissociated in Puck's saline G containing 2 mg/ml bovine serum albumin, 20 μg/ml deoxyribonuclease (DNase) and 10 units/ml nerve growth factor (NGF, all refered to as medium A). The cells were then filtered through nylon gauze, centrifuged and resuspended in medium A. The derivatised macrobeads were held in the bottom of a small glass water-jacketed column by a piece of nylon gauze held in place by a tightly fitting Teflon ring. The assembled column, without beads, was autoclaved and all manipulations were carried out in a sterile laminar flow cabinet. The beads were extensively washed with sterile phosphate-buffered saline (PBS), then medium A. The flow rate was adjusted to 1-2 ml/h and the cell suspension loaded. It was important to maintain a temperature of 0-4 °C throughout the isolation procedure for optimal viability of the neurones. The beads were washed until very few non-neuronal cells were present in the eluate. After further washing with PBS containing DNase (4 μg/ml) and

subsequently with PBS containing DNase and 0,1 % trypsin, the column was clamped off, the temperature raised to 37 °C and incubation allowed for 2-3 min. Foetal calf serum (FCS) was then added and the cells eluted into a sterile centrifuge tube. The cells were centrifuged, resuspended in 1 ml growth medium containing 10 U/ml NGF, and cultured. Neurones obtained in this way were viable.

Examples are given in Table 9.1 of the isolation of cells and of the affinity ligands used.

3.10 COMMERCIALLY AVAILABLE INSOLUBLE AFFINANTS

In parallel with the development of bioaffinity chromatography, the number of commercially available immobilized affinity ligands is also increasing. In order to obtain an idea of how and which products are mostly used in practice, commercial names of the supports and immobilized affinants used are listed in Table 9.1-3. From the references included in the table the reader can easily form an idea of how the application of commercial immobilized affinants is increasing at present; for example concanavalin A bound to Sepharose (Con A-Sepharose). It can be assumed that the development will be the same as for other chromatographic materials. Thus, in the same way as DEAE- and CM-derivatives of cellulose are prepared in very few laboratories today, it can be expected that the use of commercial biospecific sorbents will steadily increase. This is true, of course, for group-specific adsorbents or specific sorbents for substances that are currently prepared in laboratories. This is so because, in bioaffinity chromatography its specific nature should always be borne in mind. From the diversity of biologically active substances, a very wide range of necessary specific sorbents follows, and therefore a considerable number of research workers will have to prepare special, highly effective sorbents themselves.

Firms* which supply immobilized affinity ligands are mainly the following: Amicon Corporation (Lexington, Mass 02173 U.S.A.), BioCarb Chemicals (S-22370 Lund, Sweden), BioProbe International Inc. (Tustin, CA 92680-7017 U.S.A.), Bio-Rad Laboratories (Richmond, CA 94804 U.S.A.), CALBIOCHEM AG (CH-6000 Lucerne 5, Switzerland), Du Pont de Nemours GmbH (D-6380 Bad Homburg, Germany), E. Merck (D-6100 Darmstadt 1, Germany), IBF (92390 Villeneuve-La-Garenne, France),

Miles Laboratories Inc. (Elkart, Ind. 46514 U.S.A.), Millipore Corporation (Bedford, MA 01730 U.S.A.), Pharmacia LKB Biotechnology (S-75182 Uppsala, Sweden), P.L. Biochemicals Inc. (Milwaukee, Wisc. 53205 U.S.A.), SERVA Feinbiochemica GmbH Co. (D-6900 Heidelberg 1, Germany), Sigma Chemical Company (St. Louis, MO 63178 U.S.A.), SUPELCO SA (CH-1196 Gland, Switzerland), TESSEK Ltd. (CS-11001 Praha 1, Czechoslovakia). Information on immobilized affinity ligands are presented in booklets, catalogues and special bulletins. Different trademarks for the same immobilized ligand are connected with the trademarks of solid supports and therefore information about commercially available materials for bioaffinity chromatography is presented in Chapter 5.

• Only firms known to the author are mentioned in the text; therefore the list is necessarily incomplete and it should in no case be considered as implying a recommendation of any particular firm or product.

REFERENCES

Abraham, E.C., Perry, R.E and Stallings, M., J. Lab. Clin. Med., 102 (1983) 187-197.

Ackermans, F., Klein, J.P., Ogier, J., Bazin, H., Cormont, F. and Frank, R.M., Biochem. J., 228 (1985) 211-217.

Akparov, V.K. and Stepanov, V.M., J. Chromatogr., 155 (1978) 329-336.

Alberts, B. and Herrick, G., Methods Enzymol., 21 (1971) 198-217.

Babashak, J.V. and Phillips, T.M., J. Chromatogr., 444 (1988) 21-28.

Balny, C., Le Doucen, C., Douzou, P. and Bieth, J.G., J. Chromatogr., 168 (1979) 133-138.

Basch, R.S., Berman, J.W. and Lakow, E., J. Immunol. Methods, 56 (1983) 269-280.

Bayer, E.A., Ben-Hur, H., Gitlin, G. and Wilchek, M., J. Biochem. Biophys. Meth., 13 (1986) 103-112.

Bayer, E.A. and Wilchek, M., J. Chromatogr., 510 (1990) 3-11.

Beaty, N.B. and Lane, M.D., J. Biol. Chem., 257 (1982) 924-929.

Berglund, O. and Eckstein, F., Methods Enzymol., 34 (1974) 253-261.

Bergold, A. and Scouten, W.H., in W.H.Scouten (Editor), Solid Phase Biochemistry, Wiley, New York, 1983, pp.149-187.

Biellmann, J.F., Samana, H.P., Bränden, C.J. and Ekland,H. , Eur. J. Biochem., 102 (1979) 107-110.

Bloom, J.W., Wong, M.F. and Mitra, G., J. Immunol. Methods, 117 (1989) 83-89.

Böhme, H.J., Kopperschläger, G., Schulz, J. and Hofmann, E., J. Chromatogr., 69 (1972) 209-214.

Bonnafous, J.C., Dornand, J., Favero, J., Sizes, M., Boschetti, E. and Mani, J.C. , J. Immunol. Methods , 58 (1983) 93-107.

Bouriotis, V., Galpin, I.J. and Dean, P.D.G., J. Chromatogr., 210 (1981) 267-278.

Braun, R.W. and Kümel, G., Methods Enzymol., 121 (1986) 737-748.

Brehm, R.D., Tranter, H.S., Hambleton, P. and Melling, J., Appl. Environ. Microbiol., 56 (1990) 1067-1072.

Brocklehurst K., Carlsson, J., Kierstan, M.P.J. and Crook, E.M., Methods Enzymol., 34 (1974) 531-544.

Bünemann, H. and Müller, W., Nucl. Acid Res., 5 (1978) 1059-1074.

Chiancone, E. and Gattoni, M., J. Chromatogr., 376 (1986) 343-348.

Chu, F.K. and Maley, F., Arch. Biochem. Biophys., 214 (1982) 134-139.

Clonis, Y.D. and Lowe, C.R., Eur. J. Biochem., 110 (1980) 279-288.

Codogno, P., Botti, J., Font, J. and Aubery, M., Eur. J. Biochem., 149 (1985) 453-460.

Cook, G.M.W. and Buckie, J.W., Methods Enzymol., 184 (1990) 304-314.

Craven, D.B., Harvey, M.J., Lowe, C.R. and Dean, P.D.G., Eur. J. Biochem., 41 (1974) 329-333.

Cuatrecasas, P., Proc. Nat. Acad. Sci. U.S., 69 (1972) 1277-1281.

Cuatrecasas, P. and Wilchek, M., Biochem. Biophys. Res. Commun., 33 (1968) 235-239.

Cummings, R.D. and Kornfeld,S., J. Biol. Chem., 257 (1982) 11235-11240.

Dakour, J., Hansson, G.C., Lundblad, A. and Zopf, D., Arch. Biochem. Biophys., 248 (1986) 677-683.

Davies, D.R., Padlan, E.A. and Segal, D.M., Ann. Rev. Biochem., 44 (1975) 639-667.

Dean, P.D.G. and Quadri, F., in W.H.Scouten (Editor), Solid Phase Biochemistry, Wiley, New York, 1983, pp. 79-148.

De Larco, J. and Guroff, G., Biochem. Biophys. Res. Commun., 50 (1973) 486-492.

Delius,H., van Heerikhuizen, H., Clarke, J. and Koller, B., Nucl. Acid. Res., 13 (1985) 5457-5469.

Deutsch, D.G., Fogelman, D.J. and von Kaulla, K.N., Biochem. Biophys. Res. Commun., 50 (1973) 758-764.

Domen, P.L., Nevens, J.R., Krishna Mallia, A., Hermanson, G.T. and Klenk, D.C., J. Chromatogr., 510 (1990) 293-302.

Dunbar, B.S. and Schwoebel, E.D., Methods Enzymol., 182 (1990) 663-670.

Dunbar, B.S. and Skinner, S.M., Methods Enzymol., 182 (1990) 670-679.

Dvorak, D.J., Gipps, E. and Kidson, C., Nature, 271 (1978) 564-566.

Edelman, M., Methods Enzymol., 34 (1974) 499-502.

Edelman, G.M. and Rutishauser, U., Methods Enzymol., 34 (1974) 195-225.

Egorov, T.A., Svenson, A., Ryden, L. and Carlsson, J., Proc. Nat. Acad. Sci. U.S., 72 (1975) 3029-3033.

Eisenberg, S., Francesconi, S.C., Civalier, C. and Walker, S.S., Methods Enzymol., 182 (1990) 521-529.

Ersson, B., Aspberg, K. and Porath, J., Biochim. Biophys. Acta, 310 (1973) 446-452.

Feizi, T., Nature, 314 (1985) 53-57.

Filka, K., Čoupek, J. and Kocourek, J., Biochim. Biophys. Acta, 539 (1978) 518-528.

Finn, F.M. and Hofmann, K., Methods Enzymol., 184 (1990) 244-274.

Finn, F.M., Titus, G., Horstman, D. and Hofmann, K., Proc. Nat. Acad. Sci. U.S., 81 (1984) 7328-7332.

Fishel, R., Anziano, P. and Rich, A., Methods Enzymol., 184 (1990) 328-340.

Fritz, H., Gebhardt, B.M., Fink, E., Schramm, W. and Werle, E., Hoppe-Seyler's Z. Physiol. Chem., 350 (1969) 129-138.

Gahmberg, C.G. and Hakomori, S., J. Biol. Chem., 248 (1973) 4311-4317.

Gallop, P.M., Seifter, S. and Meilman, E., J. Biol. Chem., 227 (1957) 891-906.

Genaud, L., Guillot, J., Damez, M. and Coulet, M., J. Immunol. Methods., 22 (1978) 339-346.

Gerard, C., Methods Enzymol., 182 (1990) 529-539.

Gersten, D.M. and Marchalonis, J.J., J. Immunol. Methods, 24 (1978) 305-309.

Ghetie, V. and Sjöquist, J., Methods Enzymol., 108 (1984) 132-138.

Gospodarowicz, D., J. Biol. Chem., 247 (1972) 6491-6498.

Gottvaldová, M., Hrabová, H., Sillinger, V. and Kučera, J., J. Chromatogr., 427 (1988) 331-334.

Grabowski, P.J., Methods Enzymol., 184 (1990) 319-327.

Green, N.M., Biochem. J., 101 (1966) 774-779.

Green, N.M. and Toms, E.J., Biochem. J., 133 (1973) 687-700.

Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J. and Lane, M.D., J. Biol. Chem., 249 (1974) 6633-6645.

Gyka, G., Ghetie, V. and Sjöquist, J., J. Immunol. Methods, 57 (1983) 227-233.

Haeuple, M.T., Aubert, M.L., Djiane, J. and Kraehenbuhl, J.P., J. Biol. Chem., 258 (1983) 305-314.

Hanus, J., Kučera, J., Kodet, J. and Masílková, A., J.Chromatogr., 97 (1975) 270-272.

Harvey, M.J., Lowe, C.R., Craven, D.B. and Dean, P.D.G., Eur. J. Biochem. 41 (1974) 335-340.

Haugen,T., Ishaque, A., Chatterjee, A.K. and Preiss,J., FEBS Lett., 42 (1974) 205-208.

Helman, M. and Givol, D., Biochem. J., 125 (1971) 971-974.

Henrikson, K.P., Allen, S.H.G. and Malloy, W.L., Anal. Biochem., 94 (1979) 366-370.

Herion, P. and Bollen, A., Bioscience Rep., 3 (1983) 373-379.

Herman, T.M., Lefever, E. and Shimkus, M., Anal. Biochem., 156 (1986) 48-55.

Herrick, G., Nucl. Acid. Res., 8 (1980) 3721-3728.

Hey, Y. and Dean, P.D.G., Biochem. J., 209 (1983) 363-371.

Hoffman, W.L. and O'Shannessy, D.J., J. Immunol. Methods, 112 (1988) 113-120.

Hondmann, D.H.A. and Visser, J., J. Chromatogr., 510 (1990) 155-164.

Hořejší, V. and Kocourek, J., Methods Enzymol., 34 (1974) 178-181.

Hsiao, H.Y. and Royer, G.P., Arch. Biochem. Biophys., 198 (1979) 379-385.

Hughes, P., Lowe, C.R. and Sherwood, R.F., Biochim. Biophys. Acta, 700 (1982) 90-100.

Kaplan, L.J., Barr, F.G., Daims, M., Nelson, D. and Tanner, T.B., Prep. Biochem., 14 (1984) 149-161.

Kato, Y., Seita, T., Hashimoto, T. and Shimizu, A., J. Chromatogr., 134 (1977) 204-206.

Kitagawa, Y. and Okuhara, E., Anal. Biochem., 115 (1981) 102-108.

Kito, M., Tawa, R., Takeshima, S. and Hirose, S., J. Chromatogr., 528 (1990) 91-99.

Kohanski, R.A. and Lane, M.D., J. Biol. Chem., 260 (1985) 5014-5025.

Kohanski, R.A. and Lane, M.D., Ann. N.Y. Acad. Sci., 447 (1985a) 373-385.

Köhler, G. and Milstein, C., Nature, 256 (1975) 495-497.

Kopperschläger, G., Böhme, H.J. and Hofman, E., Adv. Biochem. Eng., 25 (1982) 101-138.

Kopperschläger, G., Dieze, W., Freyer, R., Liebe, S. and Hofman, E., Eur. J. Biochem., 22 (1971) 40-45.

Kraehenbuhl, J.P. and Bonnard, C., Methods Enzymol., 184 (1990) 629-641.

Kristiansen, T., Methods Enzymol., 34 (1974) 331-341.

Krug, F., Desbuquois, B. and Cuatrecasas, P., Nature New Biol., 234 (1971) 268-270.

Langone, J.J., J. Immunol. Methods, 55 (1982) 277-296.

Lee, K.B., Loganathan, D., Merchant, Z.M. and Linhardt, R.J., Appl. Biochem. Biotechnol., 23 (1990) 53-80.

Lis, H., Lotan, R. and Sharon, N., Methods Enzymol., 34 (1974) 341-346.

Lis, H. and Sharon, N., J. Chromatogr., 215 (1981) 361-372.

Lotan, R. and Nicolson, G.L., Biochim. Biophys. Acta, 559 (1979) 329-376.

Lowe, C.R., Burton, N., Dilmaghanian, S., McLoughlin, S., Pearson, J., Stewart, D. and Clonis, Y.D., in Vijayalakshmi, M.A. and Bertrand, O., (Editors)Protein-Dye Interactions: Developments and Applications, Elsevier Applied Science, London and New York, 1989, pp. 11-20.

Lowe, C.R. and Dean, P.D.G., Affinity Chromatography, Wiley, New York, London, 1974, pp. 91, 272.

Lowe, C.R., Glad, M., Larsson, P.O., Ohlson, S., Small, D.A.P., Atkinson, T. and Mosbach, K., J. Chromatogr., 215 (1981) 303-316.

Lowe, C,R. and Pearson, J.C., Methods Enzymol., 104 (1984) 97-113.

Lowe, C.R., Trayer, I.P. and Trayer, H.R., Methods Enzymol., 66 (1980) 192-208.

Maestas, R.R., Prieto, J.R., Kuehn, G.D. and Hageman, J.H., J. Chromatogr., 189 (1980) 225-231.

Manderino, G.L., Gooch, G.T. and Stavitsky, A.B., Cell. Immunol., 41 (1978) 264-275.

McLennan, B.D. and Raney, J.P., J. Biol. Chem., 258 (1983) 7592-7596.

Mendonca, M.H. and Zancan, G.T., Arch. Biochem. Biophys., 252 (1987) 507-514.

Merkle, R.K. and Cummings, R.D., Methods Enzymol., 138 (1987) 232-259.

Metcalf, E.C., Crow, B. and Dean, P.D.G., Biochem. J., 199 (1981) 465-472.

Mohr, P. and Pommerening, K., Affinity Chromatography - Practical and Theoretical Aspects, Marcel Dekker, Inc., New York and Basel, 1985, pp. 67, 78, 132, 146-147, 151-156.

Mosbach, K., Biochem. Soc. Trans. 2 (1974) 1294-1296.

Murphy, R.F., Imam, A., Hughes, A.E., McGucken, M.J., Buchanan, K.D., Conlon, J.M. and Elmore, D.T., Biochim. Biophys. Acta, 420 (1976) 87-96.

Nakano, N.I., Oshio, T., Fujimoto, Y. and Amiya, T., J. Pharm. Sci., 67 (1978) 1005-1008.

Nakatani, H., Morita, T. and Hiromi, K., Biochim. Biophys. Acta, 525 (1978) 423-428.

Nilsson, R., Myhre, E., Kronvall, G. and Sjögren, H.O., J. Immunol. Methods, 62 (1983) 241-245.

O'Carra, P., Biochem. Soc. Trans., 2 (1974) 1289-1293.

Okamura, S., Crane, F., Messner, H.A. and Mak, T.W., J. Biol. Chem., 253 (1978) 3765-3767.

O'Neil, E.A. and Kelly, T.J., J. Biol. Chem., 263 (1988) 931-937.

Oren, M., Winocour, E. and Prives, C., Proc. Natl. Acad. Sci. U.S.A., 77 (1980) 220-224.

O'Shannesy, D.J. and Hoffman, W.L., Biotechnol. Appl. Biochem., 9 (1987) 488-496.

Ostrove, S., Methods Enzymol., 182 (1990) 357-371.

Parekh, R.B., Dwek, R.A., Sutton, B.J., Fernandes, D.L., Leung, A., Stanworth, D., Rademacher, T.W., Mizuochi, T., Taniguchi, T., Matsuta, K., Takeuchi, F., Nagano, Y., Miyamoto, T. and Kobata, A., Nature, 316 (1985) 452-457.

Parikh, I. and Cuatrecasas, P., Methods Enzymol., 34 (1974) 610-619.

Pemberton, R.E., Liberti, P. and Baglioni, C., Anal. Biochem., 66 (1975) 18-28.

Phillips, T.M., in A.R. Kerlavage (Editor), The Use of HPLC in Receptor Biochemistry, Alan R. Liss, Inc., New York, 1989, pp. 129-154.

Phillips, T.M., Queen, W.D., More, N.S. and Thompson, A.M., J. Chromatogr., 327 (1985) 213-219.

Pollard, K.M. and Webb, J., J. Immunol. Methods, 54 (1982) 81-94.

Porath, J., Trends Anal. Chem., 7 (1988) 254-259.

Prisyazhnoy, V.S., Fusek, M. and Alakhov, Y.B., J. Chromatogr., 424 (1988) 243-253.

Ramadoss, C.S., Steczko, J., Uhlig, J.W. and Axelrod, B., Anal. Biochem., 130 (1983) 481-484.

Rassi, Z.E., Truei, Y. and Horvath, C., Makromol. Chem., Macromol.Symp., 17 (1988) 305-319.

Rhoads, R.E. and Hellmann, G.M., J. Biol. Chem., 253 (1978) 1687-1693.

Richardson, P.J. and Luzio, J.P., Appl. Biochem. Biotech., 13 (1986) 133-145.

Rosenfeld, P.J. and Kelly, T.J., J. Biol. Chem., 261 (1986) 1398-1408.

Šafařík, I., J. Chromatogr., 261 (1983) 138-141.

Schnaar, R.L., Anal. Biochem., 143 (1984) 1-13.

Schott, H., FEBS Lett., 41 (1974) 203-205.

Schott, H., Affinity Chromatography - Template chromatography of nucleic acids and proteins, Chromatographic Science Series, Vol. 27, Marcel Dekker, New York, Basel, 1984, pp. 1-234.

Schott, H., Eckstein, H. and Bayer, E., J. Chromatogr., 99 (1974) 31-34.

Schott, H., Eckstein, H., Gatfield, I. and Bayer, E., Biochemistry, 14 (1975) 5541-5548.

Schott, H., Rudloff, E., Schmidt, P., Roychoudhury, R. and Kössel, H., Biochemistry, 12 (1973) 932-938.

Scopes, R.K., J. Chromatogr., 376 (1986) 131-140.

Scopes, R.K., in R.K. Scopes Protein Purification, Principles and Practice, Springer - Verlag, New York, 2nd ed., 1987a, pp. 126-172.

Scopes, R.K., Anal. Biochem., 165 (1987b) 235-246.

Sharma, S.K. and Mahendroo, P.P., J. Chromatogr., 184 (1980) 471-499.

Solomon, B., Koppel, R., Schwartz, F. and Fleminger, G., J. Chromatogr., 510 (1990) 321-329.

Stead, C.V., in M.A. Vijayalakshmi and O. Bertrand (Editors), Protein - Dye Interactions: Developments and Applications, Elsevier Applied Science, London and New York, 1989, pp. 21-32.

Stellwagen, E., Methods Enzymol., 182 (1990) 343-357.

Stellwagen, E., Cass, R., Thompson, S.T. and Woody, M., Nature, 257 (1975) 716-718.

Thelohan, S., Jadaud, P. and Wainer, I.W., Chromatographia, 28 (1989) 551-555.

Thomas, T.E., and Lansdorp, P.M., J. Immunol. Methods, 112 (1988) 219-226.

Thompson, S.T., Cass, K.H. and Stellwagen, E., Proc. Nat. Acad. Sci. U.S., 72 (1975) 669-672.

Tosa, T., Sato, T., Sano, R., Yamamoto, K., Matuo, Y. and Chibata, I., Biochim. Biophys. Acta, 334 (1974) 1-11.

Trayer, I.P and Trayer, H.R., Biochem. J., 141 (1974) 775-787.

Turková, J., Beneš, M.J., Kühn, M., Stepanov, V.M. and Lyublinskaya, L.A., in J. Chaloupka and V. Krumphanzl (Editors), Extracellular Enzymes of Microorganisms, Plenum Press, New York and London, 1987, pp. 99-103.

Turková, J., Fusek, M., Maksimov, J.J. and Alakhov, Y.B., J. Chromatogr., 376 (1986) 315-321.

Turková, J., Fusek, M., Šťovíčková, J. and Králová, Z., Makromol. Chem. Macromol. Symp., 17 (1988) 241-256.

Turková, J., Hubálková, O., Křiváková, M. and Čoupek, J., Biochim. Biophys. Acta, 322 (1973) 1-9.

Turková, J. and Seifertová, A., J. Chromatogr., 148 (1978) 293-297.

Turková, J., Vavreinová, S., Křiváková, M. and Čoupek, J., Biochim. Biophys. Acta, 386 (1975) 503-508.

Updyke, T.V. and Nicolson, G.L., J. Immunol. Methods, 73 (1984) 83-95.

Vančurová, D., Turková, J., Čoupek, J. and Frydrychová, A., Collect. Czech. Chem. Commun., 44 (1979) 3405-3410.

Van Den Burg, B., Eijsink, V.G.H., Stulp, B.K., and Venema, G., J. Biochem. Biophys. Meth., 18 (1989) 209-220.

Van Etten, R.L. and Waheed, A., Arch. Biochem. Biophys., 202 (1980) 366-373.

Vijayalakshmi, M.A. and Bertrand, O., (Editors) Protein - Dye Interactions: Developments and Applications, Elsevier, Applied Science, London and New York, 1989, pp. 1-342.

Voříšek, J., Histochemistry, 92 (1989) 421-432.

Wallach, D.F.H., Kranz, B., Ferber, E. and Fischer, H., FEBS Lett., 21 (1972) 29-33.

Weber, D.V. and Bailon, P., J. Chromatogr., 510 (1990) 59-69.

Weber, D.V., Keeney, R.F., Familleti, P.C. and Bailon, P., J. Chromatogr., 431 (1988) 55-63.

Werner, S. and Machleidt, W., Eur. J. Biochem., 90 (1978) 99-105.

Wetekam, W., Mullinix, K.P., Deeley, R.G., Kronenberg, H.M., Eldridge, J.D., Meyers, M. and Goldberger, R.F., Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 3364-3368.

Wilchek, M., FEBS Lett., 7 (1970) 161-163.

Wilchek, M., Advan. Exp. Med. Biol., 42 (1974) 15-31.

Wilchek, M. and Bayer, E.A., Methods Enzymol., 184 (1990) 1-746; (1990a) 14-45.

Yamashita, K., Hitoi, A., Irie, M. and Kobata, A., Arch. Biochem. Biophys., 250 (1986) 263-266.

Yoon, J.W., Kenyon, A.J. and Good, R.A., Nature New Biology, 245 (1973) 205-207.

This Page Intentionally Left Blank

Chapter 4

General considerations on affinant - sorbent bonding

The bonding between the monomeric affinant, for example the inhibitor I, and the enzyme E is expressed by the equilibrium constant of the reaction, K_I, on the supposition that the enzyme exists in a single tertiary conformation:

$$E+I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (4.1.)$$

$$K_I = \frac{[E][I]}{[EI]} \; ; \; \Delta G_I = -RT \ln K_I$$

When the affinant is bound to the solid support, the equilibrium constant, K_I, is affected to a certain extent. An increase in K_I brings about a modification of the affinant by binding to the matrix. The steric accessibility of the affinant is limited as a consequence of this binding. On the other hand, a decrease in K_I causes non-specific adsorption of the enzyme to the solid support and to the molecules of the already adsorbed enzyme. Assuming that a single enzyme of the crude protein has an affinity for the matrix, the equilibrium between the bound affinity ligand L and the isolated enzyme E is given by the equation:

$$E+L \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} EL \qquad (4.2.)$$

$$K_L = \frac{[E][L]}{[EL]} \; ; \; \Delta G_L = -RT \ln K_L$$

The successful isolation of an enzyme by bioaffinity chromatography requires a very small K_I or K_L for the desired enzyme. Both constants should be much smaller than any dissociation constant for adsorption between the protein and matrix surface (i.e. nonspecific adsorption). The maximum K_L can be estimated as follows. Starting from a 10^{-3} mole/l concentration of inhibitor in the insoluble affinant and the requirement of

99% retention of the enzyme from the raw material which contains about 10^{-5} mole/l of enzyme in a three-fold volume of the insoluble affinant, a K_I value of 10^{-4} mole/l is obtained as the upper limit for an effective affinant. In a 3 % protein solution, where the active enzyme constitutes 10 % of the total protein, which should have a molecular weight of 10^5, about 10 % of the capacity of the matrix is utilized under the above conditions.

From this estimate, it further follows that in view of the bond that can be formed between the inhibitor and the enzyme, the whole purification process should be considered to be one of precipitation rather than chromatography. This can also be shown by means of the adsorption isotherm for bioaffinity chromatography, shown in Fig. 4.1, from which it is evident that the gross adsorption isotherm (curve 3) can be defined as the sum of the specific (curve 1) and non-specific (curve 2) adsorption isotherms. The specific adsorption isotherm characterizes the ideal specific adsorption when the adsorption energy, Δ G, for all adsorbed particles is constant and relatively large.

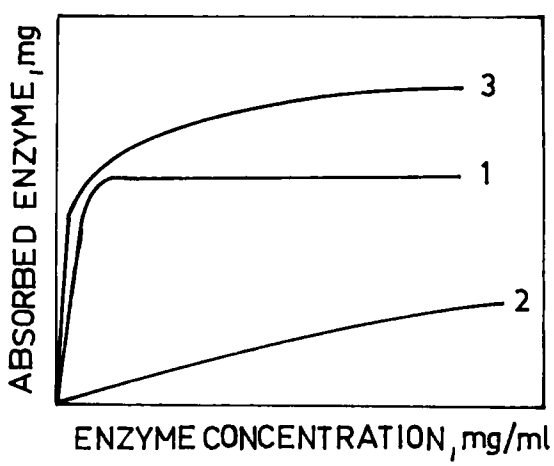


Fig. 4.1. Adsorption isotherms for bioaffinity chromatography: 1, ideal biospecific adsorption; 2, non-specific adsorption; 3, experimentally found adsorption.

Adsorption ceases when all accessible "affinant" sites are occupied. The non-specific adsorption isotherm characterizes the adsorption of proteins on non-specific sites of the matrix and on already adsorbed protein.

The ΔG_L value is the sum of ΔG_I and $\Delta G_{non\text{-}sp.}$, where $\Delta G_{non\text{-}sp.}$ is the reaction energy of non-specific complexation and hindrance. Taking a mean value of 10^{-5} mole/l for K_I, a value of about 7 kcal/mole is obtained for ΔG_I.

The adsorption energy for non-specific adsorption, $\Delta G_{non\text{-}sp.}$, results from the hydrophobic, hydrophilic or even ionic interactions and is comparable to the adsorption energy in normal chromatography. It depends very much on the nature of the solid carrier and the protein. $\Delta G_{non\text{-}sp.}$ should be as low as possible because it also includes the adsorption of molecules that form non-specific complexes with the affinant.

There are, however, instances in which the crude protein contains two or more enzymes that display affinity for the bound affinant. If the equilibrium constant of the reaction of the second enzyme, $K_I(II)$, is greater than 10^{-3} mole/l, then only minute amounts of the second enzyme will be retained together with the enzyme sought. If $K_I(II)$ is less than or equal to 10^{-4} mole/l, then a mixture of both enzymes will be adsorbed, even though the K_I value of the desired enzyme may be much less than $K_I(II)$. This follows from the specific form of the adsorption isotherm for bioaffinity chromatography, because the heat of adsorption is extremely high under chromatographic conditions. If $K_I(II)$ differs from K_I by more than 50-100, a separation can still be achieved if differential elution is applied, for example, or if the isolation is carried out by a batch process, using an amount of the insoluble affinant that corresponds exactly to the more intimately binding enzyme, or if chromatography during which equilibrium between [E(II)•I] and [E(I)•I] must be attained is very slow.

The difference between ΔG_I and ΔG_L is given by the change in steric accessibility of the affinity ligand after its immobilization, by its modification due to its binding on the carrier, by the nature of the solid support, etc.

4.1 STERIC ACCESSIBILITY

4.1.1 Nonporous and highly porous solid supports

The basic requirement for successful bioaffinity chromatography is that the formation of the complex of the macromolecular substance with the affinant covalently bound to the solid support should correspond to the formation of their complex in solution. This requires, above all, sufficient space, especially if we have to deal with the interaction of substances of high molecular weight. For this reason, using nonporous or highly porous solid supports is one of the most important requirements.

Adachie et al. (1984) compared nonporous and porous glass matrices in order to eliminate the kinetic limitation on access of the determined pyruvate, L-lactate and glutamic pyruvic transaminase to immobilized lactate dehydrogenase. Swaisgood and Chaiken (1985) have shown the utility of analytical bioaffinity chromatography with nonporous glass beads for the evaluation of thermodynamic and kinetic properties of biospecific interactions using the neurophysin-hormone interactions as a model system. A support based on pyrogeneous silicon dioxide of particle size 0.01 to 0.1 μm, modified with 3-(amino)propyltriethoxysilane and activated with glutaraldehyde was employed for the immobilization of concanavalin A, immunoglobulins, basic pancreatic trypsin inhibitor, and chymotrypsin by Fusek et al. (1987). Anspach et al. (1989) introduced nonporous, microparticulate, monodisperse silicas with particle diameters between 0,7 and 2,1 μm as stationary phases in high-performance bioaffinity chromatography. Immobilized phenylboronic acid was applied to the isolation of nucleosides, nucleotides, and glycoprotein hormones such as bovine follicotropin and human chorionic gonadotropin, while immobilized benzamidine was employed for the isolation of the serine proteases thrombin and trypsin, immobilized tri-L-alanine for the separation of pig pancreatic elastase and human leukocyte elastase, and immobilized concanavalin A for the isolation of horseradish peroxidase. In all bioaffinity chromatography systems studies, the nonporous monodisperse silicas showed improved chromatographic performance in comparison with results obtained with porous silica supports using identical activation and immobilization procedures.

Ivanov et al. (1990) used wide-porous and nonporous glass beads (Sikron and Cabosil) after the binding of 3-(amino)propyltriethoxy-silane for the immobilization of proteinase pepsin. Properties of the mineral supports and immobilized pepsin are given in Table 4.1.

Table 4.1.

Properties of the mineral supports and immobilized pepsins

Support	*Wide porous glass (WPG-C3)*	*Sikron*	*Cabosil*
Particle diameter, μm	60-80	1-2	0.01-0.1
Pore diameter, Å	2100	-	-
NH_2-group content, μmoles.g^{-1}	180	150	1100
Surface area, $m^2 . g^{-1}$	50	2.3	200
Pepsin content, nmoles . g^{-1}	7.2	6.9	22
Proteolytic activity, Units . g^{-1}	3.0	0.8	5.6
Retained activity of immobilized pepsin	99%	26%	64%

The affinity fibre has been developed as a support for HPLAC by Wikström and Larson (1987). Nonporous quartz fibre with a mean diameter of 0.5 μm was silylated with mercaptopropyltrimethoxy-silane. Tresyl chloride-activated dextran was covalently coupled to the SH groups on the fibre. Remaining active tresyl groups on the dextran were then coupled with an NAD derivative (Fig. 4.2). The affinity fibre contained 0.3 μmol NAD derivative per g and was able to bind 15 mg of lactate dehydrogenase per g of fibre. The affinity fibre was used for large-scale purification of ox heart lactate dehydrogenase and its performance was compared with other commonly used chromatographic matrices. The operational capacity was found to be 1.0 g of pure lactate dehydrogenase per hour per 100 g of fibre material. The affinity fibre was found to be particularly suitable for very rapid processing of large volumes of dilute enzyme solutions.

Extraction of circulating gastrointestinal cancer antigen (GICA) using an immunoadsorption system of monoclonal antibody immobilized on a polyolefin alloy fiber was described by Pak et al. (1984). Continuous circulation or single passage of plasma

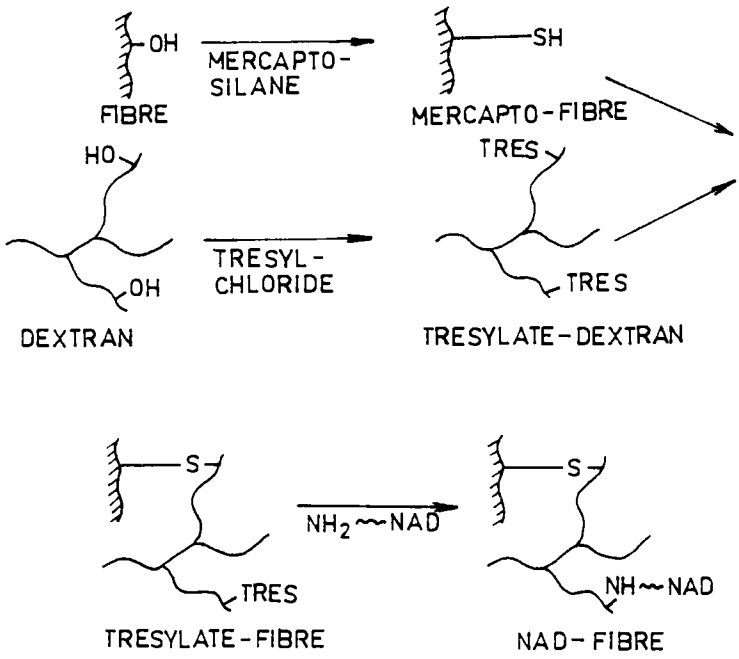


Fig. 4.2. Fibre derivatization procedure.

from gastrointestinal cancer patients through this antibody-microporous-fiber matrix resulted in 90% depletion of circulating GICA in 2 h using 0.6 mg immobilized antibody, and 90 % depletion in 5 min using 8 mg antibody. Continual circulation resulted in total GICA removal in both cases. Desorption of antibody or of antibody-containing complexes was minimal. This technique provides a selective and convenient means of removing any targeted substance by monoclonal antibody from the serum, and thus overcomes many of the shortcomings associated with conventional plasmapheresis.

Membrane filtration bioaffinity purification has been summarized by Ling and Mattiasson (1989). In this paper they used silica nanoparticles with size 12 nm and attached Cibacron Blue dye for effective purification of alcohol dehydrogenase and lactate dehydrogenase.

The effect of gel porosity on the accessibility of the immobilized affinity ligand necessary for complex formation with the complementary macromolecule is shown in Fig. 4.3.

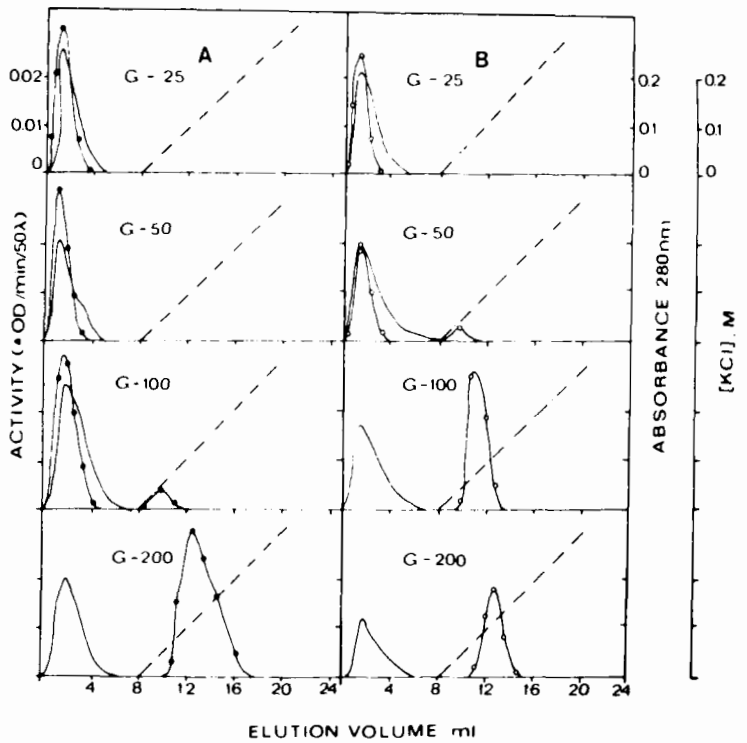
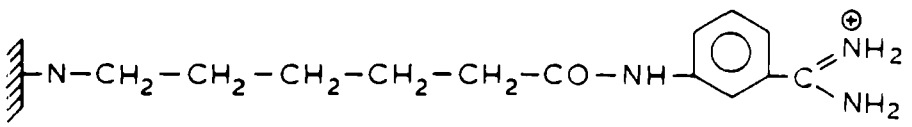


Fig. 4.3. Affinity gel filtration of synthetic mixtures of (A) lactate dehydrogenase (LDH), and (B) malate dehydrogenase (MDH), with bovine serum albumin (BSA) on NAD-Sephadex of various pore sizes.
A 50 μl sample containing 1.85 units of LDH (or 0.335 units of MDH) and 0.8 mg of BSA was applied to a 20 x 5 mm column of the appropriate NAD-Sephadex equilibrated with 10 mM phosphate buffer, pH 7.5. Non-absorbed protein was washed off with the same buffer and the column was eluted with a 0-0.5 M KCl gradient in 10 mM phosphate buffer, pH 7.5; 20 ml total. LDH (•), MDH (o) and BSA (-) were assayed in the effluent. Reproduced with permission from C.R. Lowe and P.D.G. Dean, FEBS Lett., 18 (1971) 31-34.

Lowe and Dean (1971) demonstrated the effect of the degree of porosity of the Sephadex matrix on the binding of lactate dehydrogenase and malate dehydrogenase in a mixture with serum albumin on immobilized NAD^+. On a column of a highly crosslinked Sephadex G-25 with bonded NAD^+, both dehydrogenases as well as serum albumin appear in the hold-up volume of the columns, because the immobilized ligand is inaccessible to enzymes. The NAD^+-Sephadex G-100 complex had sorbed malate dehydrogenase, while most of the lactate dehydrogenase of higher molecular weight

passed through with the serum albumin. Both dehydrogenases were then sorbed on NAD^+-Sephadex G-200. The force of interaction between the enzyme and the immobilized NAD^+ increases with increasing gel porosity, as it is evident from the potassium chloride concentration necessary for the elution of enzymes when a linear potassium chloride gradient was used. The exclusion of dehydrogenases from affinity sorbents prepared from gels of various pore sizes was used by Lowe and Dean (1971) as a microscale method for the rapid determination of apparent molecular weights by so-called affinity gel filtration.

Another example of the substantial effect of gel porosity on the capacity of the affinity sorbent is the discovery that the amount of sorbed trypsin on 6 % and 4 % agarose substituted with m-aminobenzamidine



depends on the concentration of trypsin (Nishikawa et al., 1974). Assuming a modest binding constant, Nishikawa et al. deduced, on the basis of adsorption theory, a hypothetical system in which they plotted the amount of the enzyme-ligand complex formed, [EL], as a function of the concentration of enzymes in the sorption solution, [E], for three fixed values of the ligand concentration, L_0, as shown in Fig. 4.4A.

By plotting [E] on a logarithmic scale they obtained a sigmoidal binding pattern. They made the following assumptions: (1) the ligand in the gel behaves in the same manner as a corresponding, freely dissolved, molecule; in fact the immobilization of the affinity ligand causes a loss of at least one degree of freedom in translation entropy; (2) the concentration of the gel ligand, [L], closely approximates the concentration measured per unit volume of gel; (3) the enzyme interacts freely with all accessible ligands, while the inaccessible ligands have no influence on the binding potential of the enzyme; and (4) the solid support has no effect on the enzyme-ligand bond, except for steric exclusion of some ligands. Fig. 4.4B shows that the amounts of the bound trypsin established

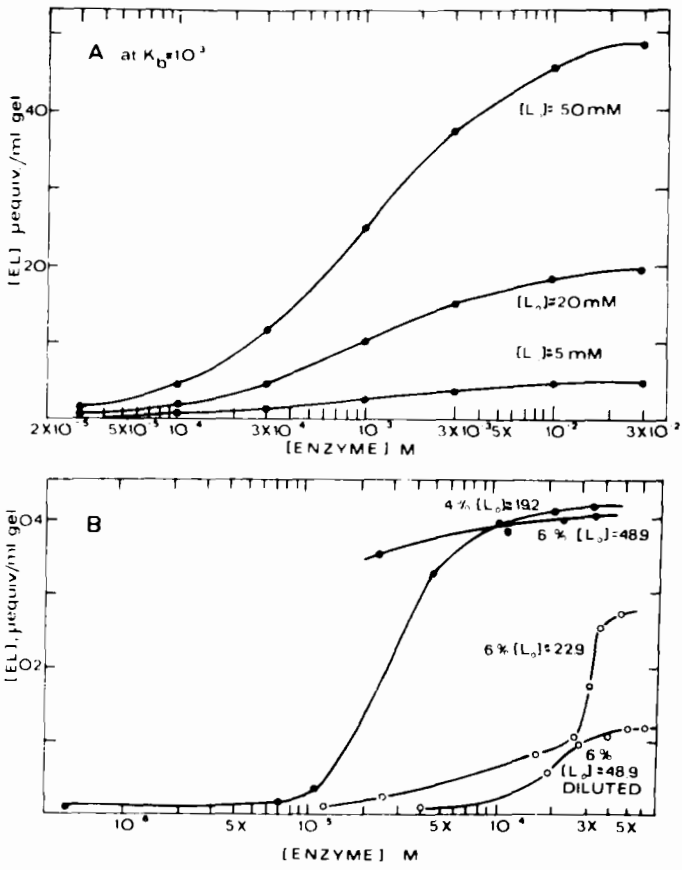


Fig. 4.4. (A) Ideal enzyme binding plot; (B) trypsin binding to different gels. Reproduced with permission from A. H. Nishikawa et al., Advan. Exp. Med. Biol., 42 (1974) 33-42.

experimentally during the equilibrium binding studies are dependent on the concentration of the starting trypsin solution (in 0.05 M bicin, pH 8.15, and 0.25 M potassium chloride solution; 4 h at 4°C). From this figure it is evident that only the bioaffinity adsorbent prepared from 4% agarose gel containing 19.2 μequiv./ml of m-aminobenzamidine approaches ideal behaviour. This carrier had a substantially higher saturation capacity than the bioaffinity adsorbent prepared from 6% agarose containing 22.9 μequiv./ml of m-aminobenzamidine; at higher concentrations of trypsin it was similar to a 6 % gel containing 48.9 μequiv./ml. Fig. 4.4B also shows the saturation

capacity of a high ligand gel (L_0 = 48.9 μequiv./ml) which was diluted with the same volume of non-modified 6% agarose. The resulting concentration of the ligand in the gel is thus L_0 = 24.45 μequiv./ml. However, the binding curve is substantially lower than in 6% gel with L_0 = 22.9 μequiv./ml. The properties of bioaffinity sorbents diluted with unmodified gels are discussed in Section 4.3.

Of course, there are instances where the gel porosity does not have any effect on the interaction of the macromolecules with an immobilized affinant, such as in systems with extremely large macromolecules, where only affinity ligands immobilized on the surface of the beads take part in complex formation. An example is the bioaffinity chromatography of polysomes, ribosomes, intact cells, organelles or membrane fractions. In these instances penetration in the bead pores can hardly be expected. As an example one might mention the high capacity of albuminated CIBA blue dextran-staphylococcal Protein A-conjugated Sepharose 6MB bioaffinity columns for the fractionation of antibody-coated lymphocytes. The capacity of the columns permitted quantitative separation of T and B lymphocytes (Duffey et al., 1981). For rapid routine analysis of cloned murine T helper cells De Bell et al. (1990) coated polystyrene latex beads with anti-T cell receptor (TCR) monoclonal antibodies. T lymphocyte aggregation with immobilized anti-TCR-antibodies is dependent on energy and microfilament assembly. Additional examples of biospecific sorption of cells and their fractions are given in Table 9.1.

4.1.2 Spacer molecules

In order to attain good accessibility for the immobilized affinity ligands and the binding sites of biological macromolecules it is necessary to have more than just a high solid support porosity. The chemical groups of the affinant that participate in the interaction with the macromolecular substance must also be sufficiently remote from the surface of the solid matrix in order to avoid steric hindrance.

The importance of the spacing of the low molecular weight inhibitor from the surface of the rigid matrix in regard to the course of bioaffinity chromatography was illustrated by Cuatrecasas et al. (1968) in one of the first successful applications of this method in

the isolation of enzymes. Fig. 4.5 represents the affinity chromatography of α-chymotrypsin, both on Sepharose coupled with ε-aminocaproyl-D-tryptophan methyl ester (A) and on Sepharose coupled with D-tryptophan methyl ester (B), in comparison with chromatography on unsubstituted Sepharose (C).

In the first instance (A), the bonded inhibitor has a high affinity for α-chymotrypsin and the enzyme can be released from the complex only by decreasing the pH of the

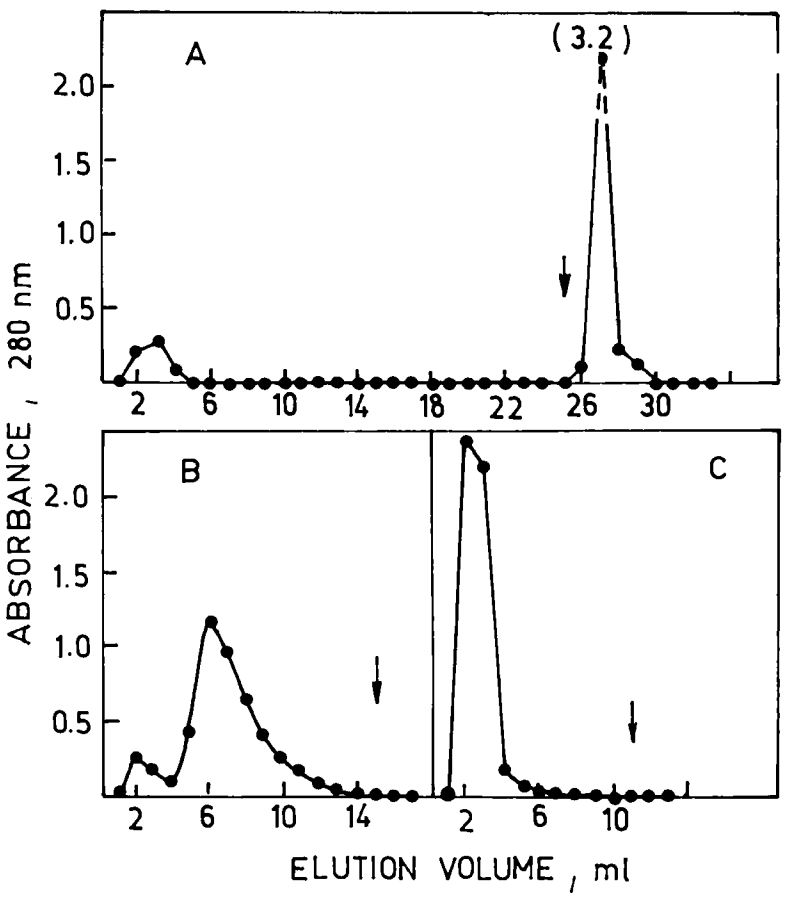


Fig. 4.5. Bioaffinity chromatography of α-chymotrypsin on inhibitor Sepharose columns. The columns (50 x 5 mm) were equilibrated and run with 0.05 M Tris-hydrochloric acid buffer of pH 8.0. Each sample (2.5 mg) was applied in 0.5 ml of the same buffer. The columns were run at room temperature with a flow-rate about 40 ml/h and fractions containing 1 ml were collected. The arrows indicate a change of elution buffer (0.1 M acetic acid, pH 3.0). (A) Sepharose coupled with ε-aminocaproyl-D-tryptophan methyl ester; (B) Sepharose coupled with D-tryptophan methyl ester; (C) unsubstituted Sepharose. The first peaks in A and B were devoid of enzyme activity. Reproduced with permission from P. Cuatrecasas et al., Proc. Nat. Acad. Sci. U.S., 61 (1968) 636-643.

eluting buffer. By using 0.1 M acetic acid of pH 3.0, the chymotrypsin fraction is eluted as a sharp peak and the volume of the eluted chymotrypsin does not depend on the volume of the sample applied to the column. In the second instance (B), the inhibitor coupled directly on Sepharose has a much lower affinity for the isolated α-chymotrypsin, owing to steric hindrance. In this instance a change of buffer is not necessary for enzyme elution and, as it can be seen from the graph, the enzyme is eluted in a much larger volume closely after the inactive material. In order to verify that non-specific adsorption on the carrier did not take place under the given experimental conditions, the chromatography of α-chymotrypsin on an unsubstituted carrier was carried out (C).

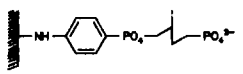
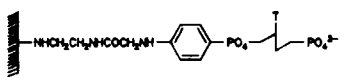
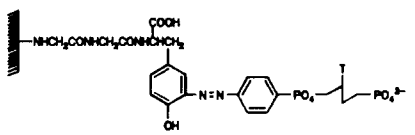
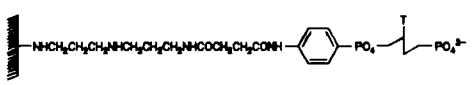
However, as it was observed, the method described for the chromatography of chymotrypsin on unsubstituted Sepharose does not provide sufficient proof of non-specific sorption. By contrast, Hofstee (1973) demonstrated for Sepharose with bonded ε-aminocaproyl-D-tryptophan methyl ester that it, for example, does sorb serum albumin or γ-globulin completely non-specifically. Thus, it was found that a series of substances, both enzymes and substances such as immunoglobulin, serum albumin and ovalbumin, contain hydrophobic regions on the surface of their molecules, by which they are capable of being bound to hydrophobic spacers, such as hexamethylenediamine or ε-aminocaproic acid. The utilization of this phenomenon for the separation of a number of biological macromolecules gave rise to the so-called hydrophobic (affinity) chromatography (Shaltiel 1974, 1984; Kennedy 1990).

In view of the different structures of the substances isolated, the affinant should be located at such a distance from the carrier surface that the bond does not require the deformation of the isolated substance. The effect of the distance of the affinant 3'-(4-aminophenylphosphoryl) deoxythymidine-5'-phosphate from the solid support surface (both Sepharose 4B and Bio-Gel P-300) on the capacity of the gel in the chromatography of staphylococcal nuclease (Cuatrecasas, 1970) is shown in Table 4.2.

In type A, the inhibitor is bound directly to the matrix, and in other types a chain of varying length is inserted between it and the carrier surface. Hipwell et al. (1974) carried out the chromatography of several dehydrogenases on N^6-ω-aminoalkyl-AMP-Sepharose. The strength of interaction can be derived from the concentration of potassium

Table 4.2.

Capacity of insoluble affinants prepared by binding 3'-(4-aminophenyl-phosphoryl)deoxythymidine-5'-phosphate on Sepharose 4B and Bio-Gel P-300 derivatives in the affinity chromatography of Staphylococcal nuclease.

Type of inhibitor bound on matrix	*Structure*	*Capacity (mg of nuclease per ml of gel) on derivative of*	
		Sepharose 4B	*Bio-Gel P-300*
A		2	0.6
B		8	2
C		8	3
D		10	

chloride necessary for the release of dehydrogenase from its complex with nucleotide. The effect of the length of the spacer arm on the binding (β) of several dehydrogenases on N^6-ω-aminoalkyl-AMP-Sepharose is evident from Fig. 4.6.

The binding (β) of two isoenzymes of lactate dehydrogenases increases rapidly from n = 2 to n = 5. When the spacer arm is lengthened further, the elution must be enhanced by a pulse produced by the addition of a small amount of NADH. The binding of malate dehydrogenase, D-glucose-6-phosphate dehydrogenase and D-glyceraldehyde-3-phosphate dehydrogenase is significantly weaker than of lactate dehydrogenase. Nonetheless, in polymers where the number of CH_2 groups k > 7, it does not seem that the binding of enzyme would change substantially. It is further evident from Fig. 4.6 that,

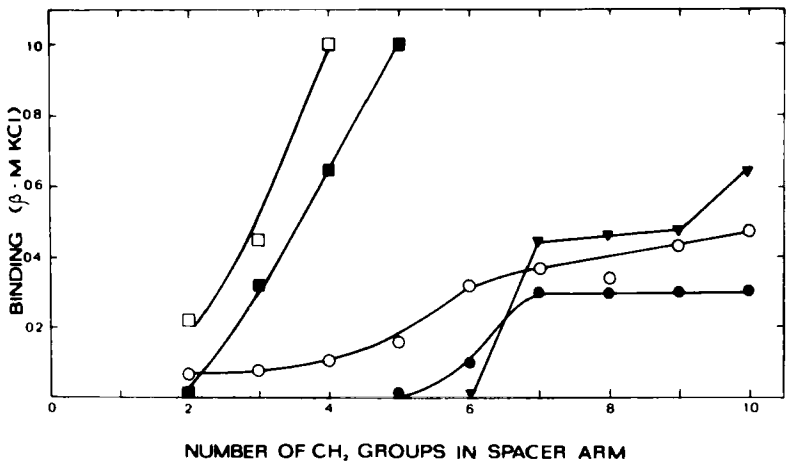


Fig. 4.6 Effect of spacer arm length on the binding of several dehydrogenases to N^6-ω-aminoalkyl-AMP-Sepharose. Columns of the modified gels (50 x 5 mm) were equilibrated at 4°C in 10 mM KH_2PO_4 - KOH at pH 7.5 containing 0.02% sodium azide. The enzyme-protein sample was run into a moist bed of each polymer and developed by washing with several bed volumes of equilibration buffer, a linear gradient of KCl (0 to 1.0 M; 20.0 ml total volume) followed by a 200 μl "pulse" of 5 mM NADH applied to the column in the same way as the enzyme-protein mixture. The column flow-rate was maintained at 8.0 - 10.0 ml/h and 1.4-ml fractions were collected. Binding represents the concentration of KCl (20°C) required to elute the enzyme. Lactate dehydrogenase-H_4 (4 U,); lactate dehydrogenase-M_4 (4 U,); D-glucose-6-phosphate dehydrogenase (2 U, ●); malate dehydrogenase (4 U, o); and D-glyceraldehyde-3-phosphate dehydrogenase (2.5 U,). Reproduced with permission from M.C. Hipwell et al., FEBS Lett., 42 (1974) 355-359.

for lactate dehydrogenase, at least four methylene groups are necessary in order to achieve binding of enzyme on the immobilized nucleotide. It is considered that the use of an extension arm with a length of at least 0.5 nm enables the nucleotide to traverse the barrier imposed by the microenvironment associated with the hydrophilic polymer (Lowe et al., 1973). This can be caused by the ordered layer of water molecules surrounding the matrix backbone and limiting diffusion in this region, or by the vibrational movement of the lattice. In any event, the region of the solvent in close proximity to the surface of the solid support represents a real barrier for the interaction of the macromolecule with the complementary affinity ligand, especially if an interaction with weak affinity is concerned. A spin-labelling investigation of a variety of long spacers was carried out by Aplin and Hall (1980). They attached nitroxide spin labels to CNBr-activated Sepharose 4B directly and through oligoglycines and ω-amino-carboxylic acids of varying length. They concluded that the ideal spacer length is 12 atoms, when the

mobility provided can be equated with accessibility of the affinity ligands without any increase in non-specific adsorption. This is in good agreement with data gained from their summary of published reports on varieties of spacer arm in bioaffinity separation. However, it should be pointed out that, where the complementary macromolecule has a low apparent molecular weight or a high affinity for the immobilized ligand, the length of the spacer arm is not as critical as in the case of large proteins or with systems of low affinity.

The use of macromolecular spacer molecules constructed by coupling denatured albumin, polylysine, polymers containing only D-amino acids (resistant to proteolysis by proteinases), polyornithine or polyvinylalanine to a suitable matrix and suitable ligand may have advantages for the bioaffinity chromatography of a number of sensitive biochemical substances (Lowe, 1979). For example, the general utility of polymeric spacer molecules may be demonstrated by the purification of the oestradiol receptor (Sica et al., 1973). A conventional multimeric spacer arm composed of several smaller units linked together permitted a 27-fold purification, while the use of albumin increased the purification to 4400-fold and a poly(lysyl-alanine) arm allowed a purification in excess of 100,000-fold.

In general terms, the effects relating to the length and nature of a prospective spacer molecule cannot be divorced either from each other or from the nature of the ligand. The presence of hydrophobic spacer molecules will generate serious problems of non-specific adsorption and O'Carra et al. (1974) have therefore recommended the introduction of polar groups, such as secondary amino, hydroxyl and peptido along the length of the spacer arm in order to break up hydrophobic enclaves. It was claimed that interference by non-biological adsorption was drastically reduced by replacement of the hydrophobic spacer molecules by more hydrophilic ones. However, Lowe (1977) synthesized a series of 8-substituted adenosine 5'-monophosphate (AMP) derivatives bearing spacer arms of the same length but differing degrees of hydrophobicity/ hydrophilicity. These derivatives exhibited similar competitive inhibition constants in free solution, although the AMP derivative attached to Sepharose 4B *via* a hydrophobic hexamethylene arm tightly bound a series of NAD^+-dependent dehydrogenases, as

well as the use of a hydrophilic arm containing a peptide linkage and a carbinol grouping, were totally ineffective. On the other hand, the use of a hydrophilic spacer prevents an undesirable possibility that can occur when a hydrophobic affinant is bonded to a long, flexible hydrophobic chain. This can itself then interact with the spacer and become masked or occluded. Such a "conformational occlusion" can be the reason for the inaccessibility of the immobilized affinant for complex formation with the substance isolated. The bioaffinity chromatography of trans-N-deoxyribosylase (Holguin and Cardinaud, 1975) is a practical example. When using Sepharose-N^6-p-amino-n-hexyladenine, Holguin and Cardinaud assumed that under the influence of the flexible chain the active part of the ligand came into close proximity to the solid support and that therefore an effective interaction with the transfer enzyme could not take place.

The importance of the character of the spacer moiety was also shown by Inoue et al. (1981). The affinity of rat liver glutathione S-transferases for an S-carbamidomethyl glutathione derivative of an ω-aminoalkyl Sepharose was found to increase with increasing carbon chain lenght of aliphatic diamine spacers employed (Fig 4.7.). These findings suggest that hydrophobic character of the spacer moiety might be important for the interaction of the adsorbent with the enzymes. Among various spacers tested, the lysyl spacer was found to be most specific for glutathione S-transferase (Fig 4.8.). This might be due to the fact that, besides the glutathione binding site, the transferases posses another binding site responsible for the interaction with hydrophobic anions.

These observations reflect the need for a more rational strategy in the design of spacer arms for bioaffinity chromatography. Each purification should be considered independently and if a spacer molecule is necessary, the length required to achieve an optimum separation efficiency should be determined. Great increases of steric accessibility of the affinity complementary binding sites of high molecular weight affinity ligands after oriented immobilization through the carbohydrate moiety or by use of biospecific complex formation with antibodies or other biologically active molecules are discussed in detail in Section 4.3 and in Chapter 10.

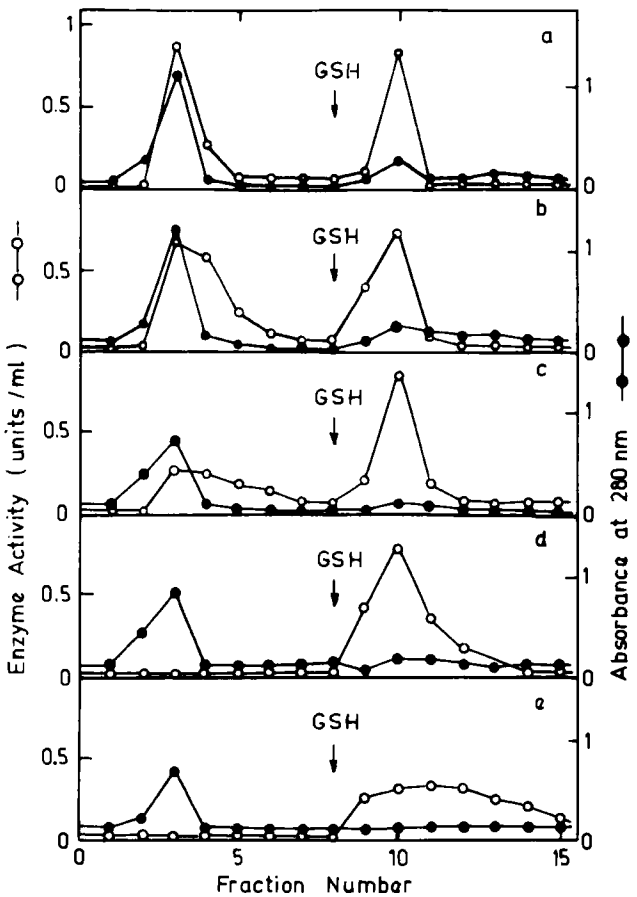


Fig. 4.7. Bioaffinity chromatography of glutathione S-transferases on glutathione-conjugated Sepharose columns containing various spacers. The enzyme samples containing 5 units glutathione S-transferase activity were applied to the column (1 x 3.8 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After washing with 16 ml buffer solution, elution of the enzyme was performed with 10 mM Tris-HCl buffer, pH 8.0/5 mM reduced glutathione (2 ml/fraction). All procedures were carried out at 4°C. Spacers used were : a, ethylenediamine; b, propanediamine; c, hexanediamine; d, octanediamine; e, decamethylenediamine. Arrows shows the change in the elution buffer solution. GSH, reduced glutathione. (o), enzyme activity; (●), absorbance at 280 nm.

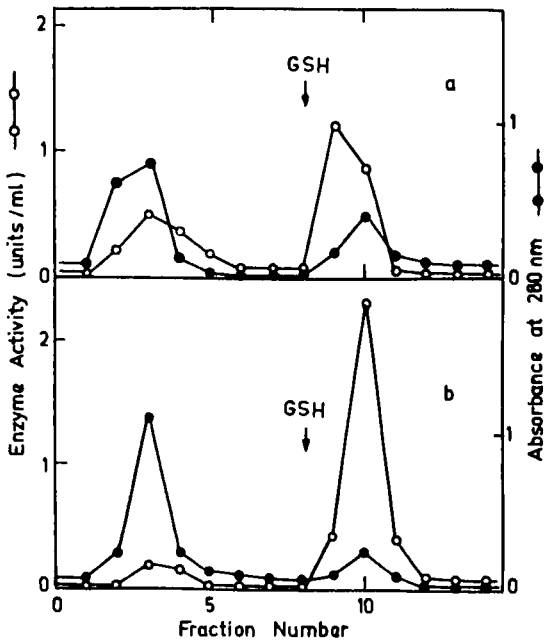


Fig. 4.8. Chromatography on the lysyl or pentanediamine spacer adsorbent. The enzyme samples (7 units) were applied to the columns of lysyl or pentanediamine spacer adsorbent (1 x 3.8 cm). Elution of the enzyme was carried out with 5 mM glutathione. Other conditions were the same as in Fig. 4.7. a, pentanediamine spacer adsorbent; b, lysyl spacer adsorbent. (o), enzyme activity; (•), absorbance at 280 nm. Both figures reproduced with permission from M. Inoue et al., Biochim. Biophys. Acta, 659 (1981) 362–369.

4.2 MICROENVIRONMENTAL EFFECT OF MATRIX NATURE

Studies on the conformation of model peptides in membrane-mimetic environments (Gierasch et al., 1982) permit us to expect that steric accessibility of the reactive group or of the affinity ligand will not only be the result of their distance from the surface of the solid support but will also depend on the interfacial water region, which does not stabilize the same conformation as does bulk water. The arrangement of the solvent layers on the surface of the solid support will also obviously be one of the main factors affecting the penetration of the bound molecules to the surface of the support. As a logical result the mode of immobilization has a greater effect on the interaction of the substances isolated with immobilized low molecular weight ligand than with high molecular weight affinity ligands.

Angal and Dean (1977) studied the effect of matrix on the binding of human serum albumin to the sulphonated aromatic dye Cibacron Blue 3 G-A immobilized on ten solid supports. The adsorption is selective since albumin from rabbit, chicken or bovine sera did not bind to the same immobilized dye. Cibacron Blue was attached to the cellulose (Whatman Biochemicals, Maidstone, Kent, U.K.), the acrylamide-cross linked agarose Sephacryl (Pharmacia, Uppsala, Sweden), the co-polymerized polyacrylamide-agarose mixed gels Ultrogels Ac A54 and Ac A44 (LKB Instruments, Croydon, Surrey, U.K.), agaroses Sepharose 6B, 4B and 2B (Pharmacia G.B. Ltd., London, U.K.) and to epichlorohydrin-crosslinked Sepharose CL6B, CL4B and CL2B. Bioaffinity column supports were compared by frontal analysis. This method supplied the information presented in Table 4.3.

A 16-fold range in ligand concentration was observed on the immobilized dye to the various matrices, despite the similarity of the reaction conditions used. The molar effectiveness of the agarose adsorbents ranged between 0.064 and 0.157 mol of human serum albumin/mol of dye and was significantly higher than the results obtained for cellulose and Ultrogels. Apparent association constants were also similar for all substituted Sepharoses and Sephacryl (24×10^3 - $47 \times 10^3 M^{-1}$), but were at least a factor of 10 lower for cellulose and the Ultrogels. This probably explains the observed lower working capacities of these adsorbents. In the same way Jessup and Dean (1981) examined the effect of the nine different support matrices on the capacity of immobilized Procion Red HE-3B dye for a higher plant 6-phosphogluconate dehydrogenase. Consideration of the respective ligand concentrations, capacities for enzyme, and flow properties of the adsorbents indicated that agarose (Sepharose 6B) or crosslinked agarose (Matrex Gel Red A from Amicon, Woking, Surrey, U.K.) were the preferred supports for the purification of the isolated enzyme.

Human immunoglobulin G (IgG) was immobilized by Taylor (1985) onto acrylate-based activated supports Eupergit C (Rohm Pharma, Weiterstadt, F.R.G.) and Separon HEMA 1000 Emax (TESSEK Ltd., Praha, Czechoslovakia), onto agarose-based activated supports ACT Ultrogel (LKB, Stockholm, Sweden), Affi-Gel 10 (Bio-Rad Labs., Richmond, CA, U.S.A.), CNBr-Sepharose 4B and Epoxy-Sepharose 6B (Pharmacia -

Table 4.3.

Comparison of the properties of different support media. (The value for human serum albumin adsorbed is calculated by difference and after elution with thiocyanate)

Support material	*Ligand concn. (μg/ml of gel)*	*HSA*[1] *adsorbed (mg/ml of gel)*	*HSA eluted by desorption (mg/ml of gel)*	*10^{-3} x Apparent association constant (M^{-1})*
Cellulose	3.2	0.8	0.2	1.1
Sephacryl	1.9	10.7	8.4	25.0
Ultrogel AcA54	0.3	0.2	0.2	3.1
Ultrogel AcA44	0.4	0.3	0.2	4.8
Sepharose 6B	2.3	32.4	20.7	45.3
Sepharose 4B	1.5	15.7	14.1	42.0
Sepharose 2B	0.9	9.1	8.4	35.0
Sepharose CL6B	0.7	5.4	4.2	30.0
Sepharose CL4B	0.4	5.0	4.6	46.8
Sepharose CL2B	0.2	1.7	1.7	24.6

[1] Human serum albumin

PL Bio - Chemicals, Piscataway, N.Y., U.S.A.) and Reacti-Gel 6X (Pierce Chemical Co., Rockford, IL, U.S.A.) and onto glass bead CDI- and NHS-Activated Glycophase (Pierce Chemical Co.). Based on the retention of antibody binding, the best supports for IgG coupling were found to be Separon HEMA (Fig. 4.9) and ACT Ultrogel. The most inefficient supports for IgG coupling were those based on glass beads and the epoxy-Sepharose product.

Thermodynamic and kinetic investigations on rigid and soft bioaffinity gels with varying particle and pore sizes were described by Anspach et al. (1990). The determination of isotherms for the adsorption of lysozyme was done on immobilized Cibacron Blue F3GA dye. As soft-gel chromatographic supports they used Cellufine GC 700 Medium (Amicon, Danvers, MA, U.S.A.), Sepharose CL6B (Pharmacia LKB, Uppsala, Sweden) and Trisacryl GF 2000 (Reactifs IBF, Villeneuve la Garenne, France). Nucleosils 1000-5 and 4000-5 (Machery-Nagel, Düren, F.R.G.) were used as the porous silica-based supports. Nonporous, used silica was made in their laboratories and is commercially available from E. Merck (Darmstadt, F.R.G.). In Fig. 4.10(a) and (b) the equilibrium capacity of lysozyme was plotted against the corresponding protein

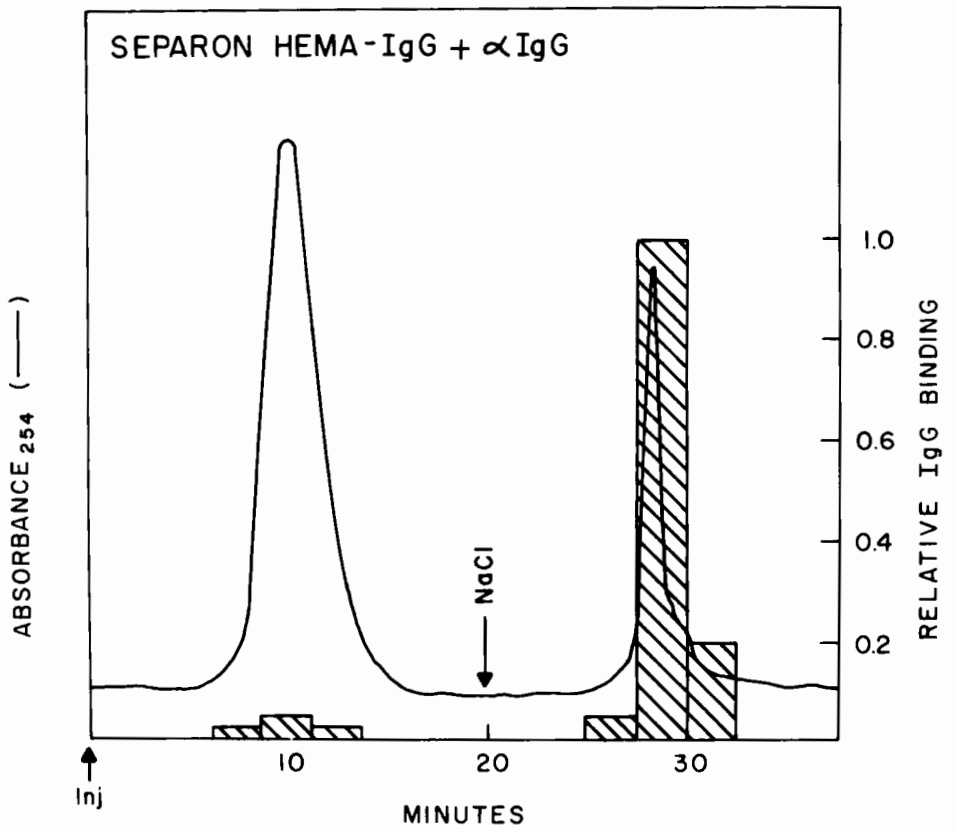


Fig. 4.9. Purification of human IgG antibody by HPLIC on a column packed with Separon HEMA containing immobilized human IgG. Hatched portions indicate isolated antibody concentrations. Reproduced with permission from R.F. Taylor, Anal. Chim. Acta, 172 (1985) 241-248.

concentration at the column inlet. As Anspach et al. (1989) described, the shape of the isotherm can be utilized to evaluate the type of interaction. Adsorption of the protein on the soft gel sorbents in Fig. 4.10(a) gives typical Langmuir-type isotherms. Adsorption on silica-based bioaffinity sorbents in Fig. 4.10(b) gives rectangular-shaped isotherms for some of the sorbents due to non-specific interactions with the silica surface.

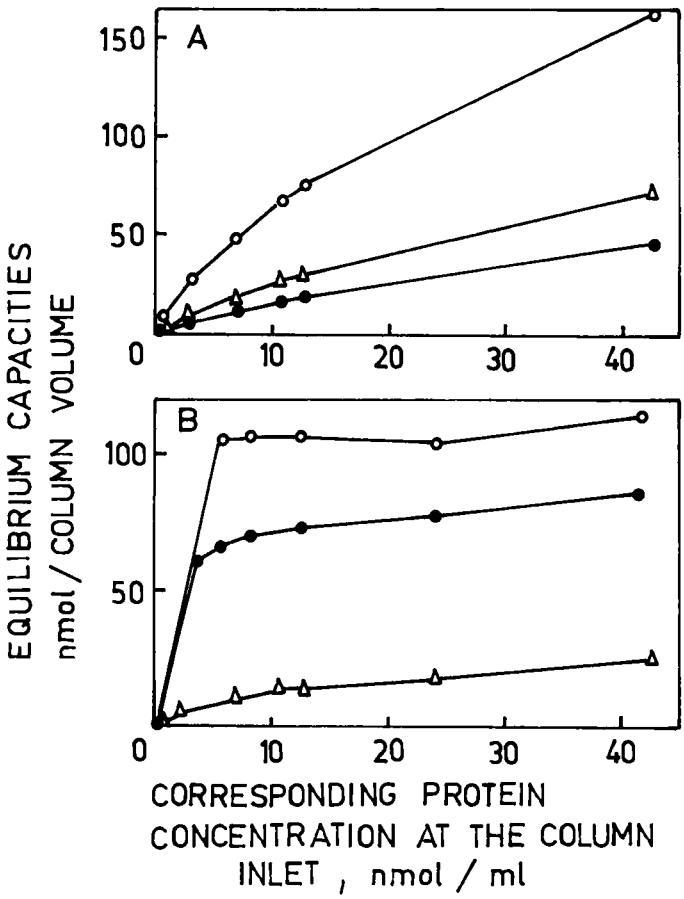


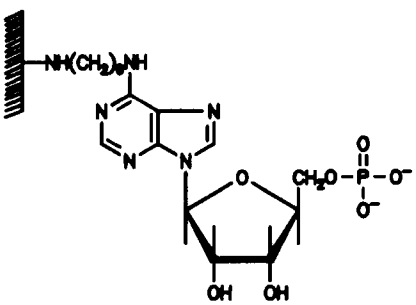
Fig. 4.10. Equilibrium isotherms for adsorption of lysozyme on Cibacron Blue F3GA. (a) Adsorption of the protein on the soft-gel sorbents; o = Cellufine GC 700; Δ = Sepharose CL6B; ● = Trisacryl GF 2000. (b) Adsorption on silica-based affinity sorbents; o = Nucleosil 1000-5; ● = Nucleosil 4000-5; Δ = nonporous glass beads (20-40 μm). Reproduced with permission from F.B. Anspach et al., J. Chromatogr., 499 (1990) 103-124.

4.3 CONFORMATION, ORIENTED IMMOBILIZATION AND STABILITY OF ATTACHED AFFINANT

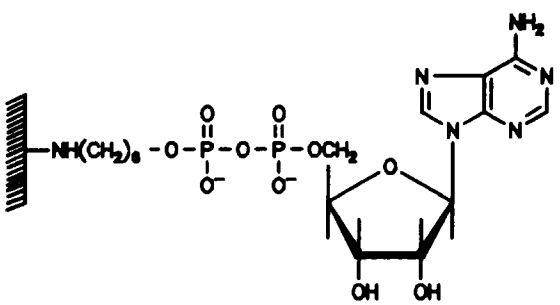
The main principle governing the specific interactions of biological macromolecules is the complementarity of the binding sites. For example, the high reactivity of specific substrates follows from the perfect interaction of configurationally and conformationally oriented groups of the substrate with the complementarily located groups or sites of the enzyme. The interaction of the substrate and the inhibitor with the enzyme

thus rises with the greater complementarity of the binding sites. This is true not only with respect to the spatial arrangement, but also with respect to the nature of the complementary parts of the molecules. However, the means by which the affinity ligand is bonded to the solid support is thus considerably limited, because the ligand must be bound by that part of the molecule which does not participate in the biospecific binding. In addition, the immobilization of the affinant should not cause a change in conformation nor should it affect the nature of its binding sites. The effectiveness of the affinity adsorbent depends on the extent to which this is achieved.

The basic importance of the attachment of nucleotides to a solid support as regards the efficiency of the affinity chromatography of kinases and dehydrogenases depending on pyridine nucleotide has been demonstrated by the studies of Harvey et al. (1974). The adsorbent N^6-(6-aminohexyl)-AMP-Sepharose contains AMP bound to Sepharose by means of the N^6-adenine moiety:



while in the sorbent P^1-(6-aminohexyl)-P^2-(5'-adenosine) pyrophosphate-Sepharose AMP is linked by 5'-phosphate:



The linking of various dehydrogenases and kinases onto these two adsorbents is mentioned in the preceding chapter in Table 3.1. Glucose-6-phosphate dehydrogenase, D-glyceraldehyde 3-phosphate dehydrogenase and myokinase were bound to P^1-(6-aminohexyl)-P^2-(5'-adenosine) pyrophosphate-Sepharose only, while alcohol dehydrogenase and glycerokinase were bound only to N^6-(6-aminohexyl)-5'-AMP-Sepharose. Lactate dehydrogenase, malate dehydrogenase, 3-phosphoglycerate kinase and pyruvate kinase were bound to both sorbents, while hexokinase and creatine kinase were bound to neither of them. These results reflect the nature of the enzyme-nucleotide interactions and it can be concluded that while the free 5'-phosphate group is essential for the binding, for example, to alcohol dehydrogenase or glycerokinase, it has a completely different role in the interaction of glyceraldehyde 3-phosphate dehydrogenase. In this instance the decisive role is played by the adenosine moiety of the affinant. The much stricter binding requirements with hexokinase and creatine kinase evidently result in these enzymes not being attached to either of the adsorbents. However, hexokinase from yeast extracts was isolated by use of ATP in complex with immobilized boronic acid (Matrex Gel phenylboronate from Amicon, Lexington, Mass., U.S.A) by Bouriotis et al. (1981).

High molecular weight affinity ligands usually offer more possibilities for the preparation of affinity adsorbents. A series of very active affinity adsorbents has been prepared by direct attachment of the protein to a solid support, and many examples are given in Chapter 9 in Tables 9.1-3. However, a very important condition in this instance is that the attachment to the solid support should not cause a change in the native conformation of the ligand.

To protect the binding site of the lectin, Clemetson et al. (1977) coupled the lectins to Sepharose 4B after CNBr-activation in the presence of 2% of the appropriate sugar. Efficient biospecific adsorbents for the purification of the proteins exhibiting high affinity to cytochrome c can be prepared according to a method reported by Azzi et al. (1982). Fig. 4.11 shows the example of the preparation of efficient biospecific adsorbents for the isolation of mitochondrial cytochrome c oxidase and reductase. Cytochrome c from *Saccharomyces cerevisiae* was attached to a thiol-Sepharose 4B through cysteine-

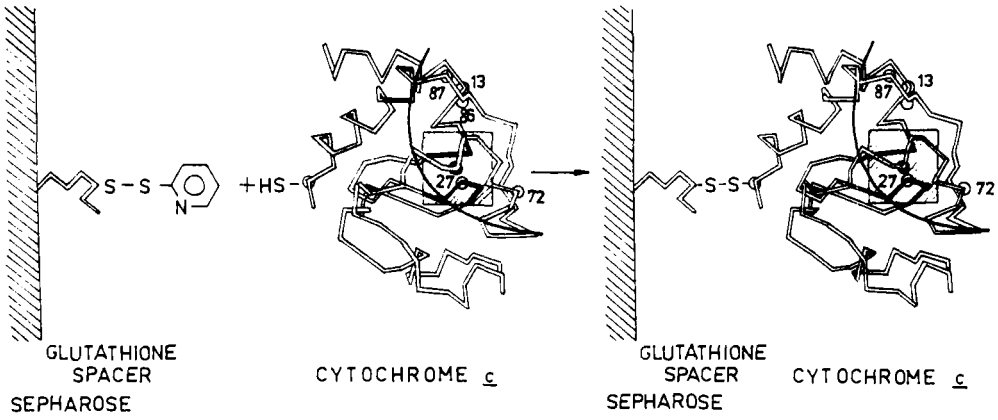


Fig. 4.11. Scheme of the reaction of S. cerevisiae cytochrome c with activated thiol-Sepharose 4B. Reproduced with permission from A. Azzi et. al., Proc. Nat. Acod. Sci. U.S., 79 (1982) 2447-2450.

107. In this way, the site of interaction of cytochrome c with cytochrome oxidase and reductase remained unmodified and available for binding to a number of partner enzymes.

4.3.1 Oriented immobilization by use of biospecific complex formation

Increased accessibility and stability of active sites of antibodies can be achieved by oriented immobilization using the sorption and the cross linkage of Fc parts of antibodies to immobilized Protein A (Fig. 3.16 and Fig. 3.19).

The use of biotinylated mouse monoclonal anti-idiotypic antibody adsorbed to streptavidin-coated beads for the isolation of active phosphorylcholine receptors from detergent-solubilized membranes is shown in Fig. 3.29. The great advantage of this oriented immobilization of monoclonal antibody is biotinylation of its periodate oxidized carbohydrate moiety by biotin hydrazide (Babashak and Phillips, 1988). The development of a biospecific sorbent for the isolation of insulin receptors based on avidin-biotin technology, where the hormone is attached in a targeted fashion, is demonstrated in Fig. 3.33. Insulin derivatives containing biotin attached either directly

or *via* a spacer arm to the terminal amino group of the B chain of insulin were prepared by Finn et al. (1984). C-Terminal biotinylation of proteins by the use of biocytinamide and a nonspecific proteinase carboxypeptidase Y was described by Schwarz et al. (1990). Further details about biotin-avidin or streptavidin interactions and their applications are discussed in Section 3.8.

A consideration of recycling must not only deal with the affinity ligand as such but also with sanitation of the column. In some bioaffinity chromatographic processes there is a need for good control of the aseptic conditions in the column. In order to achieve such conditions, as well as to reduce the costs of the purification of biologically active molecules from a crude homogenate on a large scale, Mattiasson and Olsson (1986) described the use of heterobifunctional affinity ligands. Cibacron Blue dye and soy bean trypsin inhibitor (STI) attached to Dextran T40 in a soluble form were added to a solution containing lactate dehydrogenase (LDH). After binding of LDH with Cibacron Blue-dextran-STI, the complex formed was isolated by passage through bioaffinity matrix trypsin-Sepharose. Fig. 4.12 shows the stepwise elution process. The substance of interest LDH is isolated first, followed by the affinity ligand, which can then be reused.

The theoretical and experimental evaluation of the use of heterobifunctional bioaffinity ligands in general chromatographic purification systems has been described by Olsson and Mattiasson (1986). Homobifunctional affinity ligands bis-NAD analogues were used by Mansson et al. (1983) for site-to-site directed immobilization of alcohol dehydrogenase and lactate dehydrogenase or by Norrlöw et al. (1987) for the preparation of silica particles with a prearranged distance (X) between the boronate groups.

The highly selective bioaffinity adsorbent for the rapid isolation of trypsin from an activated extract of human pancreas was prepared by oriented immobilization of bovine pancreatic trypsin inhibitor (BPTI) in the presence of guanidinated trypsin by Temler and Kägi (1977). Guanidinated trypsin was prepared by guanidination of the trypsin-BPTI complex. CNBr-activated Sepharose, used for the immobilization of BPTI, could react with the exposed lysin-15 of the trypsin-binding site of the inhibitor. In the procedure developed in this study this reaction was prevented by masking the trypsin-binding site of BPTI with modified bovine trypsin. The use of a suitable biospecific

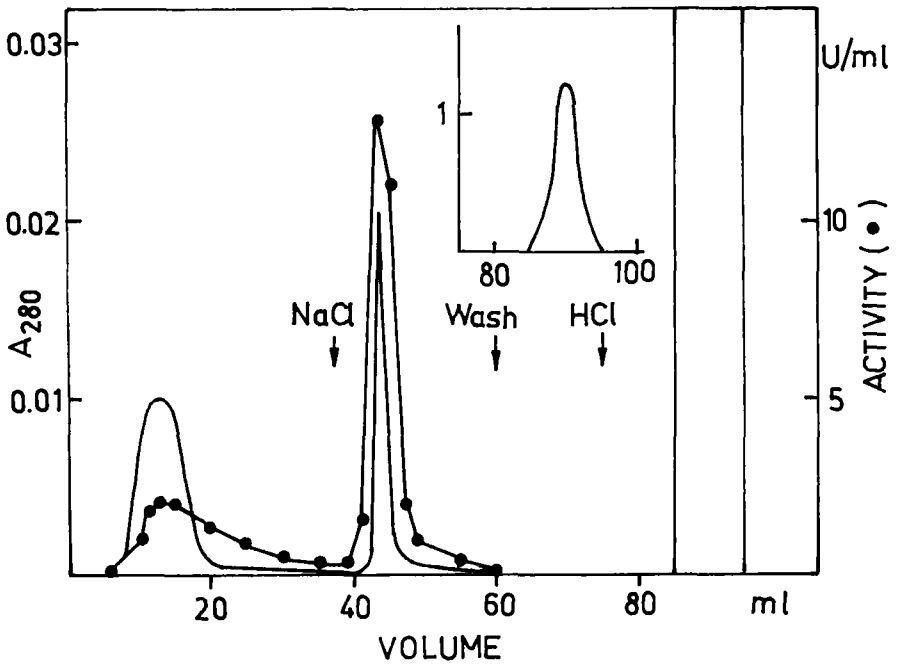


Fig. 4.12. Chromatography of a pre-equilibrated mixture of LDH and STI-dye-Dextran T40 on trypsin-Sepharose. Sample applied: LDH (88 units) and STI-dye-Dextran T40 (32.5 μM dye). Flow-rate 1.0 ml/min. LDH was eluted with a pulse of 0.5 M ethylenediamine hydrochloride buffer pH 6.0 containing 2 mM calcium chloride. The ligand was eluted by a pulse of 10 mM hydrochloric acid. Reproduced with permission from B. Mattiasson and U. Olsson, J. Chromatogr., 370 (1986) 21-28.

complex for the immobilization of affinity ligands can result not only in better accessibilities of complementary binding sites, but also may increase the stabilities of their conformations.

The use of monoclonal and polyclonal antibodies for the oriented immobilization of enzymes is discussed in Chapter 10.

Mattiasson (1988), in his chapter on affinity immobilization, discussed the possible advantage of this system in increasing the operational stability of biospecific sorbents. In practical application each biologically active preparation has a certain lifetime and each biospecific sorbent has a finite shelf life which also has to be taken into account. For example the labile enzyme can be stored under the most suitable conditions and applied to the sorbent just prior to use.

The operantional half-lives of the four immunosorbents prepared by the immobilization of the same toxin determined by Kukongviriyapan et al. (1982) are shown in Table 4.4. Toxin-albumin-Sepharose had the highest stability (half-life 108 days) in contrast to toxin-succinyl-aminoethyl-Sepharose (19 days).

Table 4.4.

Ligand density, maximal antibody binding capacity and operational half-life of various adsorbents

Affinity adsorbent [a]	*Toxin immobilized (mg/ml packed gel*[b]*)*	*Maximum binding capacity* [c] *(mg/ml immobilized toxin)*	*Half-life (days)*
Seph-T3	2.60	4.75	43.0
Seph-Ae-Suc-T3	2.01	6.0	19.0
Seph-BSA-T3	1.50[d]	2.6	108.0
Biogel-Ae-Suc-T3	2.01	5.5	25.0

[a] Abbreviations: Seph, Sepharose 4B; Ae, aminoethyl; Suc, succinyl and T3, *N.n. siamensis* toxin 3.

[b] Volume of gel was measured in 0.15 N NaCl

[c] Protein eluted at pH 2.05

[d] Determined by using ^{125}I-labelled *N. n. siamensis* toxin 3

A different line of development in bioaffinity chromatography has been taken by genetic technologists. The interaction between immunoglobulin G and Protein A was used for an efficient isolation of alkaline phosphatase by Nilsson et al. (1985). The gene of this enzyme was extended by that part of the Protein A gene which codes for the part of the protein molecule responsible for the interaction of protein A with immunoglobulin G. The lysate of *Escherichia coli* modified in this manner was chromatographed on Sepharose with immobilized immunoglobulin G. Genetic approaches to protein purification are discussed in more details in Chapter 11.

4.3.2 Stabilization of trypsin by use of multipoint attachment

The advantage of immobilized enzymes for the study of the stability of biospecific sorbents is the simple determination of enzymatic activity. The preparation of highly

efficient and stabilized immobilized trypsin developed by Blanco et al. (1989) is based on two important stabilizing effects for conserving the active protein conformation. In order to achieve an immobilized trypsin derivative which preserves 100 % catalytic activity as compared with the soluble enzyme used in its preparation, multipoint covalent attachment of the enzyme to aldehyde-agarose gels was performed in the presence of the competitive trypsin inhibitor benzamidine. Two protecting effects of benzamidine on the native structure of trypsin were described by Blanco and Guisán (1988): the inhibitor adsorbed on the active center of the enzyme protects the enzyme from distorting conformational changes induced either by heat or by multipoint covalent attachment to aldehyde agarose gels.

Enzyme inactivation under the influence of various denaturing actions is usually considered to be a two-step process (Mozhaev et al., 1990a):

$$N \rightleftharpoons D \rightarrow I$$

where N,D and I are the native, reversibly denatured and irreversibly inactivated forms of an enzyme. A general strategy of enzyme stabilization should be based on the inhibition of the first step of the inactivation mechanism, i.e. the unfolding of the protein molecule (Martinek et al., 1977). The fact that enzyme-support multipoint attachment may exert an important stabilizing effect on the immobilized enzyme derivatives is commonly acknowledged (Klibanov, 1982). These more rigid enzyme molecules should be more resistant to conformational changes than the corresponding unmodified ones. Blanco et al. (1989) have developed strategies for the stabilization of enzymes by multipoint covalent attachment on glyoxyl-Sepharose 6B gels after periodate oxidation to varying degrees. The advantages of the immobilization system described are: (a) the geometric congruence between enzyme and support surfaces is quite intense; (b) the amine-aldehyde reaction does not present problems of steric hindrance; (c) the aldehyde groups are very stable, even in quite alkaline media, and therefore prolonged multi-interaction processes between the enzyme and the activated support can be performed; and (d) the amine-aldehyde reaction is a fast but quite reversible one. These enzyme (amine)-support (aldehyde) multi- interaction processes are completed by borohydride

reduction of the derivatives. In this way, the Schiff's bases formed as a consequence of the amine-aldehyde reaction are converted into very stable secondary amine bonds. At the same time, the aldehyde groups remaining on the support surface are converted into inert hydroxyl ones.

The preparation of immobilized trypsin derivatives in respect of four main variables is presented in Table 4.5.

Table 4.5.

Preparation of trypsin (amine)-agarose (aldehyde) derivatives

Derivative	*Surface Density*[a]	*pH*[b]	*Contact Time*[c]	*Temperature*[d]
A1	0.5	10.0	24	25
B2	2.5	10.0	72	25
C2	6.0	10.0	72	25
D2	13.0	10.0	72	25
E2	20.0	10.0	72	25
E2-7.6	20.0	7.6	72	25
E2-8.4	20.0	8.4	72	25
E2-9.2	20.0	9.2	72	25
(I)-E2	20.0	10.0	0.25	25
(6)-E2	20.0	10.0	6	25
(12)-E2	20.0	10.0	12	25
(24)-E2	20.0	10.0	24	25
(48)-E2	20.0	10.0	48	25
E2 (4)	20.0	10.0	72	4
E2 (15)	20.0	10.0	72	15
E2 (35)	20.0	10.0	72	35

[a] Aldehyde residues per 1000 $Å^2$ of gel surface
[b] pH of the "insolubilization suspension"
[c] Contact time between the insolubilized enzyme and the activated support prior to borohydride reduction (hours)
[d] Temperature of the "insolubilization suspension" (^oC)

Stabilization of each derivative was calculated as the ratio between pseudo-half-life times (time necessary to reach 50 % residual activity at pH 7.6 and 50°C) and the one corresponding to the A1 derivative (one-point enzyme-support attachment). Previously,

Blanco and Guisán (1988) had found that the stability of this A1 derivative is the same as that of soluble trypsin, in the absence of autolysis phenomena, e.g. in the presence of benzamidine. In this way, stabilization of the derivatives will represent the real stabilization of their three-dimensional structures as a consequence of the multi-interaction with the activated supports.

The surface density of the active aldehyde groups seems to be the variable that plays the most important role in the enzyme stabilization through these multi-interaction processes. Fig. 4.13 shows how the increase of stabilization depends on increasing surface density of aldehyde groups in the activated gels. Fig. 4.14 represents the time courses of insolubilization and further multi-interaction between trypsin and the highest activated gel E, at pH 10.0 and 25°C. Under these experimental conditions trypsin is insolubilized on gel E in less than 15 min.

Contact time between insolubilized enzyme and the activated support was controlled by borohydride reduction of the derivatives. The enzyme preserved 100% of its catalytic

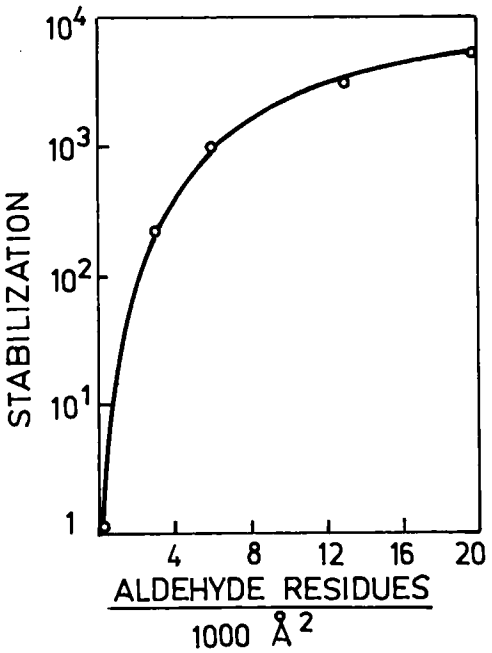


Fig 4.13. Effect of surface density of aldehyde groups in the activated gels on the stability of trypsin-agarose derivatives. Surface density is defined as aldehyde residues per 1000 $Å^2$ of gel surface. Reproduced with permission from R. M. Blanco et al., Enzyme Microb. Technol., 11 (1989) 353-359.

activity during the processes of insolubilization, subsequent multi-interaction, and final borohydride reduction. On the other hand, the stabilization of the resulting derivatives was greatly increased. The three-dimensional structure of the most stable derivative was 5000-fold more stable than the one corresponding to unmodified trypsin. Amino acid analysis of hydrolysates of this very stable derivative reveals that seven lysine residues per trypsin molecule have reacted with the activated support during the process of the immobilization of the enzyme. This trypsin (amine)-agarose (aldehyde) multi-interaction seems to be extraordinarily intense. This fact may be explained through the existence of an area of trypsin surface containing a very high density of lysine residues, as it has been demonstrated by crystallographic studies and also through the tendency of enzymes to insolubilize on these aldehyde-agarose gels at alkaline pH, by means of such areas as contain the highest density of amine groups.

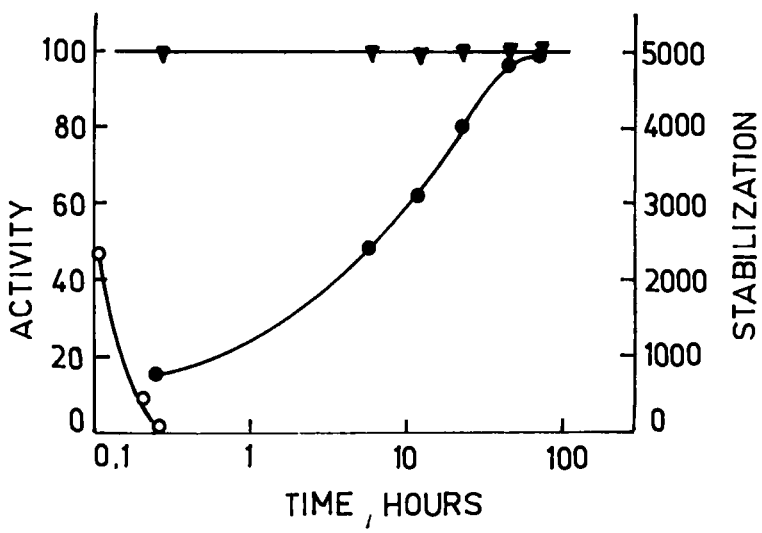


Fig. 4.14. Time course of immobilization-multi-interaction of trypsin on gel E during the preparation of (I)-E2, (6)-E2, (12)-E2, (24)-E2, (48)-E2 and E2 derivatives. Conditions for preparation of derivatives are given in Table 4.4. o : Activity of the enzyme in the supernatant of the "insolubilization suspension" (percentage corresponding to soluble enzyme used in the preparation of the derivative). ▼: Activity of whole "insolubilization suspension" (% corresponding to soluble enzyme used in the preparation of the derivative). ● : Stabilization of derivatives reduced at different times. Reproduced with permission from R. M. Blanco et al., Enzyme Microb. Technol., 11 (1989) 353-359.

The stabilization of trypsin derivatives increased from 500 to 5000 as the pH of the enzyme-support multi-interaction medium increased from pH 7.6 to 10.0. At pH values higher than 10, the enzyme inactivates quite quickly, as well as the activated support. As the pH increases, the density of unprotonated amines on the protein surface increases. Unprotonated amines are the only ones able to produce a nucleophilic attack of the aldehyde groups on the support, and hence pH defines the possibilities of the enzyme to multi-interact with the activated support. As pH increases, so do these possibilities, and therefore the stabilization of the derivatives must be greater.

The stability of the derivatives increased with the temperature at which the preparation of the derivatives was carried out. Stabilization of trypsin-agarose coupled at 4°C was 1500, 3000 at 15°C and 5000 at 25°C. Derivatives prepared at 25°C or 35°C show the same stability. These results may be due to the fact that, in the case of these very stabilized derivatives, the enzyme-support multi-interaction is nearly the most intense possible. In general, the increase of stability with temperature of preparation of derivatives may be explained if, as the authors do, we consider that, as temperature increases, the vibrational movements of the insolubilized trypsin molecules must also increase and therefore the possibility of formation of new correct alignments between new amine groups of the enzyme and new aldehyde groups of the support also increases.

However, the strategy thus developed is not universal. On the other hand, a very intense enzyme-support multipoint attachment may also promote important conformational changes in the enzyme structure and hence considerable loss of catalytic activity. So a very careful control of these enzyme-support multi-interaction processes is necessary in order to obtain derivatives with promising activity-stability ratios.

4.3.3 Stabilization by use of hydrophilization

According to X-ray crystallographic data, about one half of the surface area of many proteins is occupied by nonpolar amino acids. Nonpolar residues are very often organized into hydrophobic surface clusters. These play an important role *in vivo*, since they enable proteins to bind *via* hydrophobic interactions to other proteins (forming multienzyme complexes), to lipids in biological membranes, to polysaccharides in cell

walls, to substrates and effectors during enzymatic catalysis, i.e. to function in an optimal manner. However, the contact of nonpolar residues with water is thermodynamically disadvantageous and is harmful to protein stability *in vitro*. Hence, a reduction of the nonpolar surface area should stabilize proteins. This is in fact observed when comparing the *in vitro* structure and stability of mesophilic and thermophilic proteins and of enzymes from wild and mutant strains. The increase of the stability of natural glycoprotein as a result of differences in the content of carbohydrate moieties determined in carboxypeptidases Y from various kinds of yeast has been already discussed in Section 3.4. The stabilization of trypsin derivatives by multipoint attachment to periodate oxidized Sepharose, which has been described in Section 4.3.2 above, could also be followed from the hydrophilization of hydrophobic surface clusters of trypsin by polysaccharide agarose. Hydrophilization of nonpolar surface areas is, according to Mozhaev et al. (1990b), the most simple and reliable approach to artificial stabilization of proteins.

The idea that the contact with water of nonpolar clusters located on the surface of trypsin molecules with water destabilizes enzymes was experimentally verified by Mozhaev et al., (1988). An example of the success of hydrophilization is the incorporation of amino groups into the surface tyrosine residues of trypsin, resulting in a hundred-fold stabilization of the enzyme. Galactosylation of tyrosines on the surface of trypsin and immobilization through galactose molecules after activation by galactose oxidase has been used by Turková et al. (1992).

4.3.4 Carbohydrates as a tool for oriented immobilization of glycoproteins and RNA

A solution that may overcome both a low stability and capacity of biospecific sorbents from glycoproteins may lie in their immobilization through their carbohydrate moieties. Because the complementary binding sites of enzymes and antibodies are in their protein moieties, immobilization of these glycoproteins through their carbohydrate moieties is useful not only for stabilization of the protein conformation, it will also confer a better accessibility on the active sites.

Multiple forms of carboxypeptidase Y from various kinds of yeast had molecular weights in the range 49 000 - 65 000. They contained an identical protein and differed only in the content and composition of their carbohydrate moieties. The stabilities of the enzyme forms increased with increasing carbohydrate contents (Turková et al., 1988). Hsiao and Royer (1979) immobilized carboxypeptidase Y by use of amino, carboxyl and phenolic groups on enzyme surface. The possible explanation for the low yields they obtained could be that amino acid side chains in glycoprotein were shielded by carbohydrate spines which projected from the surface of the enzyme molecule. When carboxypeptidase Y was adsorbed by Hsiao and Royer (1979) on immobilized concanavalin A followed by crosslinking with glutaraldehyde the bound enzyme retained 96% of the native catalytic activity and showed very good operational stability.

Similar results were described by Igbal and Saleemuddin (1983) who immobilized glucose oxidase by covalent attachment to AE-cellulose as well as by binding to concanavalin A attached to cellulose. About nine times more enzyme was immobilized by coupling glucose oxidase to Con A-cellulose as compared to the direct coupling procedure. Treatment of the Con A-cellulose bound preparation with 0.5% glutaraldehyde solution resulted in a further significant increase in the stability of the enzyme.

Successful immobilization of glucose oxidase *via* activation of its carbohydrate residues by oxidation with periodic acid has been described by Zaborsky and Ogletree (1974). However, Pazur et al. (1970) reported that extensive oxidation of the carbohydrate residues in glucoamylase by periodate markedly affected the stability of the enzyme. Avigad et al. (1962) stated, that active aldehyde groups can be generated in glycoproteins containing galactose or galactopyranosides *via* oxidation catalyzed by galactose oxidase. Petkov et al. (1990) therefore used the specific oxidation of D-galactose present in the carbohydrate moiety of glucose oxidase from *Aspergillus niger* by galactose oxidase in the presence of catalase. Oxidized enzyme was coupled to hydrazide derivatives of O-α-D-galactosyl Separon H 1000 or of Sepharose 4B. Both solid supports were modified with adipic acid dihydrazide after their activation with galactose oxidase. Each immobilized preparation of glucose oxidase showed higher

activity than was achieved by other immobilizing procedures. The advantages of hydrazido-derivatized supports are described in Chapter 6.

However, no active aldehyde groups were formed by galactose oxidase in glucose oxidase from *Penicillium vitale*. Periodate oxidation of carbohydrate residues of this glucose oxidase in acetate buffer in the dark for 4 h at 4°C did not influence the catalytic activity of the enzyme. Fig. 4.15 shows the high stability of this enzyme after the attachment to the hydrazide derivative of polyacrylate-coated porous glass.

Site-specific modification of antibodies and possible oriented immobilization may be achieved by chemical or enzymatic oxidation of the carbohydrate moieties of antibodies prior to hydrazido-derivatized support attachment (O'Shannessy and Quarles, 1987; Solomon et al., 1990). As the carbohydrate residues on the Fc fragment of the antibody molecule are located remotely from the binding site of the antibody, immobilization through this region does not usually impair the immunological activity of the antibody. Further details are discussed in Section 3.3.

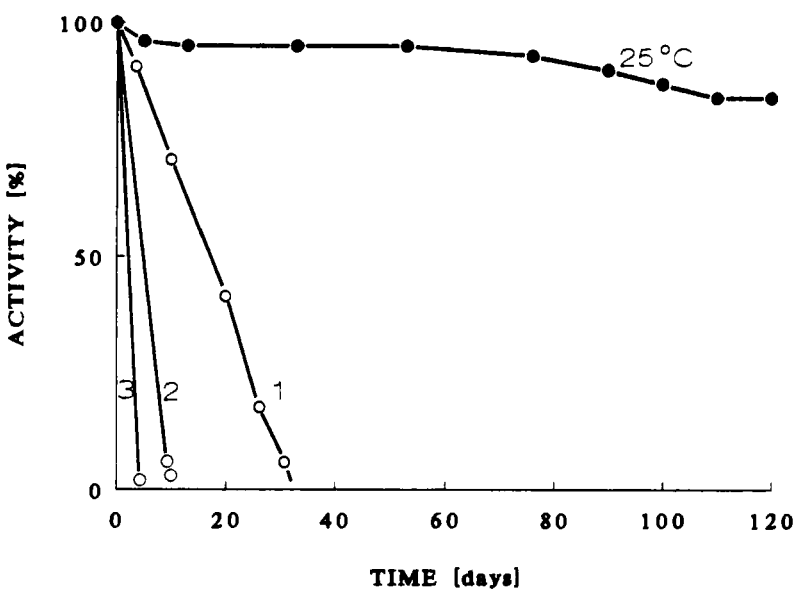


Fig. 4.15. Stability of immobilized (●) and soluble (o) glucose oxidase (GO). The enzyme was kept in phosphate buffer (pH 6.5) at 25°C. Concentration of soluble GO was 1 - 1 mg/ml, 2 - 0.1 mg/ml, 3 - 0.01 mg/ml. Reproduced from J. Turková, Technology Today, 2 (1990) 82-85.

Periodate oxidation of RNA has been shown by Zamecnik et al. (1960) to be specific for the 3'-terminal cis-diol, resulting in the formation of a reactive dialdehyde, which may then be condensed with primary amines, hydrazines or other suitable nucleophiles. Since only one reactive dialdehyde is generated per RNA molecule, this technique leads to an extremely site-specific and oriented immobilization of RNA. Bioaffinity chromatographic procedures, using ribosomal or tRNA covalently coupled after periodate oxidation to agarose through an adipic acid dihydrazide spacer, have been developed to isolate and identify the *Escherischia coli* ribosomal proteins by Burrell and Horowitz (1979). A review of the coupling of glycoproteins, nucleotides, nucleosides, RNA and miscellaneous compounds onto hydrazido-derivatized supports and their use in bioaffinity chromatography has been published by O'Shannessy (1990). The methods mentioned above are described in Chapter 6.

4.4. CONCENTRATION OF THE AFFINANT ON THE MATRIX

The theoretical deduction of mutual relationships between the amount of sorbed enzyme, the concentration of the affinity ligand and ligand-enzyme equilibrium constants is discussed in more detail in Chapter 15. The importance of the concentration of the affinity ligand may be expressed by the following equation, where the dissociation constant (K_L) of the enzyme-immobilized ligand complex (EL) is given by:

$$K_L = \frac{[E_o - EL][L_o - EL]}{[EL]} \tag{4.3}$$

where E_0 and L_0 are the total concentrations of enzyme and ligand, respectively. Rearranging and denoting $[E_0]$ - [EL] by the concentration of free enzyme, [E]

$$[EL] = \frac{[E]L_o}{K_L} / (1+\frac{[E]}{K_L}) \tag{4.4}$$

This equation demonstrates that the concentration of bound enzyme, [EL], expressed in units of moles per unit volume (or weight) of gel, is clearly limited by the concentration

of the immobilized ligand, [L_0]. In other words, if the concentration of enzyme is increased, the capacity of the adsorbent, [EL], is determined by the immobilized ligand concentration. For a fixed concentration of added enzyme, [E_0], the percentage of enzyme bound [EL]/[E_0], will be given by Eq. 4.5,

$$\frac{[EL]}{[E_0]} = \frac{[L_0]}{K_L} / \left(1+\frac{[L_0]}{K_L}\right) \tag{4.5}$$

and is a hyperbolic function of L_0 in the same way that the percentage saturation of an enzyme is determined by the substrate concentration (Lowe, 1979). This is illustrated in Fig. 4.16, which shows the dependence of the amount of bound lactate dehydrogenase from rabbit skeletal muscle, hog heart muscle and glycerokinase on the concentration of N^6-(6-aminohexyl)-5'-AMP bound to Sepharose, as determined by Harvey et al. (1974).

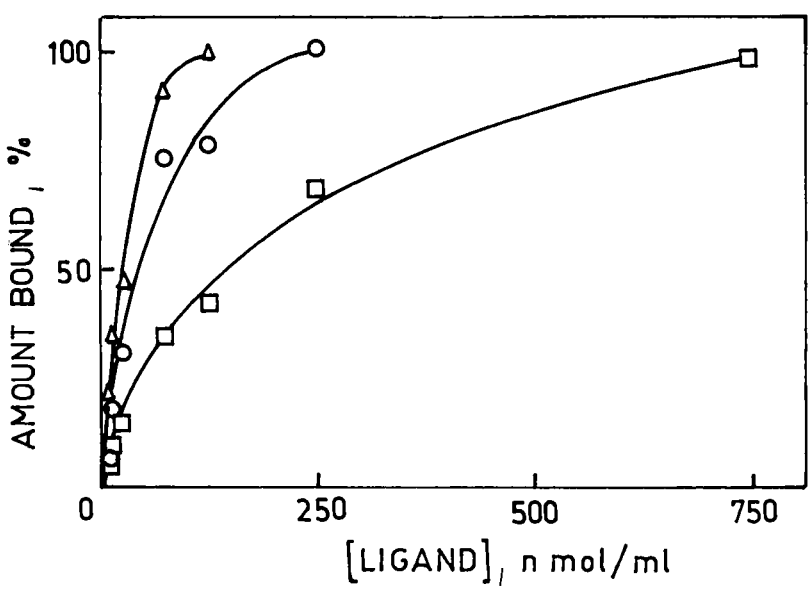


Fig. 4.16. Capacity of immobilized nucleotide adsorbent [N^6-(6-aminohexyl)-5'-AMP-Sepharose] in relation to ligand concentration. The sample (100 μl), enzyme (5 U) and bovine serum albumin (1.5 mg) were applied to a column (50 x 5 mm) containing 1.0 g of the affinity adsorbent diluted to the approriate ligand concentration with Sepharose 4B. Δ , Lactate dehydrogenase (rabbit skeletal muscle); o , lactate dehydrogenase (pig heart muscle); ❑ glycerokinase. Reproduced with permission from M. J. Harvey et al., Eur. J. Biochem., 41 (1974) 335-340.

Fig. 4.17 shows theoretical curves for several K_L values based on Eq. 4.5. However, the simple model described above has no real operational significance and serves merely to emphasise the importance of ligand concentration in bioaffinity chromatography.

In order to minimize the nonspecific sorption of inert compounds to affinity sorbents and for the greatest possible exploitation of the attached affinity ligand it should be used at the lowest possible content in the specific sorbent. The importance of the low concentration of the affinity ligand and the effect of the uneven surface of the gel are illustrated in Fig. 4.18.

This figure illustrates schematically the surface of the macroreticular hydroxyalkylmethacrylate polymer in the form of aggregated beads. After the binding of the affinity ligand *via* the spacer, well accessible, less accessible and sterically inaccessible molecules of the affinity ligand can be recognized. This steric hindrance may in many cases explain the low saturation of molecules of the immobilized ligand with the isolated compound and the heterogeneity in the affinity of immobilized ligands. For the prepa-

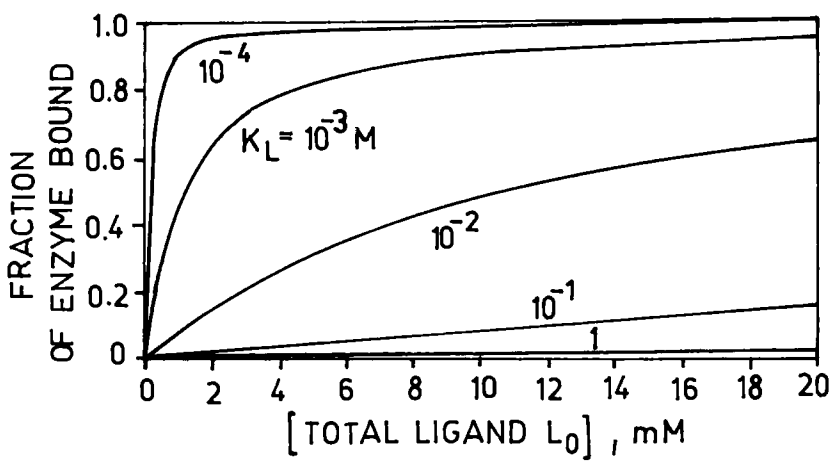


Fig. 4.17. Fractional enzyme binding for low enzyme concentrations as represented by Eq. 4.5. This general hyperbolic graph can also be used to estimate the fractional ligand saturation. Reproduced with permission from D. J. Graves and Y.-T. Wu, Methods Enzymol., 34 (1974) 140-163.

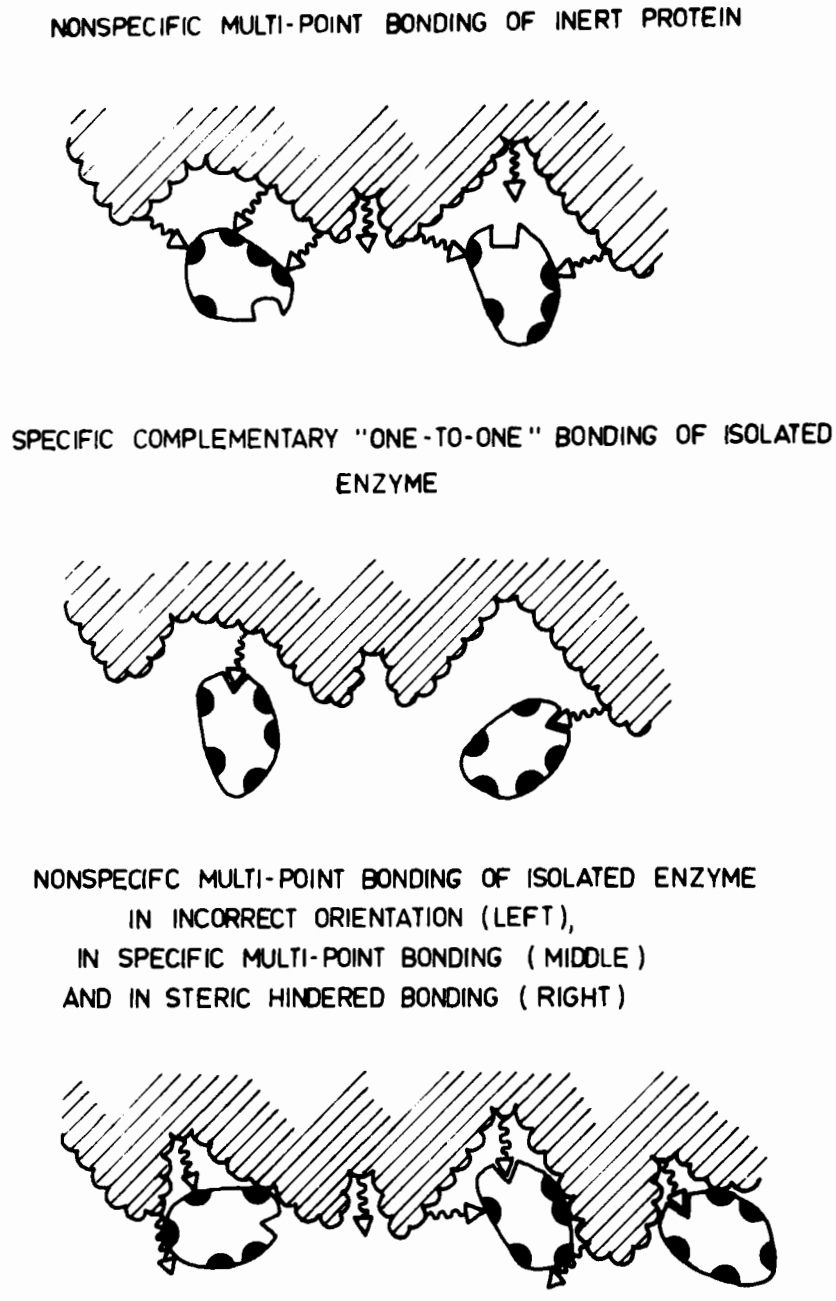


Fig. 4.18. Schematic illustration of the effect of concentration of an immobilized affinity ligand and uneven surface of a solid carrier on specific and nonspecific sorption.

ration of a homogeneous bioaffinity sorbent it is hence necessary to select conditions for the ligand-carrier binding under which the density of the affinity ligand is low and the ligand is preferentially bound only to readily accessible sites (Fig. 4.18, top).

The low density of the affinity ligand is also required to prevent nonspecific binding. The bottom part of the figure illustrates the sorption of macromolecules, e.g. enzymes, which do not have a complementary binding site for the immobilized affinity ligand. This binding is caused by the high density of the immobilized affinity ligand, permitting the formation of multiple nonspecific bonds between the macromolecules in solution and the solid phase of the sorbent. These nonspecific bonds allow the compounds present in the mobile phase to bind to molecules of the affinity ligand, the spacer and also the surface of the solid matrix. These nonspecific multiple bonds may be stronger than a single complementary bond between the isolated enzyme and immobilized complementary affinity ligand, e.g. an inhibitor. When the nonspecific multiple bonds are involved in addition to the specific complementary bond (middle part of the figure) they increase the strength of the binding in the specific complex. This results in the elution of the identical enzyme in several fractions and also gives rise to difficulties with the elution of the enzyme from the specific sorbent. The multiple nonspecific bonds may then lead to binding of the enzyme containing the complementary binding site to the immobilized ligand in an incorrect orientation (middle part of the figure left). Thus, bioaffinity chromatography may yield good results only on a sorbent containing a low concentration of the affinity ligand (top part of the figure) where the enzyme can bind only *via* the complementary bond to the immobilized affinity ligand at a ratio of 1:1. At this low concentration of the immobilized ligand, multiple nonspecific bonds with the affinant cannot occur.

In order to determine experimentally the effect of the concentration of the immobilized inhibitor on the course of affinity chromatography of proteolytic enzymes, specific sorbents for carboxylic proteinases containing different concentrations of ε-aminocaproyl-L-Phe-D-Phe-OMe were prepared by Turková et al. (1982). The inhibitor was coupled to the epoxide-containing Separon H 1000; the resulting concentrations of ε-aminocaproyl-L-Phe-D-Phe-OMe in μmol/g of dry gel were 0.85, 1.2, 2.5, 4.5, and 155.

Solutions of porcine, chicken, or human pepsin were applied continuously to the columns of the affinity sorbents mentioned above until the effluent showed the same activity as the solution applied (cf. Fig. 4.19). On columns of affinity sorbents containing the inhibitor attached in the concentration range 0.85 - 4.5 μmol/g in all cases one fraction of very active pepsin in one sharp peak was achieved. The amount of desorbed pepsin was calculated from the adsorbance at 278 nm and proteolytic activity measurement. On a contrast, using column of the affinity sorbent containing the inhibitor at a concentration of 155 μmol/g, several pepsin peaks were found. This different behaviour

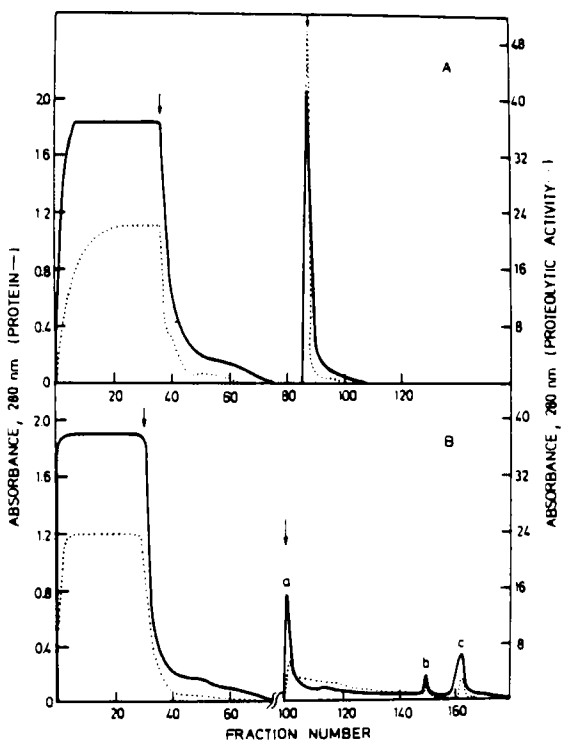


Fig. 4.19. Bioaffinity chromatography of porcine pepsin on ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$-Sepharon columns with (A) low and (B) high concentrations of the immobilized inhibitor. The solution of crude porcine pepsin was applied continuously (see text) onto the affinity columns (5 ml) equilibrated with 0.1 M sodium acetate (pH 4.5). At the position marked by the first arrow equilibrated buffer was applied to the columns to remove unbound pepsin and nonspecifically adsorbed proteins. The second arrow indicates the application of 0.1 M sodium acetate containing 1 M sodium chloride (pH 4.5). Fractions (5 ml) were taken at 4-min intervals. The inhibitor concentration of affinity sorbents were (A) 0.85 and (B) 155 μmol/g of dry support. Solid line, protein; broken line, proteolytic activity. a, b and c, fractions of pepsin of the same specific proteolytic activity. Reproduced from J. Turková et al., J. Chromatogr., 236 (1982) 375-383.

of the enzyme on affinity sorbents having low and high amounts of immobilized inhibitor could be due to multiple bonding of the enzyme molecule and inert proteins, as was illustrated in Fig. 4.18.

Fig. 4.20 shows the amounts of porcine, chicken, and human pepsins eluted depending on the concentration of ε-aminocaproyl-L-Phe-D-Phe-OMe of the individual affinity sorbents. Part B illustrates the portion of immobilized inhibitor molecules (in percent) involved in the specific complex with the isolated pepsin, again depending on the concentration of the attached inhibitor. From the comparison of the curves for the individual pepsins it is evident that ε-aminocaproyl- L-Phe-D-Phe-OMe-Separon is a very good sorbent for porcine pepsin. The specific sorbent containing 0.85 μmol of inhibitor per g of dry support sorbed 29.4 mg of porcine pepsin per g of dry sorbent. It follows from the molecular weight of pepsin (35 000) that 99% of the inhibitor molecules

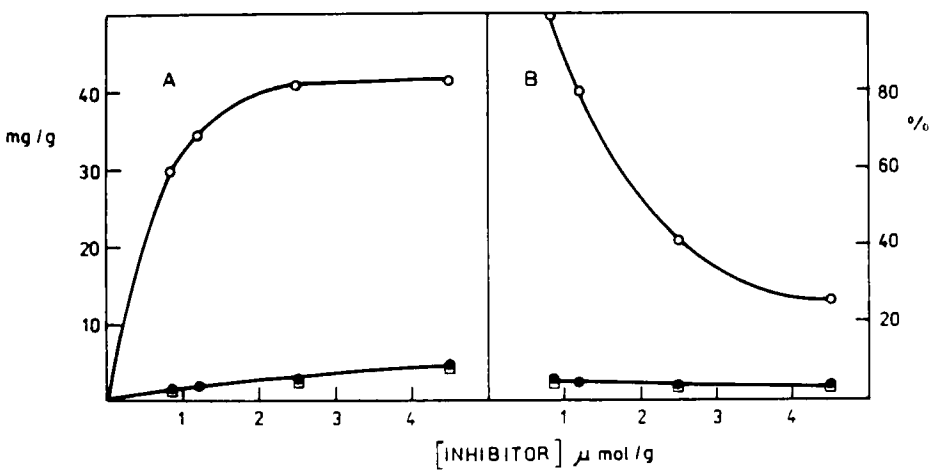


Fig. 4.20. (A) Capacity of immobilized inhibitor sorbent (ε-aminocaproyl-L-Phe-D-Phe-OMe-Separon) in mg of pepsin per 1 g of dry sorbent and (B) portion of immobilized inhibitor molecules involved in specific complex formation (in percent) with respect to immobilized inhibitor concentration (in μmol of inhibitor per 1 g of dry sorbent). o , porcine pepsin; • , chicken pepsin, and , □ human pepsin. Reproduced from J. Turková et al., J. Chromatogr., 236 (1982) 375-383.

attached were involved in the specific complex. As the amount of the affinity ligand attached increased, the portion of the inhibitor molecules involved in the specific complex with pepsin decreased sharply.

With specific sorbents containing 4.5 μmol inhibitor per g only 26% of the total number of inhibitor molecules attached take part in the sorption of pepsin. In an affinity sorbent with the lowest concentration of the affinity ligand only, all the molecules of the affinity ligand are fully available for the formation of the complex with the isolated enzyme. As shown in Fig. 4.21 both adsorption and desorption of 3-hydroxybutyrate dehydrogenase are affected by ligand concentration, as has been determined by Scawen et al. (1982). Their results also demonstrated that a low degree of substitution (3.5-4μmol of dye/g of Sepharose) has to be adopted for the purification of enzyme.

Liu and Stellwagen (1987) used immobilized Cibacron Blue F3GA of variable dye density for the determination of the different adsorptions of monomeric octopine dehydrogenase and tetrameric lactate dehydrogenase. They determined that the half-time for desorption of lactate dehydrogenase from Cibacron Blue F3GA-Sepharose CL-6B was nearly identical (27s) to the mass transfer half-time of the protein in the matrix. The change in the visible adsorbance of Cibacron F3GA accompanying its complexation with lactate dehydrogenase was used to observe the kinetics of complexation. The results of their experiments indicated that it is chromatographic mass transfer and not the chemistry of complexation that limits the zonal chromatographic results. That is why the effect of immobilized dye concentration on protein complexation was studied using zonal chromatography. (The theory and application of zonal chromatography are discussed in Chapter 14). A monomeric protein, octopine dehydrogenase, was retained by a single interaction with a Sepharose CL-6B column containing 11.6 mM immobilized Cibacron Blue F3GA. By contrast, a tetrameric protein, lactate dehydrogenase, was retained by the same column by multiple interactions. The degree of multiple interactions was found to increase systematically with increasing immobilized dye concentration. The concentration of immobilized dye that is accessible to protein was found to be inversely related to the concentration of ionic components in the solvent. Zonal chromatographic measurements of free dye and unconjugated matrix

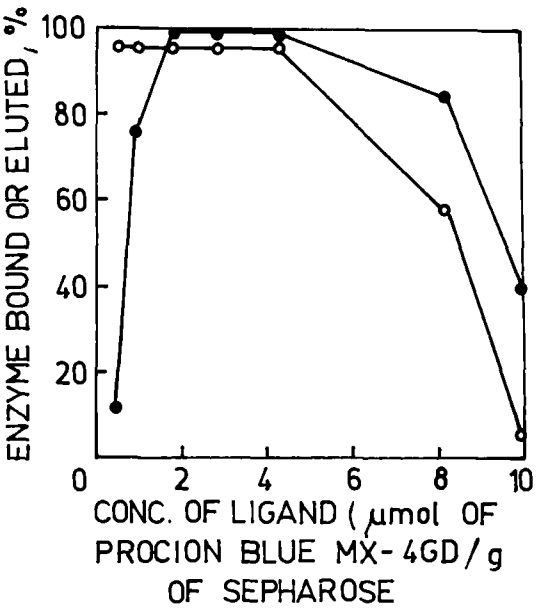


Fig. 4.21. Effect of ligand concentration on adsorption and elution of 3-hydroxybutyrate dehydrogenase on Procion Blue MX-4GD-Sepharose. Columns (1 ml, 0.9 cm x 1.6 cm high) were prepared containing Sepharose substituted with various amounts of Procion Blue MX-4GD. The columns were equilibrated in 10 mM potassium phosphate buffer, pH 7.5 at a flow rate of about 0.2 ml/min and loaded with 1 ml of a 10-unit/ml solution of 3-hydroxybutyrate dehydrogenase in 10 mM potassium phosphate buffer, pH 7.5. The enzyme used had been partially purified by chromatography on Procion Red H-3B-Sepharose. The columns were washed with 5 ml of equilibration buffer containing 1 M KCl and eluted with 5 mM NADH in equilibration buffer containing 1 M KCl. The washings and eluates were examined for 3-hydroxybutyrate dehydrogenase activity. The experiments were performed at room temperature. ●, Enzyme bound, percentage of that applied; O, enzyme eluted, percentage of that bound. Reproduced with permission from M. D. Scawen et al., Biochem. J., 203 (1982) 699-705.

suggest that increasing the concentration of the ionic components promotes the adsorption of immobilized dye to the adjacent matrix surface. This type of adsorption markedly affects both the capacity of an immobilized dye column and the multiplicity of its interaction with oligomeric proteins.

Silica matrices having different p-aminophenyl α-D-mannopyrano-side densities were used by Anderson et al. (1986) for the high-performance bioaffinity chromatography of concanavalin A. Methyl α-D-mannopyranoside was used as a competing inhibitor in the mobile phase. As the ligand density increased, retention was observed

to change from a primarily monovalent interaction of concanavalin A to a primarily divalent interaction.

4.5 EFFECT OF THE HETEROGENEITY OF THE IMMOBILIZED AFFINANTS

When the sorbed substance is released from the specific sorbent, the effect of the heterogeneity of the immobilized affinity ligand should be born in mind (Amneus et al., 1976). Fig. 4.22 shows the separation patterns of chymotrypsins and trypsins from mouse pancreatic homogenates on various preparations of Sepharose with coupled soybean trypsin inhibitors (STI). Assuming that the difference in elution conditions reflects differences in biological activities, the former can be used for the characterization of the molecule. The applicability of the adsorbent then depends on the functional homogeneity of the immobilized affinity ligand.

The heterogeneity of an adsorbent in terms of association constants can be caused by (1) the heterogeneity of the biospecific ligand used for the preparation of sorbent, (2) various changes of the ligand under the effect of immobilization, and (3) various modifications of the affinity ligand caused by the molecules present in the fractionated mixture.

(1) Heterogeneity of the ligand before immobilization

High molecular weight affinity ligands of biological origin, such as proteins, nucleic acids and carbohydrates, used for the preparation of a specific sorbent, may be genetically heterogeneous and such heterogeneity can be found in both commercial and non-commercial ligand preparations. It is evident that the presence of impurities in immobilized affinants having a similar or stronger affinity towards the isolated molecules may impair the use of a specific sorbent in gradient separations.

(2) Alteration of the affinity ligand by immobilization

The effective activity of the ligand can be changed in various ways by the effect of immobilization. The microenvironment formed by the matrix (charge density, steric hindrance, etc.) may affect the interaction of the immobilized ligand with the isolated molecules to a considerable extent and in a variety of ways, and it can also influence the

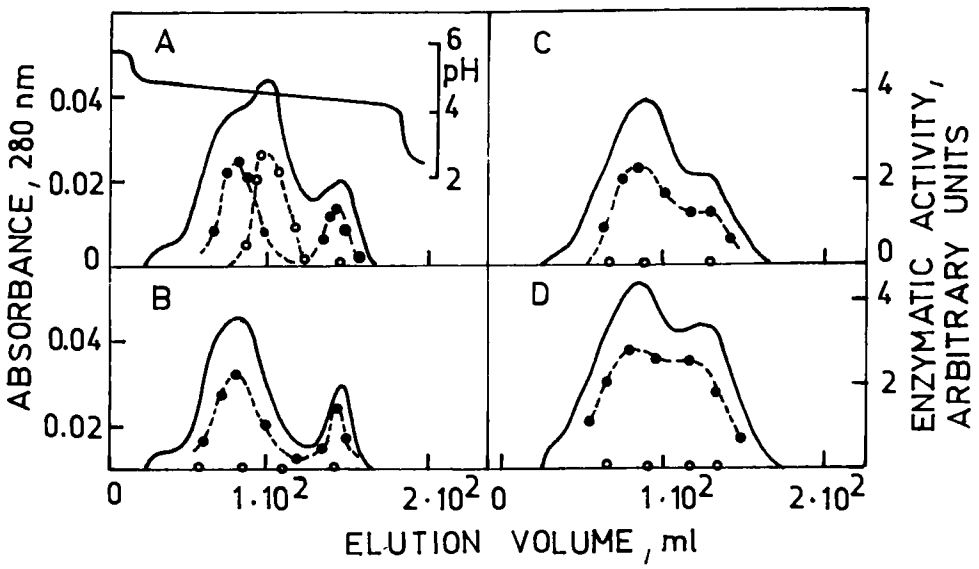


Fig. 4.22. Separation patterns of mouse chymotrypsin on Sepharose 4B substituted with different preparations of soybean trypsin inhibitor [STI; STI(W) = inhibitor obtained from Worthington Biochemical Corp., STI(S) = inhibitor obtained from Sigma]. --, Absorbance or pH; (●), chymotryptic activity; (o), tryptic activity. (A) Unmodified STI(W)-Sepharose, coupled at pH 7.2; (B) Chymotrypsin modified STI(W)-Sepharose, coupled at pH 7.2; (C) chymotrypsin-modified STI(W)-Sepharose, coupled at pH 8.5; (D) chymotrypsin-modified STI(S)-Sepharose, coupled at pH 7.2. Reproduced with permission from H. Amneus et al., J. Chromatogr., 120 (1976) 391-397.

structure of the affinity ligand itself. Immobilization also brings about changes in chemical structure, which will change the molecular properties of the affinity ligand as a consequence. Here, for example, the number of bonds between the affinity ligand and the solid support plays a considerable role. A comparison of Figs. 4.20B and 4.20C clearly shows the unfavourable effect of an increased number of bonds between the molecules of STI and Sepharose 4B, caused by the increase in pH from 7.2 to 8.5 during the binding after cyanogen bromide activation.

(3) Modification of the affinity ligand after immobilization

The fractionated material can contain components that modify the activity of the attached affinity ligand. These components may be similar to the compounds being isolated, although they need not be. The enzymes, for example proteases and nucleases, present in crude extracts may cleave the coupled affinants (proteins, nucleic acids) and thus reduce the capacity of the adsorbent for the binding of the specific complementary

compound. In addition to this non-specific degradation of the affinity ligand, the enzymes and other chemicals present in the fractionated mixture can modify the properties of the coupled affinant specifically, giving rise to forms with retained but changed activities.

All three of the above mentioned effects can be followed in Fig. 4.20. Soybean trypsin inhibitor (Kunitz) obtained from both Worthington Biochemical Corp. [STI(W)] and Sigma [STI(S)] was bound on Sepharose activated with cyanogen bromide. The attached STI was further modified with a solution of chymotrypsin: the adsorbent column was washed with a solution of chymotrypsin at pH 8 and a flow-rate of 10 ml/h for 24 h and, after incubation, chymotrypsin was eluted with a buffer of pH 2.5. From Fig. 4.20A it is evident that when the unmodified STI(W)-Sepharose attached at pH 7.2 was used, only poor resolution occurred between the two peaks with chymotryptic activity and the peak with tryptic activity. Two further peaks with tryptic activity were eluted still later, at much lower pH values. After a modification of STI(W)-Sepharose with a solution of chymotrypsin, elution of the tryptic activity simultaneously with the chymotryptic activity no longer took place. At the same time, good resolution of the two peaks with chymotryptic activities was obtained. If STI(W)-Sepharose, prepared by coupling at pH 8.5 and modified with a solution of chymotrypsin, was used for separation, the resolution of the two peaks with chymotryptic activities deteriorated considerably, in the same manner as when modified STI(S)-Sepharose coupled at pH 7.2 was used (Fig. 4.20D).

If various amounts of activated pancreatic homogenate were fractionated on modified STI(W)-Sepharose, attached at pH 7.2, then with increasing load of the adsorbent the two peaks with chymotryptic activities were eluted at higher pH values (see Table 4.6) and they were less well resolved. The gel had a capacity of 9 mg of chymotrypsin per millilitre. After the separation of a total of nine aliquots from pancreatic homogenates, this capacity decreased to 0.2 mg/ml.

Table 4.6.

Elution conditions for mouse chymotrypsin (CHT-I and CHT-II) of chymotrypsin-modified STI(W)-Sepharose, coupled at pH 7.2

Number of pancreases applied	*CHT activity not retained (%)*	*pH of solution*	
		CHT-I	*CHT-II*
0.5	0	4.75	4.30
1	0	4.90	4.35
2	3	5.05	4.50
4	57	>5.10	4.80

4.6 NON-SPECIFIC SORPTION

The efficiency of bioaffinity chromatography is mainly lowered by non-specific sorption of inert substances. This can be demonstrated by the adsorption isotherm in Fig. 4.1. Non-specific effects are generally relatively weak and are therefore manifested mainly in interacting systems with low affinities. However, enormous effects of the concentration of immobilized affinity ligands, spacer arms and uneven surface of solid supports on non-specific sorption is shown in Fig. 4.16. In Section 4.1.2 the comparison between the application of hydrophilic and hydrophobic spacers reflects the necessity to consider each preparation of suitable efficient biospecific sorbent independently. Chapters 5 and 6 show that there is neither a universal support nor a universal immobilization method, and that the conditions must be developed individually in each case with respect to the application required. The control of non-specific sorption is very useful for efficient bioaffinity chromatography.

4.6.1 Control of non-specific binding

Skjak and Larsen (1982) used epoxy-activated Sepharose for coupling of alginate. Excess epoxy groups were blocked by storing the gel in 1 M ethanolamine. When all the epoxy groups were blocked by ethanolamine the epoxy-activated Sepharose showed no non-specific binding to the column under the conditions of bioaffinity chromatography of the enzyme mannuronan C-5-epimerase.

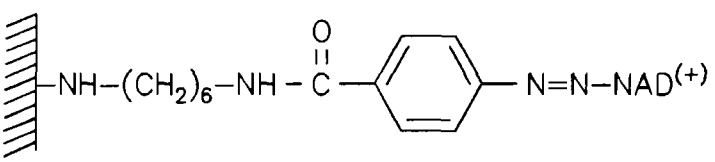
Hayunga and Summer (1986) used lectin bioaffinity chromatography for the characterization of surface glycoproteins on *Schistosoma mansoni* adult worms. They prepared control columns by treating CNBr-activated Sepharose 4B or 6MB with ethanolamine to block reactive groups, followed by alternate washing with 0.1 M $NaHCO_3$ buffer pH 8.3 and 0.1 M sodium acetate buffer pH 4.0, and final equilibration with acetate buffer at pH 6.8. This was done to control the non-specific binding of material by Sepharose, using columns that were identical to commercial columns, with the exception that they contained no immobilized lectin. Heinzel et al. (1976) also showed that native Sepharose or Sepharose blocked with ethanolamine immediately after cyanogen bromide activation did not adsorb proteins. However, if Sepharose was not blocked immediately after activation, then non-specific sorption of proteins was observed on it, which became stronger as the time between the activation with cyanogen bromide and the blocking of the active sites increased. Human serum albumin-Sepharose blocked 12 h after attachment of the human serum albumin on to Sepharose adsorbed 1.5 - 2.0 mg of proteins per millilitre of packed gel, while human serum albumin-Sepharose blocked immediately after coupling of the serum albumin adsorbed only 0.4 mg of protein per millilitre of packed gel. Jost et al. (1974) demonstrated, that by attachment of alkylamines or arylamines onto cyanogen bromide-activated agarose a strong ion exchanger is formed (resulting in an isourea bond), with an apparent pK value of about 10 for the basic amidine nitrogen. By combining hydrophobic ligands with charges, an adsorbent is obtained that functions as a detergent, which strongly sorbs some enzymes that are partly denatured during the isolation. Nilsson and Waxdal (1978) isolated lectin binding proteins from murine lymphoid cells. The different lectins were coupled to Affi-Gel 10. A bovine serum albumin - Affi-Gel 10 column prepared under identical conditions served as a control for non-specific adsorption. Davis et al. (1986) evaluated the immunosorbents for the isolation of cytokinins. The nature of both the solid support and the linkage chemistry affected the non-specific adsorption of sample contaminants and the characteristics of the immobilized antibody. In order to examine non-specific binding of cytokinins to the immobilized IgG protein, a control column was prepared with non-specific rabbit IgG coupled to CNBr-activated Sepharose 4B.

4.6.2 Ionic and hydrophobic effects and suppression of secondary interactions

Morrow et al. (1975) developed a semi-quantitative theory to explain the non-specific binding of proteins onto substituted chromatographic affinity supports, caused by electrostatic and hydrophobic interactions. They developed an extended Debye - Hückel theory which they applied to the study of the dependence of the adsorption equilibrium constant on the ionic strength and the rate of desorption of the enzyme.

Heinzel et al. (1976) described the suppression of non-specific protein adsorption of human serum albumin-Sepharose by neutralizing the basic urethane groups (which arise from iminocarbonate groups formed mainly during the coupling of serum albumin to Sepharose) by the anionic dye Blue Dextran 2000 (BD). The immunosorbent prepared in this manner adsorbed 0.7 - 1.0 mg of proteins per millilitre of packed gel, while the isolated antibody was pure and preserved its native properties. Lornitzo et al. (1974) described non-specific (ionic) binding of fatty acid synthetase subunits onto a specific sorbent prepared by binding pantetheine to ε-aminocaproyl-Sepharose by means of a larger amount of carbodiimide (ethyldimethylamino-n-propyl-carbodiimide).

O'Carra et al. (1974) studied glyceraldehyde-3-phosphate dehydrogenase sorbed on the polymer:



and observed that the amount of the elutable enzyme depends on the time during which the enzyme is in contact with the substituted support. The yield of the enzyme that can be eluted with NAD^+ becomes progressively lower the longer the enzyme remains adsorbed on the gel before elution. The adsorption of the enzyme is not appreciably affected by high concentrations of potassium chloride, the enzyme is eluted in a pure state with 2 mM NAD^+ if the elution buffer contains a 0.2 M concentration of salt. Under such conditions, the enzyme is not sorbed on the support with attached spacer, nor after replacement of NAD^+ with $NADP^+$. From this result it can be concluded

that the sorption is biospecific in nature. The decrease in the amount of elutable enzyme with the time it has in contact with the specific sorbent leads to the hypothesis that this decrease is due to nonbiospecific adsorption onto the hydrophobic spacer arm after it has been previously bound biospecifically on immobilized NAD^+. The correctness of this hypothesis is supported by the results obtained with a bioaffinity sorbent in which the hydrophobic spacer was replaced with a hydrophilic one. The sorbent prepared in this manner retained its strong affinity for glyceraldehyde-3-phosphate dehydrogenase, while the yield of the enzyme eluted with NAD^+ solution was greater than 90% and did not decrease significantly, even when the enzyme was allowed to stand in the column for 1 1/2 h before elution. After the same time, the yield of NAD^+-elutable enzyme which was sorbed on a sorbent with hydrophobic spacer decreased to almost 10%.

Bioaffinity chromatography has also been described as a very useful and successful method for the purification of integral membrane proteins by Thomas and McNamee (1990). According to these authors a high ionic strength buffer will reduce non-specific binding due to the electrostatic interactions, and the presence of detergent in the buffer should reduce non-specific hydrophobic interactions. When the affinity ligands are lectins, ionic detergents such as deoxycholate and sodium dodecyl sulfate significantly reduce the binding capacity of most commonly used lectins. In this type of bioaffinity chromatography nonionic detergents (up to 2.5 % w/v) are recommended. Since only the membrane protein portion binds directly to the bioaffinity sorbent, detergent-containing buffers may extract and elute the lipid portion of these bound complexes. This may inactivate and possibly even elute the protein. For this reason, lipids are frequently included in elution buffers (1 - 2 % w/v) in order to stabilize active proteins.

By contrast, the non-specific binding of lipids and proteins to an immunoadsorption column would shorten the effective life of the column (Keenan, 1988). To prevent this problem the lipoproteins were removed from rabbit antisera and porcine plasma before running column by dextran sulphate precipitation. The use of bioaffinity chromatography to bind the contaminating proteins, allowing the sample of interest to pass through the biospecific column in the wash volume, has been recommended by Ostrove (1990) in order to achieve a great saving of time. The solution of non-specific adsorption

has also been described in the review on "Biospecific interactions: their quantitative characterization and use for solute purification" by Winzor and De Jersey (1989).

4.6.3 Compound affinity

Although the non-biospecific adsorption (O'Carra et al., 1974) generally represents an undesirable complication in bioaffinity chromatography (in the sense of sorption), many examples have been described in which it contributed usefully to an increase in the affinity of weak bioaffinity systems. Under such circumstances a suitable choice and a careful control of the conditions may preserve the predominance of the biospecific nature of the interaction suitably supported by the non-biospecific interaction, so that the latter does not become dominant.

The use of immobilized p-aminobenzamidine for the bioaffinity chromatography of human enterokinase in comparison with the isolation of trypsin is a good example. Grant et al. (1978) attached p-aminobenzamidine to CNBr-activated Sepharose 4B through the glycylglycine spacer arm. However, it was found that cytochrome c was adsorbed at pH 8.35 by glycylglycine-substituted Sepharose which was functioning as a cation exchanger. Coupling of p-aminobenzamidine to the spacer arm completely abolished this adsorption, suggesting that residual anionic groups were minimal. In distinction from trypsin, enterokinase was only retained by adsorbents which had been synthesised with a high concentration of CNBr and spacer arm. The lability of the adsorbent for enterokinase during storage provided evidence for the necessity of a favourable orientation of the ligand in the gel polymer. In distilled water (pH 6) at 4°C after 7 and 14 days there was a 60% and almost 100% decay, respectively, of the enterokinase binding capacity; the full capacity was retained over the same period by storage in 0.1 M sodium phosphate buffer of pH 6.1. Any loss of spacer arm by hydrolysis during this short term storage was undetectable; furthermore, cytochrome c was not adsorbed, indicating that the decay in enterokinase binding was not due to hydrolysis of the ligand. Trypsin adsorption was unaltered and independent of the mode of storage. The loss of enterokinase adsorption at low ionic strength, but retention of the original trypsin binding capacity, suggests a steric alteration in which the ligand becomes inaccessible to

enterokinase but not to the smaller trypsin molecule. This could be the result of hydrogen bonding between the carbonyl group of glycylglycine and the isourea group.

From the above discussion it follows clearly that many factors affect the interaction of the immobilized ligand with the complementary molecule. Both biospecific and non-biospecific sorptions are based, in principle, on the same nature and combination of electrostatic and hydrophobic interactions. The contribution of non-biospecific interactions can best be determined from a comparison of the dissociation constants of the complex of the isolated macromolecule with the immobilized affinity ligand, and with the same ligand in the solution used for elution, as shown in Chapter 14. This characterization of the affinity system is very necessary, especially if bioaffinity chromatography is used for isolation purposes as well as for the study of the specific interactions that take place in biochemical processes.

REFERENCES

Adachi, S., Tanabe, S. and Hashimoto, K., Biotechnol. Bioeng., 26 (1984) 635-639.

Amneus, H., Gabel, D. and Kasche, V., J. Chromatogr., 120 (1976) 391-397.

Anderson, D.J., Anhalt, J.S. and Walters, R.R., J. Chromatogr., 359 (1986) 369-382.

Angal, S. and Dean, P.D.G., Biochem. J., 167 (1977) 301-303.

Anspach, F.B., Johnston, A., Wirth, H.J., Unger, K.K. and Hearn, M.T.W., J. Chromatogr., 476 (1989) 205-225.

Anspach, F.B., Johnston, A., Wirth, H.J., Unger, K.K. and Hearn, M.T.W., J. Chromatogr., 499 (1990) 103-124.

Anspach, F.B., Wirth, H.J., Unger, K.K., Stantov, P., Davies, J.R. and Hearn, M.T.W., Anal. Biochem., 179 (1989) 171-181.

Aplin, J.D. and Hall, L.D., Eur. J. Biochem., 110 (1980) 295-309.

Avigad, G., Amaral, D., Asensio, C. and Horecker, B.L., J. Biol. Chem., 237 (1962) 2736-2743.

Azzi, A., Bill, K. and Broger, C., Proc. Nat. Acad. Sci. U.S., 79 (1982) 2447-2450.

Babashak, V. and Phillips, T.M., J. Chromatogr., 444 (1988) 21- 28.

Blanco, R.M., Calvete, J.J. and Guisan, J.M., Enzyme Microb. Technol., 11 (1989) 353-359.

Blanco, R.M. and Guisán, J.M., Enzyme Microb. Technol., 10 (1988) 227-232.

Bouriotis, V., Galpin, I.J. and Dean, P.D.G., J. Chromatogr., 210 (1981) 267-278.

Burrell, H.R. and Horowitz, J., Eur. J. Biochem., 75 (1977) 533- 547.

Clemetson, K.J., Pfueller, S.L., Luscher, E.F. and Jenkins, C.S.P., Biochim. Biophys. Acta, 464 (1977) 493-508.

Cuatrecasas, P., J. Biol. Chem., 245 (1970) 3059-3065.

Cuatrecasas, P., Wilchek, M. and Anfinsen, C.B., Proc. Nat. Acad. Sci. U.S., 61 (1968) 636-643.

Davis, G.C., Hein, M.B. and Chapman, D.A., J. Chromatogr., 366 (1986) 171-189.

De Bell, K.E., Taplits, M.S., Hoffman, T. and Bonvini, E., Cell. Immunol., 127 (1990) 159-171.

Duffey, P.S., Drouillard, D.L. and Barbe, C.P., J. Immunol. Methods, 45 (1981) 137-151.

Finn, F.M., Titus, G. and Hofmann, K., Biochemistry, 23 (1984) 2554-2558.

Fusek, M., Čapka, M. and Turková, J., Biotechnol. Lett., 9 (1987) 561-566.

Gierasch, L.M., Lacy, J.E., Thompson, K.F., Rockwell, A.L. and Watnick, P.I., Biophys.J., 37 (1982) 275-284.

Grant, D.W., Magee, A.I. and Hermon-Taylor, J., Eur. J. Biochem., 88 (1978) 183-189.

Harvey, M.J., Lowe, C.R., Craven, D.B. and Dean, P.D.G., Eur. J. Biochem., 41 (1974) 335-340.

Hayunga, E.G. and Sumner, M.P., J. Parasitol., 72 (1986) 283-291.

Heinzel, W., Rahimi-Laridjani, I. and Grimminger, H., J. Immunol. Methods, 9 (1976) 337-344.

Hipwell, M.C., Harvey, M.J. and Dean, P.D.G., FEBS Lett., 42 (1974) 355-359.

Hofstee, B.H.J., Anal. Biochem., 52 (1973) 430-448.

Holguin, J. and Cardinaud, R., Eur. J. Biochem., 54 (1975) 505- 514.

Hsiao, H.Y. and Royer, G.P., Arch. Biochem. Biophys., 198 (1979) 379-385.

Inoue, M., Hara, M., Nagashima, F., Matsui, S., Mitsuyasu, N. and Morimo, I., Biochim. Biophys. Acta, 659 (1981) 362-369.

Iqbal, J. and Saleemuddin, M., Biotechnol. Bioeng., 25 (1983) 3191-3195.

Ivanov, A.E., Turková, J., Čapka, M. and Zubov, V.P., Biocatalysis, 3 (1990) 235-241.

Jessup, W. and Dean, P.D.G., J. Chromatogr., 219 (1981) 419-426.

Jost, R., Miron, T. and Wilchek, M., Biochim. Biophys. Acta, 362 (1974) 75-82.

Keenan, M.J., Biochem. Arch., 4 (1988) 239-245.

Kennedy, R.M., Methods Enzymol., 182 (1990) 339-343.

Klibanov, A.M., Adv. Appl. Microbiol., 29 (1982) 1-28.

Kukongviriyapan, V., Poopyruchpong, N. and Ratanabanangkoon, K., J. Immunol. Methods, 49 (1982) 97-104.

Ling, T.G.I. and Mattiasson, B., Biotechnol. Bioeng., 34 (1989) 1321-1325.

Liu, Y.C. and Stellwagen, E., J. Biol. Chem., 262 (1987) 583-588.

Lornitzo, F.A., Qureshi, A.A. and Porter, J.W., J. Biol. Chem., 249 (1974) 1654-1656.

Lowe, C.R., Eur. J. Biochem., 73 (1977) 265-274.

Lowe, C.R., An Introduction to Affinity Chromatography, Elsevier/North Holland Biomedical Press, Amsterdam, 1979, pp. 269-522, (322, 329-330, 418-423).

Lowe, C.R. and Dean, P.D.G., FEBS Lett., 18 (1971) 31-34.

Lowe, C.R., Harvey, M.J., Craven, D.B. and Dean, P.D.G., Biochem. J., 133 (1973) 499-506.

Månsson, M.O., Siegbahn, N. and Mosbach, K., Proc. Nat. Acad. Sci. U.S., 80 (1983) 1487-1491.

Martinek, K., Klibanov, A.M. and Berezin, I.V., J. Solid-Phase Biochem., 2 (1977) 343-374.

Mattiasson, B., Methods Enzymol., 137 (1988) 647-656.

Mattiasson, B. and Olsson, U., J. Chromatogr., 370 (1986) 21-28.

Morrow, R.M., Carbonell, R.G. and McCoy, B.J., Biotechnol. Bioeng., 17 (1975) 895-914.

Mozhaev, V.V., Melik-Nubarov, N.S., Sergeeva, M.V., Šikšnis, V. and Martinek, K., Biocatalysis, 3 (1990a) 179-187.

Mozhaev, V.V., Melik-Nubarov, N.S., Šikšnis, V. and Martinek, K., Biocatalysis, 3 (1990b) 189-196.

Mozhaev, V.V., Šikšnis, V.A., Melik-Nubarov, N.S., Galkantaite, N.Z., Denis, G.J., Butkus, E.P., Zaslavsky, B.Y., Mestechkina, N.M. and Martinek, K., Eur. J. Biochem., 173 (1988) 147-154.

Nilsson, B., Abrahmsen, L. and Uhlen, M., EMBO J., 4 (1985) 1075- 1080.

Nilsson, S.F. and Waxdal, M.J., Biochemistry, 17 (1978) 903-910.

Nishikawa, A.H., Bailon, P. and Ramel, A.H., Advan. Exp. Med. Biol., 42 (1974) 33-42.

Norrlöw, O., Månsson, M.O. and Mosbach, K., J. Chromatogr., 396 (1987) 374-377.

O'Carra, P., Barry, S. and Griffin, T., Methods Enzymol., 34 (1974a) 108-126.

O'Carra, P., Barry, S. and Griffin, T., FEBS Lett., 43 (1974b) 169-175.

Olsson, U. and Mattiasson, B., J. Chromatogr., 370 (1986) 29-37.

O'Shannessy, D.J., J. Chromatogr., 510 (1990) 13-21.

O'Shannessy, D.J. and Quarles, R.H., J. Immunol. Methods, 99 (1987) 153-161.

Ostrove, S., Methods Enzymol., 182 (1990) 357-371.

Pak, K.Y., Randerson, D.H., Blaszczyk, M., Sears, H.F., Steplewski, Z. and Koprowski, H., J. Immunol. Methods, 66 (1984) 51-58.

Pazur, J.H., Knull, H.R. and Simpson, D.L., Biochem. Biophys. Res. Commun., 40 (1970) 110-116.

Petkov, L., Sajdok, J., Rae, K., Šůchová, M., Káš, J. and Turková, J., Biotechnol. Techniques, 4 (1990) 25-30.

Scawen, M.D., Darbyshire, J., Harvey, J. and Atkinson, T., Biochem. J., 203 (1982) 699-705.

Schwarz, A., Wandrey, C., Bayer, E.A. and Wilchek, M., Methods Enzymol., 184 (1990) 160-162.

Shaltiel, S., Methods Enzymol., 34 (1974) 126-140.

Shaltiel, S., Methods Enzymol., 104 (1984) 69-96.

Sica, V., Nola, E., Parikh, I., Puca, G.A. and Cuatrecasas, P., Nature (London) New Biol., 244 (1973) 36-39.

Skjåk-Braek, G. and Larsen, B., Carbohyd. Res., 103 (1982) 137- 140.

Solomon, B., Koppel, R., Schwartz, F. and Fleminger, G., J. Chromatogr., 510 (1990) 321-329.

Swaisgood, H.E. and Chaiken, I.M., J. Chromatogr., 327 (1985) 193-204.

Taylor, R.F., Anal. Chim. Acta., 172 (1985) 241-248.

Temler, R.S. and Kägi, J.H.R., Enzyme, 22 (1977) 249-255.

Thomas, T.C. and McNamee, M.G., Methods Enzymol., 182 (1990) 499- 520.

Turková, J., Technology Today, 2 (1990) 82-85.

Turková, J., Beneš, M.J., Vohník, S., Helusová, S. and Tichá, M., J. Chromatogr., 597 (1992) 19-27.

Turková, J., Bláha, K. and Adamová, K., J. Chromatogr., 236 (1982) 375-383.

Turková, J., Fusek, M., Šťovíčková, J. and Králová, Z., Makromol. Chem., Macromol. Symp., 17 (1988) 241-256.

Wikström, P. and Larsson, P.O., J. Chromatogr., 388 (1987) 123-134.

Winzor, D.J. and De Jersey, J., J. Chromatogr., 492 (1989) 377- 430.

Zaborsky, O.R. and Ogletree, J., Biochem. Biophys. Res. Commun., 61 (1974) 210-216.

Zamecnik, P.C., Stephenson, M.L. and Scott, J.F., Proc. Nat. Acad. Sci. U.S., 46 (1960) 811-822.

This Page Intentionally Left Blank

Chapter 5

Solid matrix supports

One of the most important factors in the development of bioaffinity chromatography and immobilized enzymes is the development of solid supports. A correct choice of solid support and the covalent coupling between the matrix and the affinity ligand may be essential for the success of the desired bioaffinity chromatographic separation. The effect of solid supports on the biospecific adsorption has been already discussed in Section 4.1. Solid matrix supports can also have a considerable effect on the stability of the immobilized affinity ligand and the adsorbed material. A solid support may even be an affinity ligand itself, e.g. polysaccharides for some lectins. An increasing number of different types of supports, their activated forms and biospecific matrices prepared from them as so-called ready-to-use adsorbents are now becoming commercially available. In the text we mention only those firms known to the author. The information about commercially available solid supports and their derivatives is therefore necessarily incomplete and it should in no case be considered as implying the recommendation of any particular firm or product. Within the scope of this chapter only basic solid supports are described and briefly discussed.

5.1 REQUIRED CHARACTERISTICS

An ideal matrix for successful application in bioaffinity chromatography and for the immobilization of enzymes should possess the following properties (Porath,1974):

(1) insolubility;

(2) sufficient permeability and a large specific area;

(3) high rigidity and a suitable form of particles;

(4) zero adsorption capacity;

(5) chemical reactivity permitting the introduction of affinity ligands;

(6) chemical stability under the conditions required for the attachment, adsorption, desorption and regeneration;

(7) resistance to microbial and enzymatic attack;

(8) hydrophilic character.

Complete insolubility is essential, not only for the prevention of losses of bioaffinity adsorbent, but mainly for the prevention of contamination of the substance being isolated by dissolved carrier. The requirement of sufficient permeability of the solid support, permitting sufficient freedom for the formation of complexes of macromolecules with complementary affinity ligands, has already been discussed in detail in Section 4.1.

Table 5.1 shows the amount of chymotrypsin and glycine bound to 1 ml of hydroxyalkyl methacrylate gels of various pore sizes, depending on the exclusion molecular weights and the different specific surface areas (Turková et al., 1973). It is obvious that the amount of bound chymotrypsin depends directly on the specific surface area, which is largest with Spheron 300 and 500. The amount of bound glycine indicates that there are relatively small differences in the number of reactive groups. The relative proteolytic activity is also given in Table 5.1.

Table 5.1.

Amounts of chymotrypsin and glycin bound to hydroxyalkyl methacrylate gels (Spherons) as a function of their specific surface areas

Gel	Exclusion mol.wt	Specific surface area (m^2/ml)	Amount of bound glycine (mg/ml)	Amount of bound chymotrypsin (mg/ml)	Relative proteolytic activity (%)
Spheron 10^5	10^8	0.96	0.5	0.73	-
Spheron 10^3	10^6	5.9	3.1	7.8	44
Spheron 700	700,000	3.6	2.8	6.7	49
Spheron 500	500,000	23	2.6	17.1	37
Spheron 300	300,000	19.5	3.15	17.7	44
Spheron 200	200,000	0.6	2.3	6.9	53
Spheron 100	100,000	0.2	2.6	2.6	38

Practically the same conclusion may be drawn from the paper on the influence of the physical properties of the base matrix in the preparative bioaffinity chromatography of

proteins published by Narayanan et al. (1990). They synthesized and characterized four silica-based high performance bioaffinity media differing in pore size and surface area. A pore structure analysis of one matrix during the coupling of concanavalin A (Con A) is shown in Table 5.8. The surface characterization and coupling of Con A to four silica gels is shown in Table 5.9. In order to compare the accessible surface areas of the four studied media, besides Con A the binding capacities of lactoglobulin, horseradish peroxidase, glyceraldehyde-3-phosphate dehydrogenase and thyroglobulin were also determined. A large protein like thyroglobulin (molecular weight 670,000) was not coupled at all to a silica preparation with an average pore diameter of 88 Å, while a medium sized protein like peroxidase (molecular weight 40,000) bound less to a silica preparation with an average pore diameter of 776 Å despite the large pore size.

Con A immobilized to four silica preparations was used for bioaffinity chromatography of horseradish peroxidase. Although the Con A concentration of silica preparation 3 (mean pore diameter 408 Å) is comparable to preparation 2 (mean pore diameter 190 Å), the adsorption capacity for peroxidase on Con A-silica preparation 2 is higher. The use of the same sorbents for bioaffinity separation of small molecules (p-nitrophenyl sugar derivatives) shows that the pore diameter is much less important for binding.

The pore size effects of diol-bonded silica in high-performance bioaffinity chromatography have been studied by Walters (1982). He concluded that two factors were responsible for poor binding capacities at intermediate pore size: restricted diffusion and slow adsorption-desorption kinetics. Horstmann et al. (1986) studied the adsorption of proteins on Sepharose bioaffinity adsorbents of varying particle sizes. The maximum capacities obtained increased with decreasing particle size in biospecific sorbents. The volumetric coefficients of overall mass transfer in bioaffinity chromatography were studied by Katoh and Sada (1980). Sepharose 6B was efficient for adsorbed components of low or middle molecular weight because of the high operating velocity. The mass transfer coefficient of adsorbed components of high molecular weight in Sepharose 6B was extremely low. In this case the column productivity was lower than that in a Sepharose 4B column. Thus, an ideal bioaffinity chromatography

porous support would have sufficiently large pores accessible for molecules to penetrate, yet it would preserve the maximum surface area for binding in a suitable particle size.

By contrast bioaffinity adsorption of cells on an immobilized ligand occurs exclusively at the external surface of the support. Therefore, in this type of separation, it is suitable to select a support with an exclusion limit as low as possible in order to favour ligand immobilization on the external surface of the beads.

In order to eliminate the kinetically limited access of the biospecifically adsorbed material to the active site of affinity ligand and removal of the adsorbed material due to internal diffusion, nonporous solid supports have been increasingly used (Adachie et al., 1984; Swaisgood and Chaiken, 1985, 1986; Unger et al., 1986; Fusek et al., 1987; Anspach et al., 1988, 1989, 1990). The comparison of porous and nonporous silica in high-pressure liquid bioaffinity chromatography (HPLBAC) is discussed in Section 5.5. The use of an activated nylon membrane as a bioaffinity adsorbent for the simple purification of enzymes from yeast cell homogenate is described in Section 5.6.

The requirement of rigidity and a suitable shape of the particles is connected with the problem of flow-through rate. For a smooth course of bioaffinity chromatography good flow properties are important, i.e. the eluent should penetrate the support column at a sufficient rate, even when the affinant is bound to it. The particle size of the support should not exceed 200 μm and should not be less than 1.5 μm.

The sorbent should have a minimum non-specific adsorption. When an insoluble affinant is prepared, it is important that it should be bound to the carrier very strongly, and the molecules of the affinant that are not bound must be washed out. Similarly, when substances that form a specific and reversible complex with the bound affinant are isolated, it is important that, as far as possible, only their retention should take place on the column of insoluble affinant and only in the form of a specific complex with the bound affinant. This is one of the main reasons why carriers that contain ionogenic groups, such as the copolymer of ethylene with maleic anhydride, which sets carboxyl groups free after the affinant has been attached, have never been as widely applied in bioaffinity chromatography as neutral agarose.

The support must possess a sufficient number of chemical groups that can be activated or modified in such a way that they become capable of binding affinants. The activation or modification should take place under conditions that do not change the structure of the support. No less important are the chemical and mechanical stabilities of the carrier under the conditions of attachment of the affinant, and also at various pH values, temperatures and ionic strengths, in the possible presence of denaturating agents, etc., which may be necessary for good sorption and elution of the isolated substance. The possibility of the repeated use of a specific adsorbent depends on these stabilities.

A further requirement is connected with the above, viz. that the specific sorbents should not be attacked by microorganisms and enzymes. This requirement is best fulfilled by inorganic supports, such as glass and silica, or by synthetic polymers, such as polyacrylamide or hydroxyalkyl methacrylate gels.

A hydrophilic character of a solid support is desirable not only because of the necessity of minimizing non-specific sorption and inactivation, but also because a hydrophobic character of the support can decrease the stability of some bound affinity ligands or eluted material on the basis of denaturation analogous to that produced by organic solvents. Table 5.2 shows the characteristics required of supports as a function of the type of chromatography: low pressure (LPLBAC), medium pressure (MPLBAC) or high pressure liquid bioaffinity chromatography (HPLBAC).

5.2 BIOPOLYMERS

Water molecules are essential for the structure and function of biologically active compounds. Bioaffinity chromatography is therefore generally performed in the aqueous phase and hydrophilic biopolymers rank as very suitable solid supports. Great attention has been paid to natural polysaccharides, such as cellulose, dextran, agarose and, to a lesser extent, starch. The modification necessary for the bioaffinity chromatography can be carried out in a relatively simple way *via* the OH groups (Chapter 6).

Table 5.2.

Characteristics required of a bioaffinity support as a function of the type of chromatography

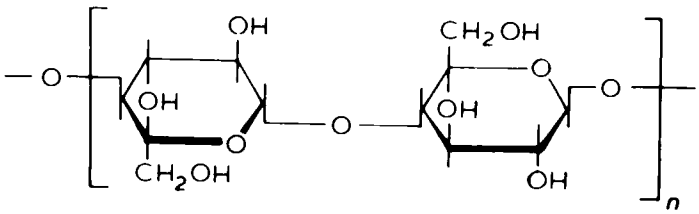
	LPLBAC	*MPLBAC*	*HPLBAC*
Rigidity	+	+	+ + +
Porosity	+ + (+)	+	+
Hydrophilic character	+ + +	+ + +	+
Secondary interaction	-	-	-
Cost	+	+	+
Activable functional groups	+ + +	+ + +	+

The conformation of the chains and the interaction between them have an influence on the use of polysaccharides as chromatographic supports. The geometry of the chains is determined by the relative orientation of adjacent sugar residues around the glycosidic bonds. But in aqueous solutions a disordered random coil form dominates, because of the flexibility of the chains. Under definable conditions, however, non-bonded energy-terms (hydrogen bridges, ionic interactions, and others) can compensate for conformation entropy and fix the polysaccharide chains in an ordered configuration. One matter of great practical importance for the optimization of polysaccharides as chromatographic support is that it is possible to influence the factors responsible for the equilibrium between an ordered and a non-ordered configuration in aqueous solution.

5.2.1 Cellulose and its derivatives

Cellulose is formed by linear polymers of β-1,4-linked D-glucose units with an occasional 1,6-bond (see next page).

Commercially available celluloses are generally crosslinked with bifunctional reagents, such as 1-chloro-2,3-epoxypropane, and they are very stable to chemical attack. Glycosidic bonds are sensitive to acid hydrolysis, and under extreme conditions an almost quantitative decomposition to pure crystalline D-glucose may take place. On interaction with oxidative reagents, such as sodium periodate, aldehyde and carboxyl groups are formed. Cellulose can be attacked, for example, by microbial cellulases.



In Chapter 2 it has been shown that cellulose is one of the standard supporting materials in bioaffinity chromatography. Cellulose is a vegetable polysaccharide. Cellulose and its derivatives are produced by a number of firms. In addition to Whatman (Maidstone, Great Britain) and Schleicher & Schüll (Zürich, Switzerland), Serva (Heidelberg, F.G.R.) lists both cellulose derivatives and bromoacetylcellulose (BA-cellulose), p-aminobenzoylcellulose (PAB-cellulose), benzoyl-DEAE-cellulose (BD-cellulose) and benzoylnaphthoyl-DEAE-cellulose (BND-cellulose). Bio-Rad Labs. (Richmond, Calif., U.S.A.) supply p-aminobenzoylcellulose under the trade name Cellex PAB and aminoethyl-cellulose under the name Cellex AE. Miles Labs (Slough, Great Britain) produce a hydrazide derivative of CM-Cellulose (Enzite-CMC-hydrazide), bromoacetylcellulose (BAC) and m-aminobenzyloxymethylcellulose (AMBC).

Macroporous reconstituted cellulose in regular beaded form is produced under the trade mark Perloza by Chemopetrol (Lovosice, Czechoslovakia). Perloza is manufactured by a special technology based on the dispersion of a solution of cellulose xanthate in an inert liquid with subsequent thermal solidification of the xanthate, followed by regeneration (Štamberg et al., 1979, Štamberg, 1988). The bead cellulose has regularly arranged layers with optimum through flow properties when used in columns. Good mechanical strength is preserved in spite of the high porosity. Due to its chemical composition it is highly hydrophilic and is well tolerated by biosystems. Its insolubility in water and a number of other solvents allows it to be used for interaction in liquid media. Perloza is a suitable material for a wide range of applications in bioaffinity chromatography, which have been summarized by Gemeiner et al. (1989). Medium-pressure Matrex Cellufine gels supplied by Amicon Co. (Danvers, MA, U.S.A.) are composed of spherical, beaded cellulose. They offer low non-specific

adsorption adsorption, outstanding physical strength and high pressure resistance capabilities, all at an economic cost. The use of Cellufine in bioaffinity chromatography has been described by Anspach et al. (1990).

To solve purification problems connected with the transfer from laboratory scale to industrial scale Hou and Mandaro (1986) have developed a radial flow chromatography method on a cellulosic media derivatized by vinyl polymers. The method employed for grafting the polymer onto the cellulose provides the matrix with a high charge density and thus a very high adsorption capacity. A schematic representation of radial flow Zeta cartridge is shown in Fig. 5.1. The cartridge was constructed by rolling up the thin sheet matrix around a central rod with webs between the layer of the matrix to allow the swelling of the media and to provide an even distribution of the fluid throughout the matrix. The fluid flows radially from the outside to the inside of the cartridge through the layers of the grafted media (ion exchange or bioaffinity), and it is then collected by the flow paths engraved on the central core, and exits through the ports located at the top of the cartridge. Mandaro and Hou (1987) have used this type of zeta chromatography for bioaffinity chromatography.

Zeta cartridges and several Zeta affinity products are produced by the French firm CUNO Inc. (Cergy-Pontoise, France). Zeta-Prep. cartridges are also producted by LKB (Broma, Sweden). Mem Sep TM Chromatography Cartridges, which are ready to use and provide a microporous network of pure regenerated cellulose in a column-type housing configuration, containing several bioaffinity membrane cartridges are producted by MILLIPORE (Bedford, MA, U.S.A.). Membrane formation, characterization and applications are in detail described in monograph "Affinity Membranes, Their Chemistry & Performance in Adsorption Separation Processes" by Klein (1991).

5.2.2 Dextran gels

Dextran is a branched-chain glucose polysaccharide produced in solutions containing sugar by various strains of *Leuconostoc mesenteroides*. Soluble dextran, prepared by fractional precipitation with ethanol of partially hydrolysed crude dextran, contains more than 90% of α-1,6-glucosidic linkages, and it is branched by 1,2-, 1,3- and

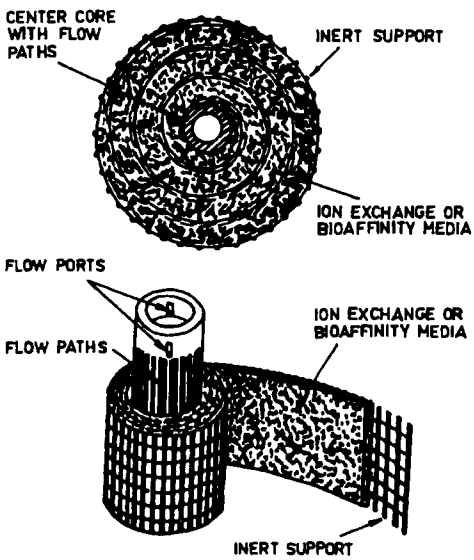
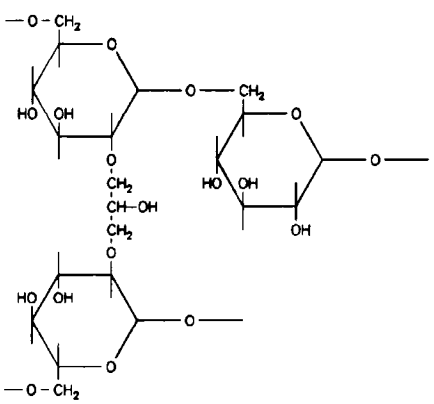


Fig. 5.1. Radial flow Zeta cartridge.

1,4-glucoside bonds. When crosslinked with 1-chloro-2,3-epoxypropane in alkaline solution, dextran affords a three-dimensional gel with the partial structure



The most important producer of dextran gels, supplied under the trade name Sephadex, is Pharmacia (Uppsala, Sweden). The gels are very stable to chemical attack; for example, exposure for 2 months to 0.25 M sodium hydroxide solution at 60°C has no influence. The glucosidic bond is sensitive to hydrolysis at low pH, although it is stable

for 6 months in 0.02 M hydrochloric acid, or for 1-2 h in 0.1 M hydrochloric acid or 88% formic acid (Lowe and Dean, 1974). Aldehyde or carboxyl groups are formed under the effect of oxidizing agents. As regards thermal stability, dextran gels can withstand heating in an autoclave at 110°C (in solution) for 40 min, or at 120°C when dry. Drying and swelling is reversible. The gels swell to a certain extent, even in ethanol, ethylene glycol, formamide, N,N-dimethylformamide and dimethyl sulphoxide.

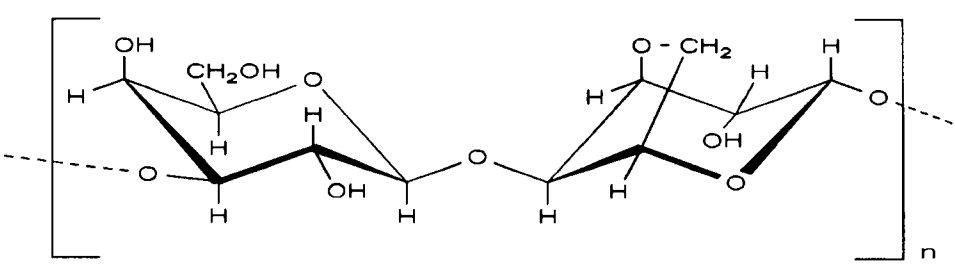
The molecular sieve, produced by covalent crosslinking of allyl-dextran with N,N'-methylene-bis-acrylamide, has been developed by Pharmacia (Uppsala, Sweden) under the trade name Sephacryl (Johansson and Lundberg, 1979). The big advantage of this kind of matrix is the excellent flow rate, because this support is exceptionally rigid. However, on the other hand the unspecific adsorption is increased.

The use of dextran gels is partly restricted by their rather low porosity. Examples of their use in bioaffinity chromatography are given in Table 9.1. They are widely used without any modification as specific sorbents for the isolation of a series of lectins.

5.2.3 Agarose and its derivatives

Agarose is a polysaccharide present in agar that contains agaropectin in addition to agarose. The separation of agarose from the polysaccharide mixture is based on differences in solubility and chemical reactivity, which is associated with the anionic character of agaropectins. Cooling aqueous agarose solutions below 50°C allows the development of agarose gels in bead, pellet, or spherical forms. Practical preparation methods have been described by Hjerten (1964) and Bengtsson and Philipson (1964).

Agarose is a linear polysaccharide consisting of alternating 1,3-linked β-D-galacto-pyranose and 1,4-linked 3, 6-anhydro-α-L-galactopyranose residues:



Arnott et al. (1974) postulated on the basis of X-ray studies that the polysaccharide chains form a double helix, then aggregate *via* hydrogen bridges and hydrophobic interactions into fibers or bundles with ordered structures. This network phase in a gel which may contain up to 100 times more water than agarose means that the structure contains relatively large voids through which large macromolecules can diffuse. In contradistinction, a gel network comprising a comparable concentration of crosslinked soluble polymer, such as the crosslinked dextrans, would lead to a lattice in which the mean pore size would be considerably smaller. These relationships are shown diagrammatically in Fig. 5.2 and suggest that agarose should exhibit special properties as a chromatographic medium. The exclusion limit may be varied within wide ranges because the pore size is inversely proportional to the agarose concentration (Mohr and Pommerening, 1985; Table 5.3).

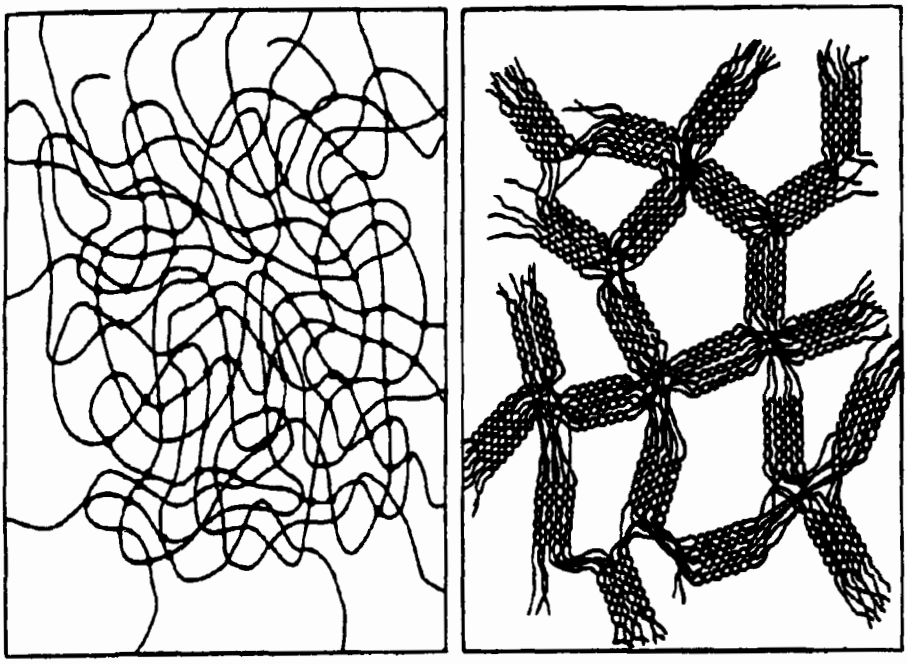


Fig. 5.2. A comparison between an agarose gel matrix (right) with a cross-linked dextran (Sephadex) matrix at equivalent polymer concentration. The aggregates in agarose gels may contain 10 - 10^4 bundles of polysaccharide helices. Reproduced with permission from Arnott et al., J. Mol. Biol., 90 (1974), 269-284.

Table 5.3.

Some commercial types of agarose gels

Trade name	%[a]	*Fractionation range (molecular weight)*
Bio-Gel A-0.5 m	10	1×10^4 to 5×10^5
Bio-Gel A-1.5 m	8	1×10^4 to 1.5×10^6
Bio-Gel A-5 m	6	1×10^4 to 5×10^6
Sepharose 6B/CL-6B	6	1×10^4 to 4×10^6
Ultrogel A6	6	2.5×10^4 to 4×10^6
Bio-Gel A-15 m	4	4×10^4 to 1.5×10^7
Sepharose 4B/CL-4B	4	6×10^4 to 2×10^7
Ultrogel A4	4	5.5×10^4 to 2×10^7
Bio-Gel A-50 m	2	1×10^5 to 5×10^7
Sepharose 2B/CL-2B	2	7×10^4 to 4×10^7
Ultrogel A2	2	1.2×10^5 to 5×10^7
Bio-Gel A-150 m	1	1×10^6 to 1.5×10^8

[a] Agarose in gel.

The main producers of agarose are Pharmacia (Uppsala, Sweden), under the trade name Sepharose, Bio-Rad Labs. (Richmond, Calif., U.S.A.), under the trade name Bio-Gel A and Reactifs IBF (Villeneuve la Garenne, France) under the trade name Ultrogel A. Agarose gels under the name SAG (Ago-Gel) -10, -6, -4 and -2 with molecular weight exclusion limits of 25×10^4 - 15×10^7 are supplied by Seravac Labs. (Maidenhead, Great Britain) and Mann Labs. (New York, N.Y., U.S.A.). Recommended solvents are dilute aqueous solutions of salts containing 0.02% of sodium azide as a bacteriostatic agent.

Supports with large beads are advantageous for the bioaffinity chromatography of cells, so that they can pass through the column without being physically trapped. For this purpose an agarose gel has been developed with the trade name Sepharose 6MB. The macrobeads have a large diameter (250 - 350 μm), uniform shape, and low nonspecific adsorption of cells. Derivatives of agarose (Bio-Gel A15 m, 74 - 149 μm) modified for use in bioaffinity chromatography are produced by Bio-Rad Labs. under

the trade name Affi-Gel, and are listed in Table 9.4. Various derivatives of agarose modified especially for application as bioaffinity resin are offered commercially.

The stability of an agarose matrix can be considerably increased by crosslinking with epichlorohydrin, 2, 3-dibromopropanol or divinyl sulphone (Kristiansen, 1974). In 1975, Sepharose CL(2B, 4B, 6B) was introduced, prepared from appropriate types of Sepharoses by crosslinking with 2, 3-dibromopropanol in strongly alkaline medium, and further desulphurization by alkaline hydrolysis under reductive conditions. The crosslinking of Sepharose CL does not decrease the effective pore size, thus suggesting that crosslinks take place mainly between chains within a single gel fiber, probably between oxygen in position 2 of the anhydrogalactose residue (Mohr and Pommerening, 1985). The increased stability of Sepharose CL is shown in Table 5.4.

The structure of agarose makes it inadvisable to dry and re-swell the gels. When agarose is not in use it should be stored in the wet or moist state and protected from microbial growth by means of a suitable bacteriostat. A number of antimicrobial agents are in common use: 0.02% sodium azide, 0.5% butanol, trichlorobutanol and saturated toluene. Other bacteriostatic agents should only be used if they are known to be innocuous to the structure of agarose. In general, agarose gels should be stored for long

Table 5.4.

Comparison of stability between normal and covalent cross-linked agarose gels

	Sepharose	*Sepharose CL*
pH	4-9	3-14
Temperature (°C)	0-40	< 70
Solvents	Agueous solutions containing high concentrations of salts, urea, guanidine.HCl, detergents	Aqueous solutions, 6 M guanidine.HCl, 8 M urea and detergents in a pH range 3-11
	Dimethylformamide-H_2O (1:1)	Organic solvents
	Ethyleneglycol-H_2O (1:1)	
Sterilization	Not autoclaving	Autoclaving at pH 7 and 110-120 °C
Chaotropic ions (KSCN)	Low stability	High stability

periods in the presence of a suitable bacteriostat in a refrigerator below 8°C but without freezing. Freezing results in irreversible structural disruption of the gel beads. Freeze drying can be carried out only after the addition of protective substances, for example dextran, glucose and serum albumin.

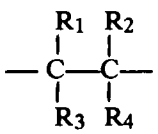
Despite some limitations in stability, the beaded derivatives of agarose exhibit many of the properties of the ideal matrix and have been widely acclaimed as the matrix of choice. Their loose structure allows ready penetration by large macromolecules and their hydrophilic polysaccharide nature and nearly complete absence of charged groups precludes the adsorption or denaturation of sensitive biochemical substances. Furthermore, the polysaccharide backbone can readily undergo substitution reactions to yield products with a moderately high capacity for further derivatisation (Lowe, 1979).

Dean et al. in their book (1985) described Superose 6B, which is a highly cross-linked agarose matrix, resulting in a very rigid gel. Both the crosslinking and narrow particle size (20 - 40 μm) contribute to its performance as a chromatography support for high performance separations. It is produced by Pharmacia LKB Biotechnology (Uppsala, Sweden). Their new names for Sepharose high performance bioaffinity columns are Hi Trap Columns which cover a wide range of life science applications. Reactifs IBF (Villeneuve la Garenne, France) produces Magnogel A4R which is a support composed of agarose (4%,w/v) cross-linked with epichlorohydrin. Its magnetic nature results from the incorporation of 7% (w/v) Fe_3O_4 in the interior of the gel beads. It has applications in situations where column operation is not favoured, e.g. in viscous solutions or in the presence of partly soluble materials such as cell debris.

5.3 SYNTHETIC COPOLYMERS

The advantages of high-performance liquid bioaffinity chromatography, which are shown in Fig. 1.2, and the usefulness of the preparation of biologically active compounds on a pilot or industrial scale, are the impetus behind the continual development of synthetic polymers. The main appeal of solid supports for these applications is their inherent mechanical stability which provides good flow characteristics even under high

pressures. The first synthetic polymers have been introduced for low- or medium-pressure bioaffinity chromatography. At present some of synthetic copolymers can be operated with pressure up to 100 p.s.i., and usually tolerate a wide pH range. They are suitable for affinity ligand immobilization and provide bioaffinity supports with high capacities. They are biologically inert and, because of their inert structure, they are not subject to enzymatic or microbial degradation. In most cases they are crosslinked macroporous vinyl polymers in beaded or spherical form having a defined size and porosity. The properties can be obtained by proper choice of monomers and conditions of polymerization (Mohr and Pommerening, 1985). The chemical structure of these supports can be characterized by the polyethylene backbone, which influences chemical stability and physical or structural stability, and the modifiable side chains R_1, R_2, R_3, R_4:



Only a limited number of synthetic polymers is characterized in this Section 5.3. However, many synthetic copolymers exist, and their intermediates for bioaffinity chromatography and biospecific sorbents prepared from them are produced by many firms. Commercial materials used in the examples of bioaffinity chromatography in Chapter 9 are summarized in Table 9.4. The table, however, should not be considered to be comprehensive. Full details are available from individual firms.

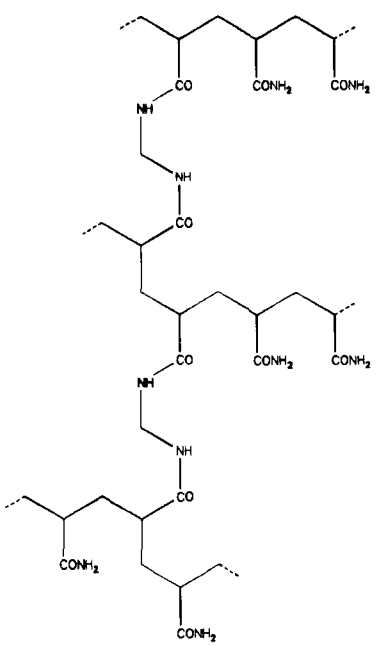
5.3.1 Polyacrylamide supports and their derivatives

Polyacrylamide gels are produced by copolymerization of acrylamide with the bifunctional crosslinking agent N,N'- methylenebisacrylamide (Hjerten and Mosbach, 1962). The monomers used in this synthesis are highly toxic and thus should be handled with care.

The main producer of polyacrylamide gels is Bio-Rad Labs. (Richmond, CA, U.S.A.) under the trade name Bio-Gel P. This gel is produced with a range of pore sizes, from

Bio-Gel P-2 with a molecular weight exclusion limit of 1800, up to Bio-Gel P-300 with a molecular weight exclusion limit of 400,000. All brands are available in 50 - 100, 100 - 200, 200 - 400 and 400 mesh size. Commercial polyacrylamide beads are purchased in the dry state and are swollen by mixing with water or aqueous solutions for periods of 4 - 48 h depending on the porosity. Bio-Gel P products are stable to most eluants used in biochemical studies, including dilute solutions of salts, detergents, urea and guanidine hydrochloride, although high concentrations of these reagents may alter the exclusion limits by up to 10%. The use of media with pH values outside the range 2 - 10 is to be avoided since some hydrolysis of the amide side groups may occur with the consequent appearance of ion-exchange groups. The use of oxidising agents such as hydrogen peroxide is also inadvisable.

Polyacrylamide gels are composed of a hydrocarbon skeleton on to which carboxamide groups are bound:



Non-specific adsorption to the matrix backbone is restricted to very acidic, very basic and aromatic compounds and is evidenced by delayed emergence from the chro-

matographic bed. Ionic groups on the matrix are almost non-existent and ion exchange is of no practical significance unless ionic strengths less than 0.02 are used. The properties of polyacrylamide suggest that in certain circumstances polyacrylamide gels may prove superior to polysaccharide based gels. For example, should degradation of the polyacrylamide matrix occur, there is unlikely to be any confusion between the reaction products and sample molecules since polyacrylamide is an entirely synthetic polymer. These advantages of polyacrylamide gels are particularly apparent in applications involving the purification of carbohydrates or carbohydrate-binding macromolecules.

Polyacrylamide gels are biologically inert and they are not attacked by microorganisms. As the gel particles adhere strongly to clean glass surfaces, Inman and Dintzis (1969) recommend the use of siliconized glass or polyethylene laboratory vessels. On reaction with a suitable compound, they can be converted into solid carriers suitable for the binding of a series of affinants (Inman and Dintzis, 1969). Their aminoethyl derivatives can be prepared by using a large excess of ethylenediamine at 90°C, and hydrazide derivatives by using an excess of hydrazine at 50°C. Aminoethyl derivatives of polyacrylamide gels can be converted into their p-aminobenzamidoethyl derivatives by reaction with p-nitrobenzoylazide in the presence of N,N-dimethylformamide, triethylamine and sodium thiosulphate.

Chemical derivatization of polyacrylamide may be also achieved by copolymerization of acrylamide and a crosslinking agent, with a functional group-bearing acrylic or vinyl monomer. For example Schnaar and Lea (1975) have synthesized acrylic acid esters of N-hydrosuccinimide and N-hydroxyphthalimide and copolymerized them with acrylamide and methylenebisacrylamide. The resulting "active" gels react readily with ligands containing primary amino groups.

For the linking of affinants, mainly enzymes, Koch-Light (Colnbrook, Great Britain) produces Enzacryls. Enzacryl AH is a hydrazide derivative of a polyacrylamide gel, and Enzacryl AA is a polyacrylamide gel containing aromatic acid residues. Enzacryl Polyacetal is a copolymer of N-acryloylaminoacetaldehyde dimethyl acetal with N, N'-methylenediacrylamide, which binds proteins through their NH_2 groups. Enzacryl

Polythiol is a crosslinked copolymer of acrylamide and N-acryloylcysteine. In the presence of oxidants this binds proteins through their -SH groups.

5.3.2 Hydroxyalkyl methacrylate supports

Hydrophilic hydroxyalkyl methacrylate gels, introduced by Wichterle and Lim (1960) were prepared by Čoupek et al. (1973). The copolymerization of hydroxyalkyl methacrylate with alkylene dimethacrylates gives rise to heavily crosslinked microparticles of a xerogel, which subsequently aggregate and yield macroporous structures of spheroids. Their structure is shown in Fig. 5.3 (Turková and Seifertová , 1978). Because of this structure, the gels have some chemical properties in common with the most commonly used support, agarose. Thus, for example, the hydroxyl groups of the gel can be activated with cyanogen bromide (Turková et al., 1973), in a similar manner to the hydroxyl groups of agarose. Amino acids, peptides and proteins can be bound to the activated gels through their amino groups. The hydroxy groups of the copolymer react in an alkaline medium with epichlorohydrin. Coupling of proteins after this activation is discussed in Chapter 6.

Hydroxyalkyl methacrylate supports have been marketed under the trade name Separon HEMA, produced by TESSEK Ltd. (Prague, Czechoslovakia), or Spheron, produced by Lachema (Brno, Czechoslovakia). Regular beads have good chemical and mechanical stability. They do not change their structures after heating for 8 h in 1 M sodium glycolate solution at 150°C or after boiling in 20% hydrochloric acid for 24 h. They are biologically inert and are not attacked by microorganisms. They can be employed in organic solvents, a property which was used to advantage during the binding of peptides on to these gels (Turková et al., 1976). The inner structure, pore size, and distribution, specific surface, and quantity of reactive OH groups can be varied, with a molecular weight exclusion limit ranging from 20,000 to 20,000, 000. A comparison of some properties of seven types of Spherons has already been shown in Table 5.1. A general description of Spheron was given by Janák et al. (1975) and a detailed characterization of the Spheron matrix was published by Mikeš et al. (1978). Because of their high rigidity this matrix shows excellent flow properties. Fig. 2.3 demonstrates the high

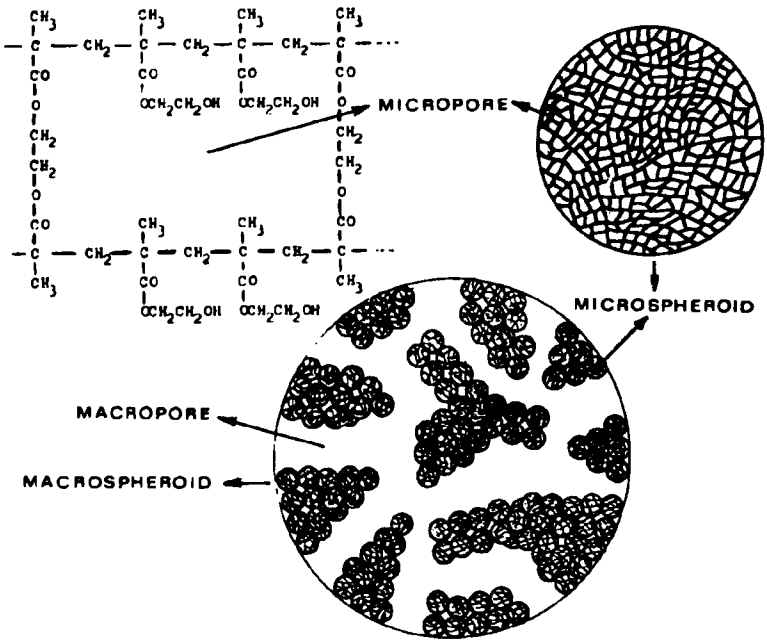


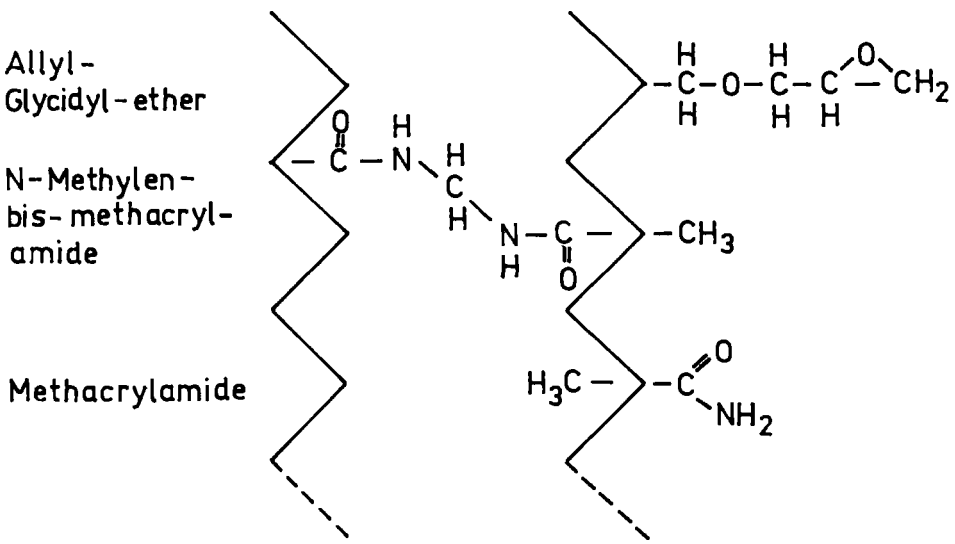
Fig. 5.3. Structure of hydroxyalkylmethacrylate copolymer (Separon, Spheron). Data from Turková and Seifertová, J. Chromatogr. 148, (1978), 293-297.

performance liquid bioaffinity chromatography (HPLBAC) of porcine pepsin on Separon H1000 modified with ϵ-aminocaproyl-L-phenylalanyl-D-phenylalanine methylester in comparison with analogous chromatography on unmodified Separon (Turková et al., 1981). The high stability of this matrix allows the application of this support to large scale operations and to industrial production (Mohr and Pommerening, 1985). However, the hydroxyethylmethacrylates have a greater nonspecific adsorption, attributable largely to hydrophobic interactions. This hydrophobic property has been used in the presence of salts for the hydrophobic interaction chromatography of proteins and peptides by Štrop et al. (1978, 1983).

Another hydroxyethylmethacrylate support is produced under the trade name Dynospheres by Dyno Particles (Lillestrom, Norway). Their monodisperse microparticles with size range 0.3-5 μm are promising synthetic polymers for HPLBAC application.

5.3.3 Oxirane acrylic beads

Krämer et al. (1978) described the preparation and properties of oxirane acrylic beads. These are obtained by copolymerisation of methacrylamide, methylene-bis-methacrylamide, glycidyl-methacrylate and or allyl-glycidyl-ether:



Due to the nature of monomers the copolymer shows an electroneutral and mostly hydrophilic matrix, with a slight hydrophobic component due to the methyl groups along the polymer backbone. The oxirane group content is 1000 μmol/g dry beads. The beads are macroporous and they show a water regain of 2.5 ml/g of dry beads. This water regain is independent of pH (0.5 to 12.5) and ionic strength. The beads are morphologically and chemically stable under these conditions, even if exposed to them for several weeks. The mechanical stability upon stirring is very good. At present these chemically very stable oxirane acrylic beads are produced under the trade mark Epergit C by Röhm Pharma GMBH (Darmstadt, F.R.G.).

The high-performance immunoaffinity chromatography (HPIAC) using Eupergit C beads was developed and optimized by Fleminger et al. (1990). They immobilized antibodies against carboxypeptidase A onto Eupergit C beads of sizes150 μm (standard, porous), 30 μm (C30N, porous) and 1 μm (C1Z, nonporous). The chemical and immunological properties of various Eupergit C beads are shown in Table 5.5. The amounts of bound antigen carboxypeptidase A (CPA) were determined by enzymatic assay of CPA. The amounts of bound anti-CPA protein (mg/g matrix) are given in parentheses. The high-performance liquid chromatographic-based immunopurification system using C390N and C17 Eupergit C beads was also used for the isolation of immunoglobulin G from serum and of human decidua proteins from the decidua tissue and from seminal plasma. These proteins were obtained at high purity in a single purification step.

Table 5.5.

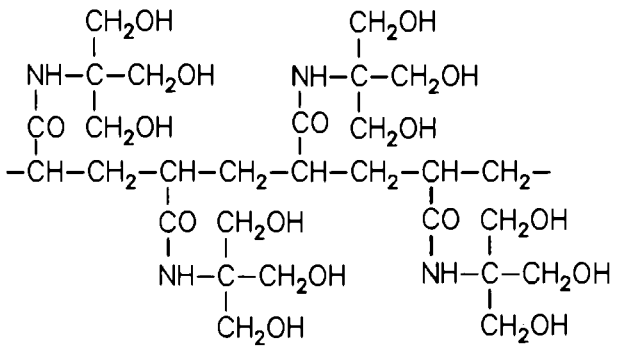
Chemical and immunological properties of various Eupergit C beads and matrix-conjugated antibodies

Eupergit C	*Size (μm)*	*Porosity*	*Oxirane content (mmol/g)*	*Protein-binding capacity (mg/g)*	*Antigen-binding capacity (mol/mol)*
Standard	150	Porous	> 1000	> 1000	0.75(1.4) 0.24 (14.2)
C30N	30	Porous	> 1000	> 100	0.8 (1.2) 0.4 (11.5)
C1Z	1	Non-porous	20	6.5	0.95 (2.0)

5.3.4 Hydrophilic acrylate-based supports

Non-ionic synthetic supports are obtained by copolymerization of N-acryloyl-2-amino-2-hydroxymethyl-1, 3-propane diol with hydroxylated acrylic bifunctional monomer. Hydrophilic supports under the trade name Trisacryl GF are manufactured by IBF

Reactifs (Villeneuve la Garenne, France). The chemical structure of the polymer Trisacryl GF05 is:



Mastering the support porosity by a strict control of polymerization, it is possible to synthesize a complete line of products covering a wide range of molecular weights from 3,000 (Trisacryl GF05) to about 20 million (Trisacryl GF 2 000).

The gel is characterized by a high degree of hydrophilicity, especially due to the presence of primary alcohol groups and also of a secondary amide function. It can be used under pressures up to 2 - 3 bar. It is not affected by organic solvents such as alcohols, ketones, dioxane, or chlorinated solvents. It is non-biodegradable, and stable to low (-20°C) and high (121°C) temperatures. Denaturing agents have no effect on the gel because its structure involves no hydrogen bonds. It is also stable to acidic pH, but less stable to high pH because of the slow hydrolysis of the amide linkage. Oligo(dT)-substituted Trisacryl was used for the purification of polyadenylated mRNA by Sene et al. (1982). Bonnafous et al. (1983) described cell bioaffinity chromatography with using of Trisacryl and ligand immobilized through cleavable mercury-sulphur bonds.

5.3.5 Hydrophilic vinyl polymers

The Japanese firm Toyo Soda Manufacturing Co. (Tokyo, Japan) developed a semi-rigid gel, a copolymer of oligoethyleneglycol, glycidylmethacrylate and pentaerythrol dimethacrylate under the trade name Toyopearl. Kato et al. (1981) described the packing of Toyopearl columns for gel filtration. The identical copolymer under the

name Fractogel TSK HW Type was described as a new hydrophilic support for gel filtration by Wernicke (1982). The supplier of Fractogel TSK is the firm E. Merck (Darmstadt, F. R. G.).

The optimal conditions for the activation of free hydroxyl groups on the gel matrix by epichlorohydrin and subsequent immobilization of ligands were investigated by Matsumoto et al. (1982). They prepared bioaffinity adsorbents which were successfully used for the bioaffinity chromatography of lectin and trypsin. Shimura et al. (1984) used Toyopearl HW-65S with attached p-aminobenzamidine for the high-performance bioaffinity chromatography of plasmin and plasminogen.

The advantage of this gel is its pressure stability up to 7 bar. Swelling of dry gels in water is 3 - 4 ml/g for Fractogel TSK HW-40, HW-65 and HW-75, 4 - 5 ml/g for Fractogel TSK HW-50 and HW-55. The most suitable gel for bioaffinity chromatography is Fractogel TSK HW-65, which is produced in four forms of activated affinity gels. The particle size of this support, moistened with water, is 0.032 - 0.063 mm. Its molecular exclusion limit is $5x10^6$ for proteins and 10^6 for polyethylene glycols or dextrans. The negligible change in swelling volume with change in eluent results in a high constancy of gel bed volume. It may be used from pH 1 to 14. The high chemical stability has led to applications at high temperature and therefore it may be autoclaved. It is resistant to microorganisms. Its properties render Fractogel TSK particularly suitable for large scale industrial application.

Ito et al. (1985) used a new micro-particulate polyvinyl alcohol gel Asahipak GS-gel (from Asahi Chemical Industry Co., Tokyo, Japan) for the coupling with p-amino-benzamidine and HPLBAC of trypsin instead of Toyopearl HW-65S (Shimura et al., 1984).

5.4 COMBINATION OF BIOPOLYMERS WITH SYNTHETIC POLYMERS

In order to minimize the reduction of porosity of polyacrylamide during activation, copolymers of agarose and polyacrylamide have been produced. This support combines the advantages of each individual polymer, while extending the potential range of

derivatisation procedures by virtue of the availability of both amide and hydroxyl groups of activation (Doley et al., 1976). Polyacrylamide-agarose gels with different and standardized porosities are produced under the name Ultrogels AcA by IBF Reactifs (Villeneuve la Garenne, France). A schematic representation of the Ultrogel AcA matrix is shown in Fig. 5.4.

Ultrogel AcA is available in four types, each comprising a three-dimensional polyacrylamide lattice enclosing an interstitial agarose gel. The gels are pre-swolen and calibrated within a narrow size range of 60 - 140 μm. The technical specifications of Ultrogel are given in Table 5.6 (Lowe, 1979).

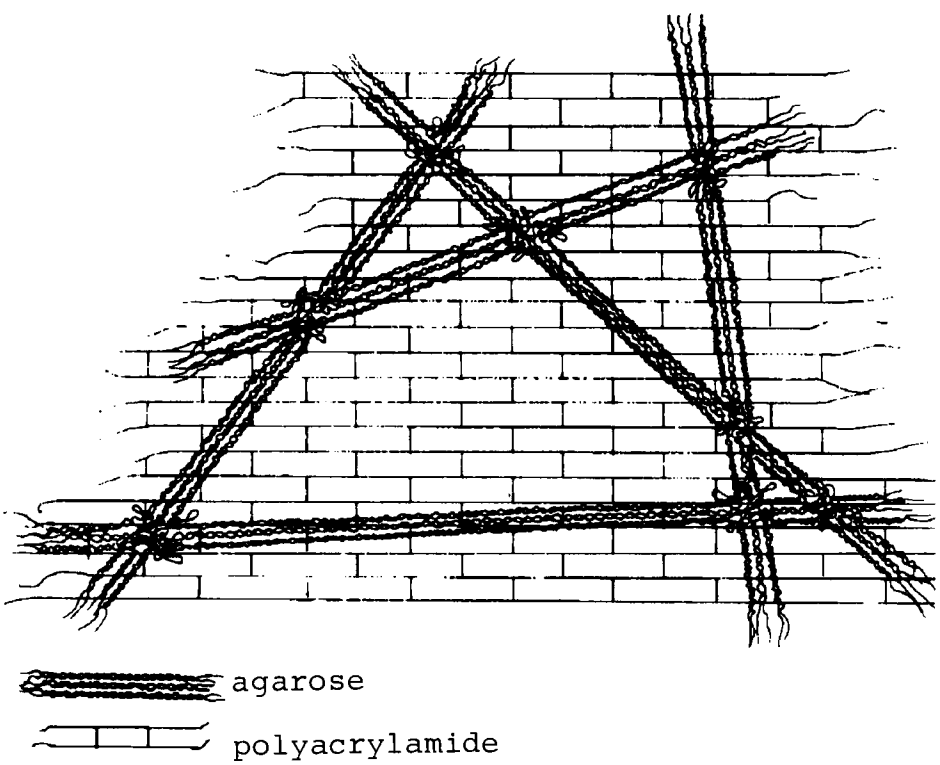


Fig. 5.4. Schematic representation of the Ultrogel AcA matrix.

During chemical reactions involving one of the polymers, certain precautions must be taken in view of the other polymer properties. Thus the gel must not be exposed to strongly alkaline media, since the amide groups will be hydrolyzed to carboxylic acids.

Table 5.6.

Technical specifications of Ultrogel

	AcA22	*AcA34*	*AcA44*	*AcA54*
Acrylamide concentration (%)	2	3	4	5
Agarose concentration (%)	2	4	4	4
Bead diameter (pre-swollen)(μm)	60 - 140	60 - 140	60 - 140	60 - 140
Fractionation range	60,000-	20,000-	12,000-	6,000
(globular proteins)	1,000,000	400,000	130,000	70,000

The limited heat resistance of the agarose must equally be respected: Ultrogel AcA must not be exposed to temperatures greater than about 40°C.

Immunosorbents prepared with glutaraldehyde-activated Ultrogel AcA are of special interest for the one-step purification of antibodies from whole serum (Guesdom and Avrameas, 1976; Lazdunski et al., 1975).

Coupling of polyacrylic hydrazide to CNBr-activated Sepharose 4B as a stable and high capacity spacer was described by Wilchek and Miron (1974). Polyacrylic hydrazido-Sepharose containing an average capacity of 15.25 μmole of available hydrazide per millilitre of agarose were used for further substitutions by the same method as described for the monovalent coupled hydrazides. Procedures for preparing derivatives of Sepharose hydrazide are shown in Fig. 5.5.

Polyacrylic hydrazido-Sepharose derivatives possess the advantages of agarose and acrylamide gels. At neutral pH they carry no charge and they contain a large number of modifiable groups. The preparation of polyacrylhydrazido-agarose based on the periodate oxidation of Sepharose followed by reaction with polyacrylhydrazide was also described by Miron and Wilchek (1981). All the preparations yielded columns which were colourless and stable after reduction with sodium borohydride. Polyacrylhydrazido-agarose could be used either directly or after further modification with different

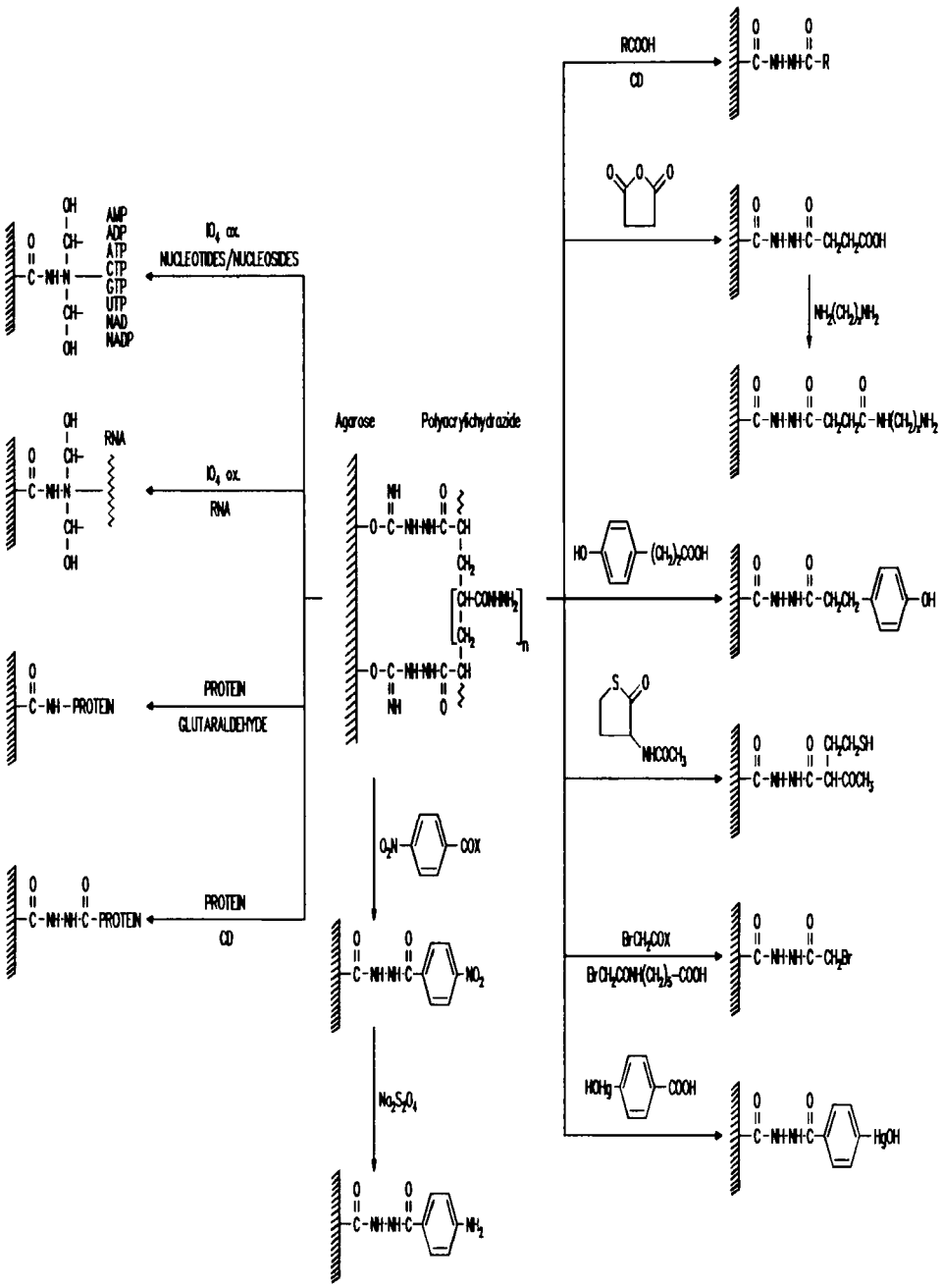


Fig. 5.5. Procedures for preparing derivatives of Sepharose hydrazide; CD = carbodiimide. Reproduced with permission from M. Wilchek, Advan. Exp. Med. Biol., 42 (1974) 15-31.

reactive groups. A support having similar properties is dextran crosslinked with N,N'-methylenebisacrylamide (Belew et al., 1978). This is produced by Pharmacia (Uppsala, Sweden) under the trade name Sephacryl.

Magnogel Ac A44 produced by IBF Reactifs (Villeneuve la Garenne, France) is a magnetized support, derived from Ultrogel Ac A44 (Guesdon and Avrameas, 1977). The polyacrylamide-agarose beads contain 7% Fe_3O_4 in the interior, thus enabling them to be attracted by a magnetic field. The gel may be activated either on the agarose or the polyacrylamide moieties. This gel is suitable for magnetic bioaffinity chromatography (Groman and Wilchek, 1987). This material offers apparent advantages where easy and rapid manipulation of particles are desirable, such as when unstable materials are being isolated, when the extract is viscous or contaminated with solid particles, or in the separation of cell types. In the field of immunodiagnostics magnetic solid phase enzyme-immunoassays (Guesdon and Avrameas, 1977) can be used successfully. As an example of the application of Magnogel for the separation of cells we may cite the lymphoid cell fractionation described by Antoine et al. (1978).

5.5 INORGANIC SUPPORTS

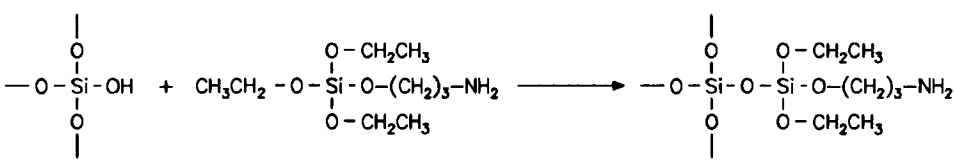
Inorganic supports have been reviewed by Weetall and Lee (1989). They classified these supports into a few major categories: metals, metal oxides, ceramics, and glasses. They described the preparations, properties and applications (for the immobilization of antibodies) of porous glasses, porous silica, titania, aluminina and zirconia bodies, and iron and nickel oxides. In their review several general considerations are mentioned, which must always be born in mind. Since inorganic particles are friable materials, one should never use a stirring bar when working with these materials. Stirring in this manner will "grind" the particles. Particles can be separated for washing or assay by several convenient methods. These include: filtration, centrifugation, settling, aspiration, or magnetic separation. Several of these methods can be utilized for the further sizing of inorganic particles particularly when clumping has occurred or when one wants to utilize particles of only a specific size range. Particles may be sized on the basis of settling times, centrifugation speed or filter porosity. One must generally decide on the best method

by considering what particle range is desired and choosing the method most likely to yield the desired particle range.

5.5.1 Controlled pore glass

Controlled pore glass (CPG) is synthesised by heating certain borosilicate glasses to 500 - 800°C for prolonged periods of time. These glass mixtures separate on such heat treatment into borate- and silicate-rich phases. The borate phase can be dissolved by treatment with acid, leaving a network of extremely small tunnels with pore diameters 30 - 60 Å. Subsequent treatment with mild caustic soda removes some siliceous material from the pore interiors and thus enlarges the pore diameter. Careful control of the various treatments can lead to a porous glass with an extremely narrow pore size distribution in the range 45 - 2500 Å (Lowe, 1979). Glass derivatives have outstanding stability. The rigidity of the beads permits high flow rates and thus facilitates a fast and efficient separation. Glass beads are resistant to microbial attack and may be readily sterilised by disinfectants or autoclaving. The latter is a prime consideration in the purification of pyrogen-free enzymes destined for *in vivo* or clinical studies.

However, in some instances they cause undesirable non- specific adsorption (Cuatrecasas and Anfinsen, 1971) and they are very slightly soluble at alkaline pH (8.0). Corning Glass Works have demonstrated that glass, when treated with γ-aminopropyltriethoxysilane, becomes a suitable support for a series of affinants (Weetal and Filbert, 1974). The silanization process was found to be a reaction between the glass surface hydroxyl groups and the amino-functional silane coupling agent:



The reaction proceeds by organic or aqueous silanization and the resulting alkyl-amino-glass can subsequently be used to immobilize affinity ligands. Some commercially

available porous glass packing materials are listed in Table 5.7, together with the names of their producers. Similar CPGs are also supplied by Electro-Nucleonics, Inc. (Fairfield; NY, U.S.A.) and Pierce Chemical Company (Rockford, Il, U.S.A.).

Glyceryl-CPG is a controlled pore glass whose surface has been chemically modified to produce a hydrophilic, non-ionic coating which shares most of the same operating characteristics as conventional CPG. The degree of adsorption of glutamate dehydrogenase on various derivatized glass surfaces was studied by Du Val et al. (1984). The glycerolpropyl glass was the weakest adsorber of the protein. Glyceryl-CPG is distributed by Electro-Nucleonics Inc. (Fairfield, NY, U.S.A.) Another supplier of glycophase-coated supports under the name glycophase G/CPG is the Pierce Chemical Company (Rockford, Il, U.S.A.). This company uses triethoxypropyl glycidosilane as the alkylsilane.

Table 5.7.

Porous glass packing materials

Type	*Molecularweight exclusion limit*		*Producer*
	Dextran standards	*Polystyrene standards,* ,	
Bio-Glass 200		30,000	Bio-Rad Labs.,
Bio-Glass 500		100,000	Richmond, Calif.
Bio-Glass 1000		500,000	U.S.A.
Bio-Glass 1500		2,000,000	
Bio-Glass 2500		9,000,000	
CPG 10-75	28,000		Waters Assoc.,
CPG 10-125	48,000		Milford, Mass.,
CPG 10-175	68,000		U.S.A.; Corning
CPG 10-240	95,000	120,000	Glass Works,
CPG 10-370	150,000	400,000	Corning, N.Y.,
CPG 10-700	300,000	1,200,000	U.S.A.
CPG 10-1250	550,000	4,000,000	
CPG 10-2000	1,200,000	12,000,000	

Ivanov et al. (1985) compared the epoxy-containing porous glass prepared with γ-glycidoxypropyltriethoxysilane and carbonylchloride-containing matrices prepared by grafting copolymers of N-vinylpyrrolidone and acryloylchloride onto the surface of γ-aminopropylsilylated porous glass for the isolation of neuraminidase from the influenza virus. A biospecific sorbent prepared from the polymer-containing glass enabled the isolation of neuraminidase with a 3 - 4 times higher specific activity as compared with a sorbent prepared from epoxy-containing glass. The use of nonporous glass beads with particle diameter $< 10\,\mu$m to eliminate the diffusional effects due to the pore structure has been described by Phillips et al. (1985).

Nonporous glass beads, porous silicas and soft gels with attached Cibacron Blue F3GA were used by Anspach et al. (1989) for the investigation of equilibration times for the adsorption of lysozyme on the different dye-affinity sorbents. In the batch experimental mode equilibration times varied from 20 s for the nonporous glass beads with a size range of 20 - 30 μm to more than 60 min in the case of a porous sorbent with a particle diameter of 100 - 300 μm and 600 Å pore size. The additional utilization of glass in bioaffinity chromatography is shown in Tables 9.1, 2, 3.

5.5.2 Porous and nonporous silica

The structure of silica is amorphous and its composition can be expressed as $SiO_2.xH_2O$. Its basic unit is tetrahedral (SiO_4) and the particle porosity, depends on the mode of preparation. Silica gel is formed from silicic acid sols by polycondensation of orthosilicic acid. Submicroscopic elemental particles are formed, which retain micelles of the starting acid and in their interior are bound by Si-O-Si siloxane bonds. Every elemental particle touches the surfaces of several neighbouring particles and in this way a conglomerate is formed containing capillary spaces (pores) of various diameters. The density of these inner spaces is very high and after drying they form large specific inner surface areas of hundreds of m^2/g. Owing to the presence of Si-OH groups at the surface, some siloxane bonds are formed during the activation of silica gel at elevated temperatures (e.g. 200 - 400°C). Various types of hydroxyl groups at the surface of silica gel

particles, together with a certain amount of water, influence the sorption properties of silica gel (Mikeš , 1988).

The main advantage of silica is its inherent mechanical stability which provides good flow characteristics even under high pressure. The use of silica with pore sizes ranging from 60 to 4000 Å or nonporous silica in small particles 1.5 - 10 μm is advisable when performing protein separations in order to provide good mass transfer. Silica is the most commonly used support for HPLC to date. Slightly acidic silanol groups on the silica surface act as centres for non-specific adsorption and denaturation. Under alkaline working conditions (pH > 8) the surface of silica gel exhibits a high solubility.

Consequent ligand leakage would cause contamination of the purified product. The problems of non-specific adsorption and high solubility have been largely overcome by derivatization of the silanol groups with silanes yielding silica derivatives coated with a hydrophilic layer. Fig. 5.6. shows the most common reaction of silanol groups with 3-glycidoxypropyltrimethoxy silane. The resulting epoxide silica can be used for the direct attachment of affinity ligands or can become the starting material for the synthesis of other products. Diol, aldehyde and primary hydroxyl silica are very often more suitable for ligand immobilization (Ernst-Cabrera and Wilchek, 1988). The preparation of epoxy-, diol- and aldehyde-silica has been described by Larsson (1984), and that of primary hydroxyl-silica by Ernst-Cabrera and Wilchek (1987). They used this material for the coupling of affinity ligands for high-performance liquid bioaffinity chromatography.

Four silica-based high-performance bioaffinity media differing in pore size and surface area were synthesized by Narayanan et al. (1990). Concanavalin A was covalently immobilized on silica gels treated with hydrophilic polymer to which glutaraldehyde was covalently attached. Table 5.8 lists the physical properties of silica preparation 2 before and after treatment with polymer, with glutaraldehyde and after binding of Con A. After the bonded phase was treated with glutaraldehyde (Glutaraldehyde-P preparation 2) there is a 42% reduction in pore volume, a 35% reduction in pore surface area and a 13% reduction in the average pore diameter. The binding of

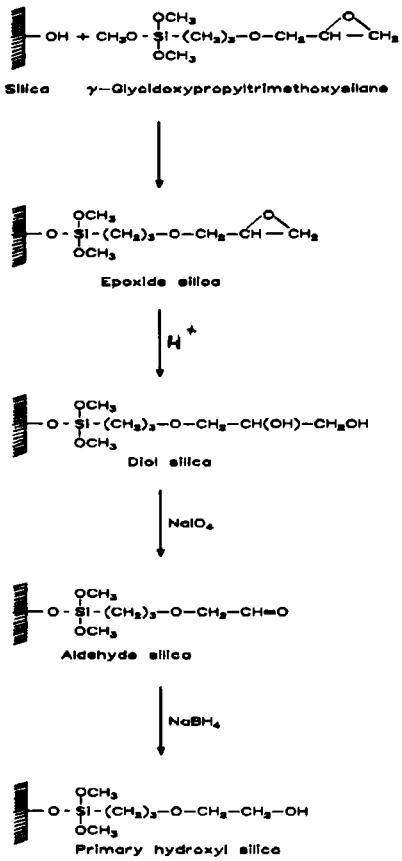


Fig. 5.6. Reaction of silanol groups with 3-glycidoxypropyltrimethoxy silane, hydrolysis of the epoxide with acid, oxidation of the diol with periodate, and reduction of the aldehyde with sodium borohydride to yield primary hydroxyl silica.

Con A (a large protein of molecular weight 102,000) is associated with very low subsequent change in the pore diameter.

Table 5.9 gives the pore diameter and surface area of four silica-based Glutaraldehyde-P preparations. The carbon surface coverage, determined by elemental analysis, correlates fairly well with the experimentally determined amount of glutaraldehyde, which in turn correlates with the ligand binding capacity of the matrices for Con A, indicating that the functional groups on the activated matrices are generally accessible to the protein. The influence of the physical properties of these silica-based supports in bioaffinity chromatography has been already discussed in Section 5.1.

Table 5.8.

Pore structure analysis of glutaraldehyde-P (preparation 2).

The physical properties were determined by mercury intrusion porosimetry and corrected for interparticle voids.

Sample	*Pore volume (ml/g)*	*Pore surface area (m^2/g)*	*Median pore diameter (Å)*
Starting material	0.95	182	211
After polymer treatment	0.76	150	204
After glutaraldehyde treatment	0.66	143	190
After binding of Con A	0.55	122	183

Table 5.9.

Surface characterization and properties of glutaraldehyde-P preparations

Pore volume, pore surface area, and pore diameter were determined by mercury intrusion porosimetry and corrected for interparticle voids.

Preparation	*1*	*2*	*3*	*4*
Property				
Pore volume (ml/g)	0.26	0.66	0.83	0.77
Pore surface area (m^2/g)	130	143	85	46
Pore diameter (median)(Å)	88	190	408	776
Carbon coverage (m^2/g)	385	424	460	280
Aldehyde (μmol/m^2)	1.3	1.8	1.53	1.3
Con A (mg/g)	80	140	104	86

The evaluation of nonporous monodisperse silica beads has been described by Unger et al. (1986). Decreasing the particle diameters from 10 μm to 1.5 μm, or even smaller values, increases the geometrical surface area within a unit column. From both theoretical and practical considerations nonporous particles of small particle diameter should thus have advantages, especially for very rapid analytical and micropreparative bioaffinity chromatography (Anspach et al., 1988). The improvement of bioaffinity chromatographic performance using the biospecific sorbents prepared by Anspach et al. (1990) from the nonporous monodisperse silicas in comparison with results obtained with porous silica supports with identical activation and immobilization procedures, has already been discussed in Section 4.1.1.

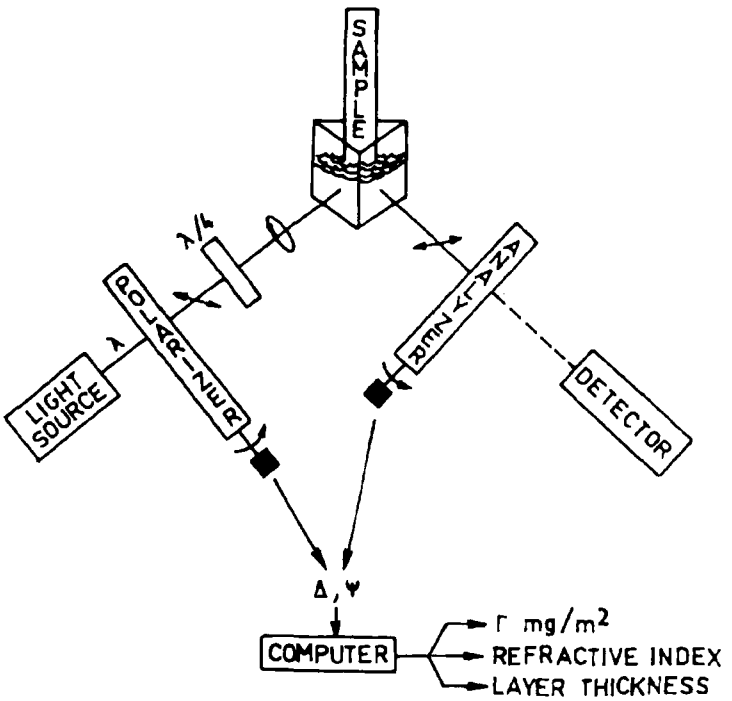


Fig. 5.7. In situ ellipsometry. A beam of laser light is reflected from the solid sample surface at an angle of incidence of 60°. Before reaching the surface the light passes through a polarizer and a quarter-wavelength plate. After reflection the light passes through a second polarizer and is detected by a photodiode. The polarizers are automatically rotated in such a way that the resulting light intensity reaching the detector is kept at a minimum. The position of the polarizers gives the Δ and ψ angles, which may be translated into the amount of adsorbed or interacting proteins on the surface. Reproduced with permission from U. Jönsson et al., Methods Enzymol., 137 (1988) 381 - 388.

Several difficulties, such as nonuniformity of the deposited silane film thickness, codeposition of polymeric silane particles, and hydrolytic removal of the deposited film, occur using methods mentioned above, and these are not acceptable for conferring an optimum performance on surface-sensitive measuring devices. Vapour phase silanization, elaborated by Jönsson et al. (1988), overcomes some of these difficulties. The technique was exemplified by the covalent binding of Protein A to silanized silica surfaces and the interaction of the immobilized Protein A with immunoglobulins. Fig. 5.7 shows *in situ* ellipsometry, an optical technique based on the reflection of polarized light from a surface, which was used for the evaluation of the results. The measurement principle that governs the ellipsometry has been described by Jönsson et al. (1985). After

the immobilization of immunoglobulins on chemically modified silica surfaces by the use of thiol-disulphide exchange reactions, they showed that the covalently bound IgG has a superior stability when the pH was lowered or incubation was carried out with detergents, urea or ethylene glycol. The quantification of the amount of immobilized IgG was determined by ellipsometry. On the basis of results obtained in the protein system tested (Protein A-IgG interaction) Jönsson et al. (1988) emphasized that ellipsometry, combined with welldefined surfaces, is a fast and sensitive method for the study of biomolecular interaction. Examples of the use of ellipsometry for the study of biomolecular interactions between receptor and cell and enzyme and coenzyme after the coupling of biomolecules to silicon surfaces were published by Mandenius et al. (1988). Their coupling of biomolecules to silicon surfaces includes the formation of a hydrocarbon layer on top of the silicon oxide present on all silicon surfaces. The three methods described comprise the coupling of ligands by the use of silane, dextran or albumin. The introduction of the hydrocarbon layer significantly limits the nonspecific adsorption and thus protects the coupled biomolecules. The preparation and application of dextran- or agarosecoated silica supports as potentially good stationary phases for high-performance bioaffinity chromatographic separation of proteins have been described by Zhou et al. (1989). The method described has been used to coat silica beads with a polysaccharide, substituted by a calculated amount of positively charged diethylaminoethyl functions in order to neutralize the negatively charged silanol groups of silica and to facilitate the formation of a hydrophilic polymeric layer on the inorganic surface.

Many silica gel supports are available commercially in both irregular or spherical shapes, either uncoated or glycophase coated. They are reviewed by Mikeš (1988), Ohlson et al. (1989) and they are described in the catalogues of many companies. From among the many examples of silica we can mention Porasil A-F with pore diameter from 80 Å to 3000 Å and surface area from 400 m^2/g to 10 m^2/g produced by Waters Associates (Milford, MA, U. S. A). The E. Merck firm (Darmstadt, F. R. G.) supplies silica-based supports under the trademark LiChrospher Si, LiChrospher Diol and LiChrosorb Diol (particle diameter 5 - 10 μm, pore diameter 100 - 400 Å and surface

area 250 - 6 m^2/g) and nonporous Monospher (particle size 2.1 μ and surface area 2.5 m^2/g), the Du Pont Instrument Products Division (Wilmington, DE, U.S.A.) suplies the SE Series or Zorbax, Macherey Nagel (Düren, F. R. G.) under the trademark Nucleosil; Rhöne-Poulenc (Usine de Salindres, France) uses the name Spherosil, TESSEK Ltd. (Prague, Czechoslovakia) uses the trademark Separon SGX, Chrompack International (Middelburg, The Netherlands) uses the trademark Chrom Spher Si. The Toya Soda Manufactur Co. Ltd. (Tokyo, Japan) produce silica gels under the name TSK, Bio-Rad Labs. (Richmond, CA, U. S. A.) supply spherical silicas under the name Bio-Sil, etc. The polyhydroxy silica support Nugel P GP-500, from Diagnostic Specialties (Metuchen, N.J., U.S.A.) was used by Roy et al. (1984) for the high-performance immunosorbent purification of recombinant leucocyte A interferon. The surface of fluid-imperrious 2μm silica microspheres from Glycotech (Hamden, CT, U. S. A.) were used after coupling of Protein A or lectins for fast separation and quantitation of immunoglobulins and glycoproteins by Várady et al. (1988).

Among the commercially available epoxide silicas belongs Ultraaffinity-EP, available as prepacked columns (Beckmann Instruments, Berkeley, CA, U.S.A.). Immobilization of the ligand onto such a matrix can be obtained by simple passage of the material through the column for a predetermined time period. The use of anti-chaotropic anions such as phosphates and sulphates is recommended since they stabilize the protein during derivatization and increase the interaction with the active support. According to Ernst-Cabrera and Wilchek (1988) this particular activated matrix exhibits low reactivity and requires basic conditions for efficient ligand coupling. Columns, cartridges or kits of a silica also activated with epoxide functional bonded phase under the name Durasphere AS are produced by Alltech Associates, Inc. (Deerfield, Il, U.S.A.)

5.5.3 Iron and nickel oxides

Iron oxide can be prepared by several methods. One method involves the use of commercially available iron oxide powders. These powders can be silanized directly and used for the attachment of affinity ligands (Weetall and Lee, 1989). Commercially available series of particles consisting of an iron oxide core coated with a silane polymer

terminating in either amine (particle size 1 - 2 μm), carboxyl (particle size 0.5 - 1 μm), or sulphydryl (particle size 1 - 3 μm) functional groups are supplied by Advanced Magnetics Corp. (Cambridge, MA, U.S.A.) The same firm also offers its particles pre-reacted with a variety of proteins including Protein A, avidin and goat anti-mouse antiserum (Groman and Wilchek, 1987). Magnogel, which consists of iron oxide entrapped in a polyacrylamide-agarose support, has been discussed in connection with magnetic bioaffinity chromatography in Section 5.4. Latex particles impregnated with iron oxide are also commercially available from Seradyn Inc. (Indianapolis, IN., U.S.A.). These materials show paramagnetic properties and have been successfully used in immunoassays. Nickel oxides produced by precipitation, or directly from a chemical supply house, have been used in a manner similar to the iron oxides (Weetall and Lee, 1989).

5.6 NYLON MEMBRANES AND TUBES

Synthetic polyamides, known as nylons, are a family of condensation polymers of dicarboxylic acids (or their diacid chlorides and α,ω-diamines (Kennedy and Cabral, 1983).

Several types of nylon, differing only in the number of methylene groups in the repeating alkane segments, are available in a variety of physical forms, such as fibres, hollow fibres, foils, membranes, powders, and tubes. Membrane formation and characterization are descriebd in more detail by Klein (1991).

The advantage of the purification of phosphofructokinase from yeast cell homogenate by use of the selective adsorption-desorption with Immunodyne nylon membrane has been described by Huse et al. (1990). Immunodyne, Biodyne A and Loprodyne nylon membranes are supplied by Pall Filtrationstechnik (Dreilich, F. R. G.). Immunodyne membranes are pre-activated nylon membranes developed for the covalent fixation of molecules via hydroxyl, carboxyl or amino groups. After alkaline inactivation of the reactive groups Immunodyne membrane has the ability to selectively bind phosphofructokinase and phosphoglycerate kinase from yeast cell extract. The time course of phosphofructokinase binding to different nylon membranes is shown in Table

5.10. The decrease in the phosphofructokinase activity in the homogenate with the Immunodyne membrane can be explained only by the action of the group used for substitution of the nylon matrix of Immunodyne to preactivate this membrane as an affinity ligand. Elution of phosphofructokinase from the nylon sorbent by adenine nucleotides is in agreement with this opinion.

The membrane technique applied is extremely simple and does not require sophisticated equipment. The membrane was cut and transferred to a polyethylene bottle. After filling with 10 mM sodium hydroxide solution the membrane adhered loosely to the wall of the bottle. Fig. 5.8. shows the bottle with the membrane fixed with rubber bands to a rotating shaft in an horizontal position. This arrangement allows the use of small volumes and utilization of the whole membrane area. The potential use of particle-containing cell homogenate, biospecific sorption on membranes may be superior to other methods as the first step in the extraction of cell homogenates, at least on a small scale of up to a few milligrams.

Table 5.10.

Time-course of phosphofructokinase binding to different nylon membranes

Aliquots of 6 ml of a yeast cell homogenate were incubated with the respective nylon membranes and assayed for phosphofructokinase (PFK) activity at the indicated times. An incubation without a membrane was performed as a control. Values in parentheses are the specific activities at the end of incubation determined in the supernatant after centrifugation at 25 000 g.

Time of incubation (min)	*PFK activity (U/ml) in the cell homogenate*			
	Control	*Immunodyne*	*Biodyne A*	*Loprodyne*
0	15.7	-	-	-
60	14.2	7.6	14.7	15.3
120	14.9	4.8	14.2	15.8
	(0.42)	(0.46)	(0.46)	(0.42)

Acetylcholine receptor was coupled without significant loss of biochemical properties to the inner surface of nylon tubes (supplied by Portex, Hythe, Kent, U. K.) by Yang et

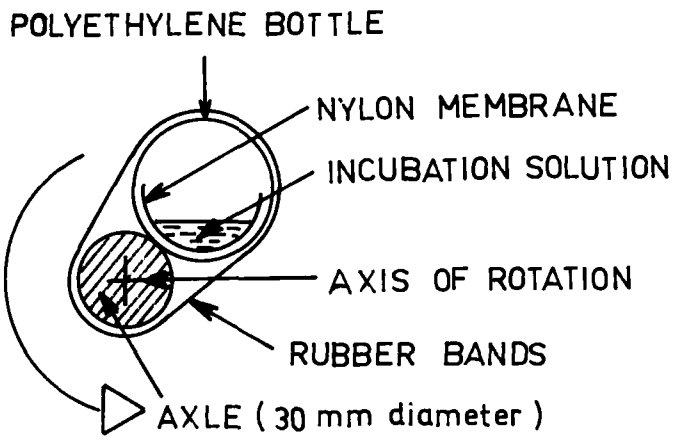


Fig. 5.8. Treatment of the nylon filters with a small volume of incubation solution within a bottle fixed to a rotating shaft.

al. (1981). The resulting tubes could be used repeatedly for the bioaffinity chromatography of receptor-specific immunoglobulins. The hydrophobic nylon surface appears to be of particular advantage when solubilized membrane proteins are used as affinity ligands. With these properties, receptor-linked nylon tubes can be used for titre determination and chromatography of antibody-containing fluids, including blood, ascites fluid and cell culture media.

5.7 OTHER SUPPORTS

Saito and Nagai (1983) coupled antibody on a plastic surface using polystyrene tubes (Falcon, Div. of Becton, Dickinson and Co., Cockeysville, MD, U. S. A.) treated with 0.5% toluene 2,4-diisocyanate dissolved in carbon tetrachloride. The polystyrene tube coated with anti-rabbit γ-globulin goat serum allowed a simple separation of the free tracer in a radioimmunoassay of thyroid-stimulating hormone and prolactin. Fluorescence-labelled latex spheres (Polysciences, Warrington, PA, U.S.A.) were used by Kieran and Longenecker (1984) for the immobilization of monoclonal antibodies

specific for erythroid differentiation (B-G) antigens. The chromatography paper Whatman 31ET (Whatman Inc., Clifton, NJ, U.S.A.) with immobilized specific antibodies was used by Zuk et al. (1985) for the elaboration of the enzyme immunochromatographic test strip assay. Whatman 540 paper was used for the preparation of cyanuric chloride-activated matrix (CCA paper) by Hunger et al. (1986). CCA paper binds nucleic acids and proteins. When stored under nitrogen at -20°C it retains its binding activity for at least a year.

A non-interactive polymer (hydrophilic polyvinylidene difluoride) is the base material of Immobilon AV Affinity Membrane (IAV) produced by Millipore (Bedford, MA, U.S.A). A variety of ligands containing amines or thiols can be covalently immobilized to chemically activated hydrophilic microporous membrane under a range of pH conditions (pH 4 - 10), ionic strength (0.01 - 1.0 M) and temperature (0 - 37°C). By varying these parameters, the user can immobilize nanogram to milligram amounts of proteins ($150\,\mu g/cm^2$ is approximately a protein monolayer). The Affinity-15 Membrane Chromatography System is produced by Sepracor Inc. (Marlborough, MA, U.S.A.). Affinity ligands are covalently bound to the membrane using a stable, secondary amine linkage, and therefore ligand leaching is virtually eliminated. Membrane technology for protein purification increases the speed, lowers the costs, and simplifies the protein purification.

Avid Plate-HZ microwell plates are designed for site-specific antibody immobilization. They utilized hydrazide chemistry to immobilize antibodies through the carbohydrate moieties of the Fc region. A stable antibody linkage is obtained with low nonspecific binding and increased assay sensitivity. They are produced by Bio Probe International Inc. (Tustin, CA, U.S.A.).

Quartz fibre (Q 106, supplied by Johns-Manville, Denver, CO, U. S. A.) was silylated with mercaptopropyltrimethoxysilane by Wikström and Larsson (1987). The use of bioaffinity fibre for large-scale purification of ox heart lactate dehydrogenase has already been discussed in Section 4.1.1. A further support for high-performance liquid bioaffinity chromatography is Affi-Prep 10 matrix, which consists of an N-hydroxysuccinimide ester covalently bound to rigid polymer beads of unknown composition

through a neutral spacer arm 10 atoms long. The Affi-Prep matrix produced by Bio-Rad Labs. (Richmond, CA, U.S.A.) is non compressible to 1,000 psi (60 bar).

The rapid evolution of many new unmodified or activated supports, biospecific sorbents in beads, columns, cartriges etc. has been described in the catalogues of many companies and in The International Product Magazine for Biotechnology (BPI, Elsevier, B. P. 214, B-1210 Brussels, Belgium). Commercially available carriers and biosorbents listed in Tables 9.1,2,3 are characterized in Table 9.4. Details can be found in the original literature cited.

REFERENCES

Adachi, S., Tanabe, S. and Hashimoto, K., Biotechnol. Bioeng. 26 (1984) 635-639.

Anspach, F.B., Johnston, A., Wirth, H.J., Unger, K.K. and Hearn, M.T.W., J. Chromatogr., 499 (1990) 103-124.

Anspach, F.B., Unger, K.K., Davies, J. and Hearn, T.W., J. Chromatogr., 457 (1988) 195-204.

Anspach, F.B., Wirth, H.J., Unger, K.K., Stanton, P., Davies, J.R. and Hearn, M.T.W., Anal. Biochem., 179 (1989) 171-181.

Antoine, J.C., Ternynck, T., Rodrigot, M. and Avrameas, S., Immunochemistry, 15 (1978) 443-452.

Arnott, S., Fulmer, A., Scott, W.E., Dea, I.C.M., Moorhouse, R. and Rees, D.A., J. Mol. Biol., 90 (1974) 269-284.

Belew, M., Porath, J., Fohlman, J. and Janson, J.C., J. Chromatogr., 147 (1978) 205-212.

Bengtsson, S. and Philipson, L., Biochim. Biophys. Acta, 79 (1964) 399-406.

Bonnafous, J.C., Dornand, J., Favero, J., Sizes, M., Boschetti, E. and Mani, J.C., J. Immunol. Methods, 58 (1983) 93-107.

Čoupek, J., Křiváková, M. and Pokorný, S., J. Polymer. Sci. Symp., 42 (1973) 185-190.

Cuatrecasas, P. and Anfinsen, C.B., Ann. Rev. Biochem., 40 (1971) 259-275.

Dean, P.D.G., Johnson, W.S. and Middle, F.A. (Editors), Affinity Chromatography Practical Approach, IRL Press, Oxford, 1985, pp. 1-30.

Doley, S.G., Harvey, M.J. and Dean, P.D.G., FEBS Lett., 65 (1976) 87-91.

DuVal, G., Swaisgood, H.E. and Horton, H.R., J. Appl. Biochem, 6 (1984) 240-250.

Ernst-Cabrera, K. and Wilchek, M., J. Chromatogr., 397 (1987) 187-196.

Ernst-Cabrera, K. and Wilchek, M., Trends Anal. Chem., 7 (1988) 58-63.

Fleminger, G., Wolf, T., Hadas, E. and Solomon, B., J. Chromatogr., 510 (1990) 311-319.

Fusek, M., Čapka, M. and Turková, J., Biotechnol. Lett., 9 (1987) 561-566.

Gemeiner, P., Beneš M.J. and Štamberg, J., Chem. Papers, 43 (1989) 805- 848.

Groman, E.V. and Wilchek, M., Trends Biotechnol., 5 (1987) 220- 224.

Guesdon, J.L. and Avrameas, S., J. Immunol. Methods, 11 (1976) 129-133.

Guesdon, J.L. and Avrameas, S., Immunochemistry, 14 (1977) 443- 447.

Hjerten, S., Biochim. Biophys. Acta, 79 (1964) 393-398.

Hjerten, S. and Mosbach, R., Anal. Biochem., 3(1962) 109-118.

Horstmann, B.J., Kenney, C.N. and Chase, H.A., J. Chromatogr., 361 (1986) 179-190.

Hou, K.C. and Mandaro, R.M., Biotechniques, 4 (1986) 358-367.

Hunger, H.D., Coutelle, C., Behrendt, G., Flachmeier, C., Rosenthal, A., Speer, A., Breter, H., Szargan, R., Franke, P., Stahl, J., Cuong, N.V. and Barchend, G., Anal. Biochem., 156 (1986) 286-299.

Huse, K., Himmel, M., Gartner, G., Kopperschlager, G. and Hofmann, E., J. Chromatogr., 502 (1990) 171-177.

Inman, J.K. and Dintzis, H.M., Biochemistry, 8 (1969) 4074-4082.

Ito, N., Noguchi, K., Shimura, K. and Kasai, K.I., J. Chromatogr., 333 (1985) 107-114.

Ivanov, A.E., Zhigis, L.S., Chekhovskykh, E.A., Reshetov, P.D. and Zubov, V.P., Bioorg. Khim., 11 (1985) 1527-1532.

Janák, J., Čoupek, J., Krejčí, M., Mikeš, O. and Turková, J., in Deyl, Z., Macek, K. and Janák, J. (Editors), Liquid Column Chromatography (Journal of Chromatography Library, Vol. 3), Elsevier, Amsterdam, 1975, pp. 187-202.

Johansson, J. and Lundberg, H., J. Biochem. Biophys. Methods, 1 (1979) 37-44.

Jönsson, U., Malmqvist, M., Olofsson, G. and Ronnberg, I., Methods Enzymol., 137 (1988) 381-388.

Jönsson, U., Malmqvist, M. and Ronnberg, I., Biochem. J., 227 (1985) 363-371.

Kato, Y., Komiya, K., Iwaeda, T., Sasaki, H. and Hashimoto, T., J. Chromatogr., 205 (1981) 185-188.

Katoh, S. and Sada, E., J. Chem. Eng. Japan, 13 (1980) 151-154.

Kennedy, J.F. and Cabral, J.M.S., in Scouten, W.H. (Editor), Solid Phase Biochemistry, Analytical and Synthetic Aspects, Willey, New York, 1983, pp. 253-391.

Kieran, M.W. and Longenecker, B.M., J. Immunol. Methods, 66 (1984) 349-356.

Klein, E., Affinity Membranes, Their Chemistry & Performance in Adsorptive Separation Processes, John Wiley & Sons, Inc., New York 1991, pp.1-152.

Krämer, D.M., Lehmann, K., Pennewiss, H. and Plainer, H., Enzyme Eng., 4 (1978) 153-154.

Kristiansen, T., Biochim. Biophys. Acta, 362 (1974) 567-574.

Larsson, P.O., Methods Enzymol., 104 (1984) 212-223.

Lazdunski, C.J., Pages, J.M. and Louvard, D., J. Mol. Biol., 97 (1975) 309-335.

Lowe, C.R., An Introduction to Affinity Chromatography, Elsevier/North-Holland Biomedical Press, Amsterdam 1979, pp. 293- 319.

Lowe, C.R. and Dean, P.D.G., Affinity Chromatography, Wiley, New York, London, 1974, pp. 272.

Mandaro, R.M. and Hou, K.C., Biotechnology, 5 (1987) 928-935.

Mandenius, C.F., Welin, S., Lundström, I. and Mosbach, K., Methods Enzymol., 137 (1988) 388-394.

Matsumoto, I., Ito, Y. and Seno, N., J. Chromatogr., 239 (1982) 747-754.

Mikeš, O., High-performance Liquid Chromatography of Biopolymers and Biooligomers, Elsevier, Amsterdam, 1988, pp. A127-A238.

Mikeš, O., Štrop, P. and Čoupek, J., J. Chromatogr., 153 (1978) 23-36.

Miron, T. and Wilchek, M., J. Chromatogr., 215 (1981) 55-63.

Mohr, P. and Pommerening, K., Affinity Chromatography - Practical and Theoretical Aspects, Marcel Dekker, Inc., New York and Basel, 1985, pp. 19-39.

Narayanan, S.R., Knochs, J.S., and Crane, L.J., J. Chromatogr., 503 (1990) 93-102.

Ohlson, S., Hansson, L., Glad, M., Mosbach, K. and Larsson, P., Trends Biotechnol., 7 (1989) 179-186.

Phillips, T.M., Queen, W.D., More, N.S. and Thompson, A.M., J. Chromatogr., 327 (1985) 213-219.

Porath, J., Methods Enzymol., 34 (1974) 13-30.

Roy, S.K., Weber, D.V. and McGregor, W.C., J. Chromatogr., 303 (1984) 225-228.

Saito, T. and Nagai, F., Clin. Chim. Acta, 133 (1983) 301-310.

Schnaar, R.L. and Lee, Y.C., Biochemistry, 14 (1975) 1535-1541.

Sene, C., Girot, P., Boschetti, E., Plassat, J.L., Bloch, J. and Egly, J.M., J. Chromatogr., 248 (1982) 441-445.

Shimura, K., Kazama, M. and Kasai, K.I., J. Chromatogr., 292 (1984) 369-382.

Štamberg, J., Separ. Purif. Methods, 17 (1988) 155-183.

Štamberg, J., Peška, J., Paul, D. and Philipp, B., Acta Polymerica, 30 (1979) 734-739.

Štrop, P., Čechová, D. and Tomášek, V., J. Chromatogr., 259 (1983) 255-268.

Štrop, P., Mikeš, F. and Chytilová, Z., J. Chromatogr., 156 (1978) 239-254.

Swaisgood, H.E. and Chaiken, I.M., J. Chromatogr., 327 (1985) 193-204.

Swaisgood, H.E. and Chaiken, I.M., Biochemistry, 25 (1986) 4148- 4155.

Turková, J., Bláha, K., Horáček, J., Vajčner, J., Frydrychová, A. and Čoupek, J., J. Chromatogr., 215 (1981) 165-179.

Turková, J., Bláha, K., Valentová, O., Čoupek, J. and Seifertová, A., Biochim. Biophys. Acta, 427 (1976) 586-593.

Turková, J., Hubálková, O., Křiváková, M. and Čoupek, J., Biochim. Biophys. Acta, 322 (1973) 1-9.

Turková, J. and Seifertová, A., J. Chromatogr., 148 (1978) 293- 297.

Unger, K.K., Jilge, G., Kinkel, J.N. and Hearn, M.T., J. Chromatogr., 359 (1986) 61-72.

Várady, L., Kalghatgi, K. and Horvath, C., J. Chromatogr., 458 (1988) 207-215.

Walters, R.R., J. Chromatogr., 249 (1982) 19-28.

Weetall, H.H. and Filbert, A.M., Methods Enzymol., 34 (1974) 59- 72.

Weetall, H.H. and Lee, M.J., Appl. Biochem. Biotech., 22 (1989) 311-330.

Wernicke, R., Kontacte (Merck), 1982, pp. 3-8.

Wichterle, O. and Lím, D., Nature, 185 (1960) 117-118.

Wikström, P. and Larsson, P.O., J. Chromatogr., 388 (1987) 123- 134.

Wilchek, M., Advan. Exp. Med. Biol., 42 (1974) 15-31.

Wilchek, M. and Miron, T., Methods Enzymol., 34 (1974) 72-76.

Yang, B.H., Sundaram, P.V. and Maelicke, A., Biochem. J., 199 (1981) 317-322.

Zhou, F.L., Muller, D., Santarelli, X. and Jozefonvicz, J., J. Chromatogr., 476 (1989) 195-203.

Zuk, R.F., Ginsberg, V.K., Houts, T., Rabbie, J., Merrick, H., Ullman, E.F., Fischer, M.M., Chung Sizto, C., Štiso, S.N. and Litman, D.J., Clin. Chem., 31 (1985) 1144-1150.

Chapter 6

Survey of the most common coupling procedures

When selecting the method of attachment, the primary consideration is which groups of the affinity ligand can be used to form the linkage to a solid support without affecting its binding site. The attachment should not introduce non-specifically sorbing groups into the specific sorbent. From this point of view, it is better to couple the spacer to an affinant and, only after it has been modified in this manner, subsequently to attach it to a solid support. The linkage between the surface of a solid support and an affinant should be stable during adsorption, desorption and regeneration. When choosing an appropriate method, one should bear in mind the dependence of the stability of the affinant on pH and the other reactionary conditions, as should the fact that the coupling procedure may be greatly influenced by both the nature of the solid matrix and the substance to be attached.

When bifunctional compounds are used for the coupling, complications arising from the crosslinking of both the carrier and the proteins with one another can be expected, and therefore suitable reaction conditions, such as pH, temperature and time, should be maintained carefully during the coupling.

When chemically inert solid supports are available the preparation of biospecific adsorbents is a two-step reaction, consisting of (1) activation or functionalization of the chemically inert support, and (2) coupling of the ligand to this modified matrix. The chemistry employed in the activation of inert support matrices is dictated mostly by the nature and the stability of the matrix itself. Thus, for example, conditions applicable to the functionalization of glass would totally destroy agarose. Currently many activated solid supports made for the coupling of various chemical groups of ligands or comprising spacer molecules are available commercially, all of which are suitable for the direct attachment of appropriate ligands.

When the immobilized affinity ligand is a glycoprotein, e.g. antibody or enzyme, the low stability and capacity of the biospecific adsorbent may be overcome by the immo-

bilization of glycoproteins through their carbohydrate moieties to the hydrazide containing solid supports, as has been already discussed in Section 4.3.3.

Active aldehyde groups in all diol-containing glycoproteins can be prepared by periodate oxidation. Different methods for the coupling of hydrazide reagents to individual oxidized sugar residues have been described by Gahmberg and coworkers (Gahmberg and Hakomori, 1973; Gahmberg et al., 1976; Gahmberg and Andersson, 1977). Fig. 6.1 shows their enzymatic activation by galactose oxidase, the oxidation of sialyl groups of glycoconjugates by the use of combined enzymatic action of neuraminidase and galactose oxidase, or selective periodate oxidation. Both types of enzymatic activation of glycoproteins have already been used for the immobilization of enzyme (Petkov et al., 1990) or antibodies (Solomon et al., 1990). Further discussion about of activation under these mild conditions is presented in Section 6.2.2, which deals with hydrazide-containing supports.

Descriptions of many methods for the attachment of proteins to solid supports have been published by Kennedy and Cabral (1983). In the same work there is a chapter on reagents used for protein immobilization (Lewis and Scouten, 1983). A precise account of the principal methods of immobilization has been published in the book "Affinity Chromatography - A Practical Approach" edited by Dean et al. (1985). Practical guides to many coupling methods are presented in booklets published by Réactifs IBF (Villeneuve - la Garenne, France), Pharmacia LKB Biotechnology (Uppsala, Sweden) etc. Bioseparations using bioaffinity techniques have recently been summarized by Sii and Sadana (1991). The extreme diversity of the ligands being immobilized is the reason behind the fact that no method can be considered to be a universal one.

6.1 EFFECT OF THE NATURE OF PROTEINS AND SOLID SUPPORTS

The amount of protein to be coupled, the stability and the biological properties of immobilized biomolecules can be affected by the choice of the solid support and the method of coupling. In order to investigate the effect of the nature of the support and the character of the proteins coupled, serum albumin, trypsin, chymotrypsin, papain

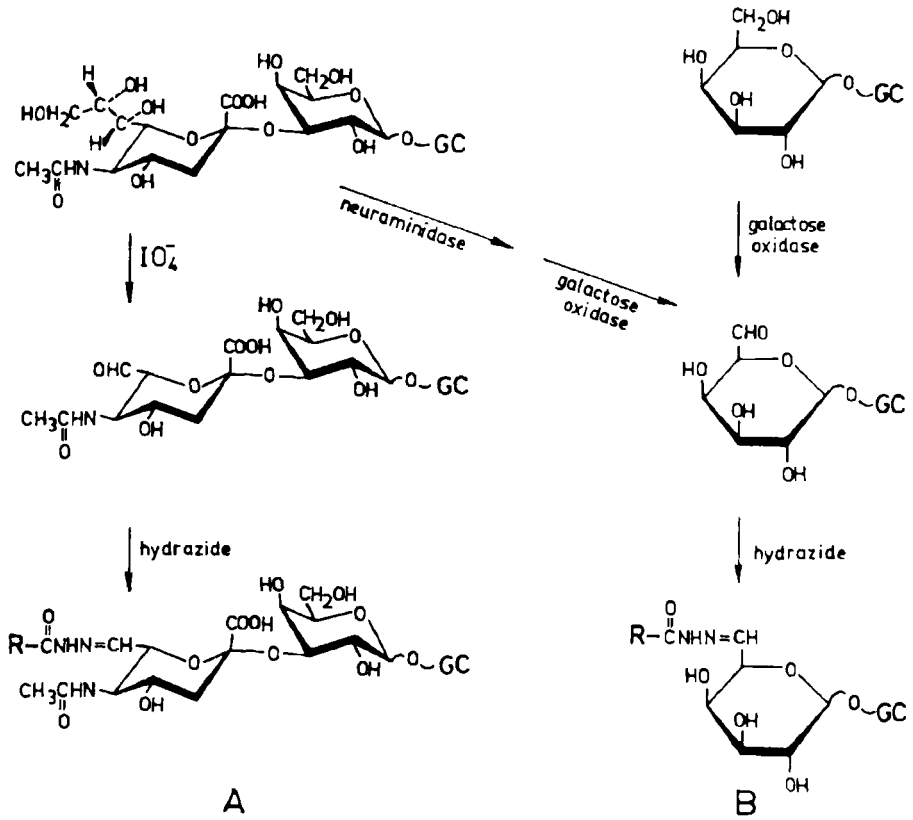


Fig 6.1. Two major reaction schemes for coupling hydrazide reagents to (oxidized) sugar residues. In (A), sialyl groups of glycoconjugates are oxidized chemically with periodate and the resultant aldehyde may be reacted with an appropriate hydrazide. In (B), galactose (or N-acetylgalactosamine) residues are oxidized enzymatically, using galactose oxidase, to the respective C-6 aldehydoderivative which undergoes subsequent interaction with an appropriate hydrazide. In cases where galactose (or N-acetylgalactosamine) residues appear penultimate to terminal sialyl residues, hydrazides may be coupled to the sialoglycoconjugate by the combined enzymatic action of neuraminidase and galactose oxidase, as shown.

and trypsin inhibitor antilysine were attached to glycidyl methacrylate copolymer, oxirane-acrylic beads, epoxy-activated Sepharose and 2,3-epoxypropoxy-propyl derivatives of glass and silica. The epoxide groups of the derivatized supports may react with the amino, carboxy and sulphydryl groups and with some aromatic nuclei, such as indole and imidazole (Zemanová et al., 1981).

Fig. 6.2 (A and B) show the difference in the coupling of proteins (in mg per g of dry support) to methacrylate copolymer and agarose. It is evident from the figures that the amount of the protein attached as a function of the pH of the reaction mixture is

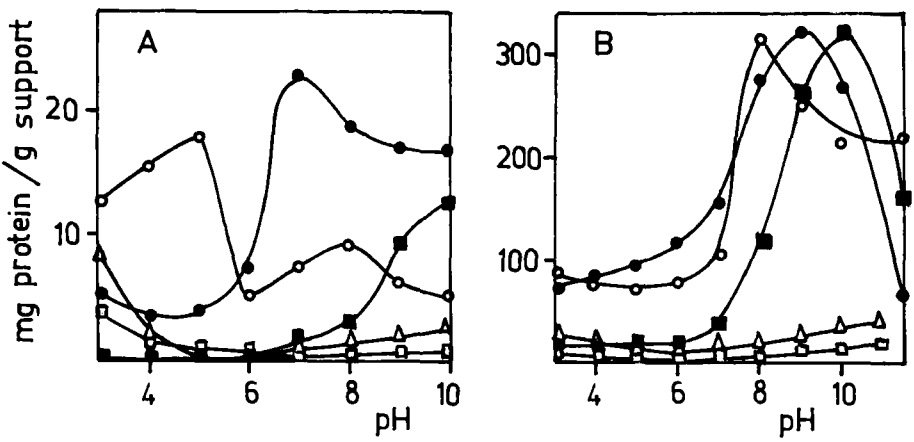


Fig. 6.2. Effect of pH on the coupling of: o, serum albumin; ●, papain; ■, antilysin; ❑, trypsin and Δ, chymotrypsin (A) to the glycidyl methacrylate copolymer and (B) to the epoxy-activated Sepharose 6B in dependence on pH. Data from I.Zemanová et al., Enzyme Microb. Technol., 3 (1981) 229-232.

considerably affected by both the character of the coupled proteins and the nature of the solid support.

The effect of the solid support was much less pronounced when various proteins were immobilized using benzoquinone. The mild effect of the solid support was determined when the effect of pH was investigated on the benzoquione-mediated immobilization of trypsin, chymotrypsin and serum albumin, either on hydroxyalkyl methacrylate copolymer (Stambolieva and Turková, 1980) or agarose (Brandt et al., 1975). Benzoquinone probably shaded the matrix.

6.2 SUPPORT MODIFICATION AND AFFINITY LIGAND IMMOBILIZATION

6.2.1 Epoxide-containing supports

A great improvement in bioaffinity chromatography resulted from the change from the soft gel supports to porous or nonporous silica, as has already been discussed in the Introduction. According to the opinion of Small et al. (1981), the modification of the surface silanol groups of silica with γ-glycidoxypropyltrimethoxysilane yields a neutral hydrophilic surface that neither adsorbs nor repels proteins and thus decreases the potential denaturation of sensitive biological substances by silica. The reaction is shown

in Fig. 5.8. The epoxide group of the carrier may react with amino, carboxy, hydroxy and sulphydryl groups, with some aromatic nuclei, such as indole, imidazole, etc. (Turková, 1980), saccharides (Uy and Wold, 1977), polynucleotides (Potuzak and Dean, 1978), adipic acid dihydrazide (Ustav et al., 1979) etc. The S-C, N-C and O-C bonds formed are extremely stable (Egly and Porath, 1978; Turková et al., 1981; Koelsch et al.,1984). Small et al. (1981) used epoxide groups of silica for the coupling of 6-aminohexyl-Procion Yellow or of Procion Blue MX-R directly *via* the triazine ring. These triazine dye-silica adsorbents have been used for high-performance liquid bioaffinity chromatography of protein mixtures containing enzymes such as lactate dehydrogenase, hexokinase, alkaline phosphatase, carboxypeptidase G2 and L-tryptophanyl-tRNA synthetase. Porous silica with γ-glycidoxypropyltrimethoxy silane was used after coupling of tomatine by Csiky and Hansson (1986) for the HPLBAC of sterols. They hydrolyzed epoxy-substituted silica in 0.1M sulphuric acid-sodium hydroxide buffer (pH 2, 50°C, 5h) and the diolsilica was used as a blank material to study the influence of the matrix in the separations.

Fig. 2.3 shows HPLBAC of pepsin on hydroxyethyl methacrylate copolymer Separon H 1000 modified with ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (Turková et al., 1981). The inhibitor was attached to epoxide groups of Separon HEMA, which were prepared by the reaction of hydroxy groups of copolymer in an alkaline medium with epichlorohydrin. The same paper has supplied Fig. 6.3, which shows the pH profile of the coupling glycyl-D-phenylalanine to the hydroxyethyl methacrylate copolymer Separon derivatized by epichlorohydrine to both a high and a low degree of epoxidation. The coupling rate was also affected by the structure of the bound amino component. The quantity of the substance immobilized and the reaction rate increased with the increasing hydrophobicity of the compounds immobilized. The coupling reaction of Separon H 1000 E_{max} with 1,6-diaminohexane was very fast, being complete after 6 h.

The same Separon was used by Smalla et al. (1988) for the study of the influence of salts on the coupling of different enzymes. Fig. 6.4 shows the dependence of the activity and protein binding of aminoacylase - Separon HEMA E conjugate on the salt used for

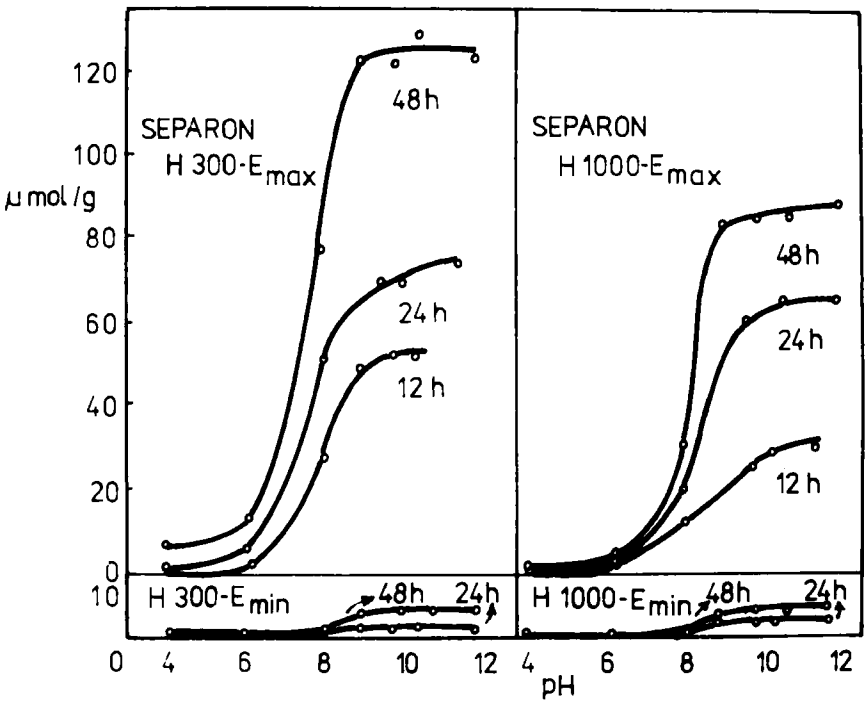


Fig. 6.3. Coupling of glycyl-D phenylalanine on various types of derivatized Separons as a function of pH and time. The content of epoxide groups: Separon H 300 E_{max} = 1 550 μmol/g of solid support, Separon H 1000 E_{max} = 1 770 μmol/g, Separon H 300 E_{min} = 240 μmol/g, Separon H 1000 E_{min} = 140 μmol/g. Data from J. Turková et al., J. Chromatogr., 215 (1981) 165 - 179.

the immobilization reaction. Aminoacylase-Separon H 1000 E_{max} with the highest activity was achieved using ammonium sulphate. However, as Fig. 6.5 shows, the optimum concentration of this salt depends on the concentration of immobilized protein. The study of the effect of different concentrations of $(NH_4)_2SO_4$ on the coupling of thermitase, trypsin, chymotrypsin, pepsin, elastase, subtilisin and carboxypeptidase A showed a different influence of added salts on the immobilization efficiency.

Epoxide-containing supports can be obtained directly by copolymerization. As an example we may mention glycidyl methacrylate copolymer (Švec et al., 1975) or EupergitC (Krämer et al., 1978). The disadvantage of biospecific sorbents prepared from glycidyl methacrylate copolymer (Turková et al., 1978) was the formation of new,

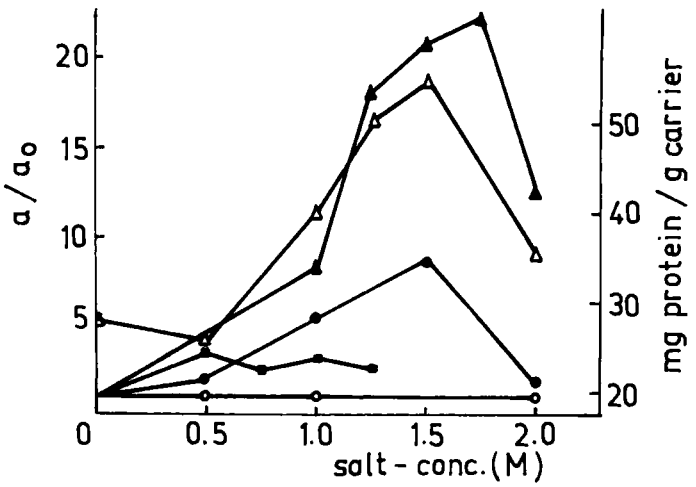


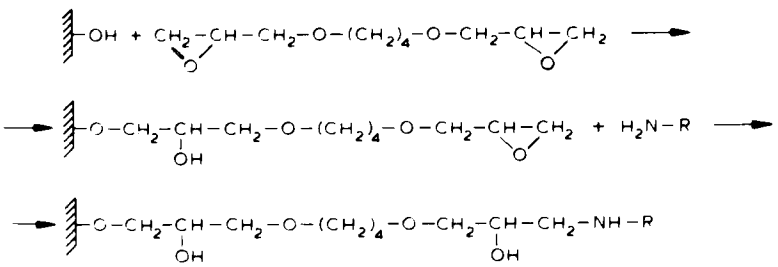
Fig. 6.4. Dependence of the activity and protein binding of aminoacylase-Separon HEMA E conjugate on the salt added during the immobilization reaction. Aminoacylase (5 mg) in 2.5 ml 0.2 M phosphate buffer (pH 8.0); 50 mg Separon HEMA E, 20 h at 5°C. Salts added: (▲) $(NH_4)SO_4$, (■) Na_2SO_4, (●) $(NH_4)_2HPO_4$, (o) NaCl. a_o, activity of the enzyme-carrier conjugate in the absence of salts; a, activity of the enzyme-carrier conjugate in the presence of salts as indicated. (Δ) $(NH_4)_2SO_4$ addition and protein binding (right axis). Aminoacylase (10 mg) in 2.5 ml phosphate buffer (pH 8.0); 50 mg Separon HEMA E, 30 h at 5°C. Data from K. Smalla et al., Biotechnol. Appl. Biochem., 10 (1988) 21 - 31.

active epoxide groups during long storage times, as determined by the coupling of different dipeptides.

Fleminger et al. (1990b) described β-mercaptoethanol as an efficient blocker of residual oxirane groups of Eupergit C. Blocking may be applied to the matrix at almost neutral pH (pH 8.0) and, as it does not form charged groups with the matrix (in contrast to ethanolamine), non-specific ionic adsorption of proteins is eliminated.

Bioxyranes (e.g. 1,4-butanediol diglycidyl ether) were used by Sundberg and Porath (1974) for the introduction of reactive oxirane groups into agarose. These groups can react further with compounds that contain amino, hydroxyl or thiol groups (see next page).

The method is suitable for the binding of sugars that form ether linkages through their hydroxyl groups. With proteins and peptides the method forms alkylamine linkages through their primary amino groups. Thioether linkages are formed with substances



that contain thiol groups. When 1,4-bis(2,3-epoxypropoxy)butane is used, a spacer corresponding to a 12-carbon chain is introduced between the ligand and the agarose chain. Through the effect of bisoxirane, crosslinking of carbohydrate chains of the gel matrix takes place, thus increasing its stability. This permits the application of more drastic conditions for the binding and elution steps. These gels are available commercially under the trade name epoxy-activated Sepharose.

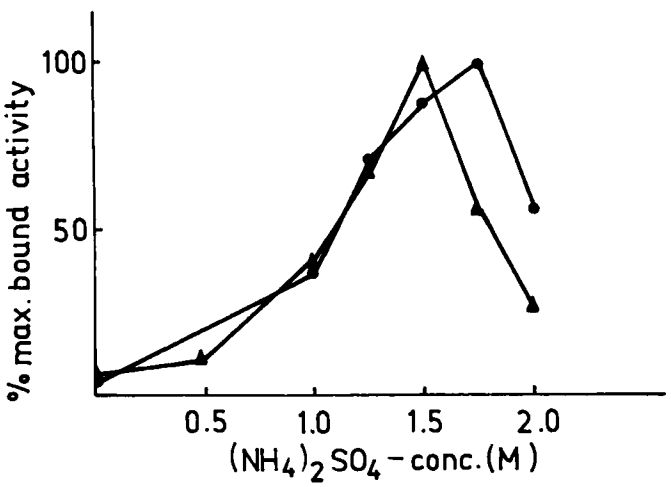


Fig. 6.5. Dependence of the activity of aminoacylase-Separon HEMA E conjugate on the salt and protein concentrations in the medium during immobilization. Aminoacylase, 5 mg (●) or 10 mg (▲), in 2.5 ml 0.2 M phosphate buffer (pH 8.0); 50 mg Separon HEMA E, 30 h at 5o C. 100% activity: (●) 4.1x10-2 μmol.min-1.mg conjugate -1; (▲) 7.0x10-2 μmol.min-1.mg conjugate-1. Data from K. Smalla et al., Biotechnol. Appl. Biochem., 10 (1988) 21 - 31.

The influences of pH, temperature, ligand concentration and reaction time on the coupling of aniline to epoxy-activated Sepharose 6B have been studied by Gelsema et al. (1981). Aniline was coupled in 100% yield at pH 6 - 7 by performing the reaction at 55°C for 17 h using an 8.5-fold excess ligand concentration. Presumably the same holds for other ligands containing aromatic amine groups at a pH about 2 units higher than the corresponding pK value. Coupling of antibiotic novobiocin *via* its phenolic hydroxy group to the epoxy-activated Sepharose was performed and the product used for the bioaffinity chromatography of DNA gyrase by Staudenbauer and Orr (1981). Epoxy-activated Sepharose 6B was also used by Andersson et al. (1983) for the immobilization of labile hydroxyl-group containing ligands in dipolar organic solvents in the presence of different kinds of catalyst. Epoxy-activated Sepharose 6B was further used for coupling of the outer membrane of *E. coli* in the presence of dioxane and used for the isolation of immunoadsorbent-purified antibodies (Henriksen and Maeland, 1986).

Hydrolysis of the epoxide with acid, oxidation of the diol with periodate, and reduction of the aldehyde with sodium borohydride shown in Fig. 5.8, can result in suitable solid supports for ligand immobilization (Ernst-Cabrera and Wilchek, 1988). Matsumoto et al. (1980) converted epoxy-activated agarose into amino derivatives using ammonia solution which, after succinylation, were activated with N-hydroxysuccinimide. Kanamori et al. (1986) used Sepharose 4B or Toyopearl HW-65 (Fractogel TSK) after activation with epichlorohydrin for the preparation of two formyl carriers, as shown in Fig. 6.6.

A large amount of m-aminobenzamidine was efficiently immobilized on both types of carriers. Formyl groups remaining on the adsorbents were converted into hydroxymethyl groups by reduction with sodium borohydride. All types of adsorbents prepared were successfully used for both low- and high-performance liquid bioaffinity chromatography of trypsin-family proteases.

6.2.2 Hydrazide derivatized solid supports

Section 3.3 has already described the 1 : 2 optimum molar ratio of immobilized antibody to purified antigen reached by Domen et al. (1990). The authors used antibody

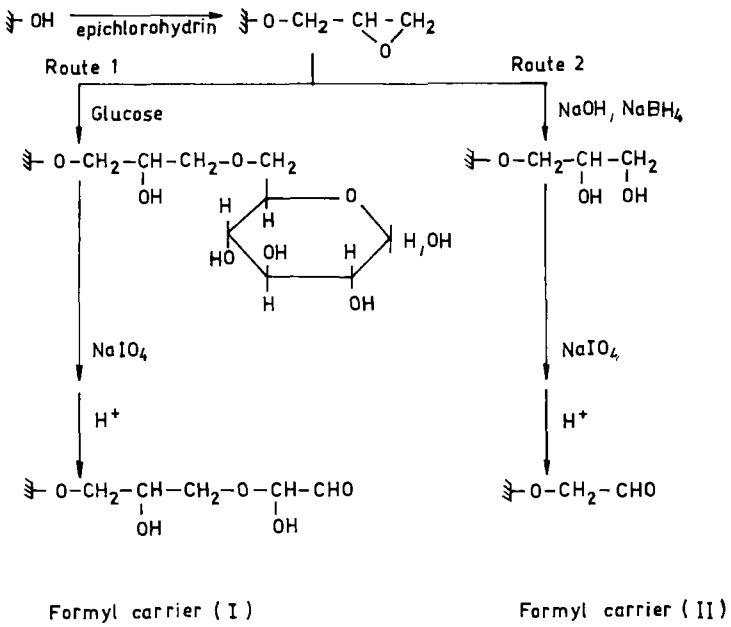


Fig. 6.6. Preparation of formyl carriers.

attached through its carbohydrate moieties to Carbo Link Gel containing hydrazide groups. The advantages of hydrazide derivatized solid suports are also discussed in Section 4.3.4. Procedures for preparing derivatives of polyacrylic hydrazido-Sepharose are shown in Fig. 5.5. Modification of Sepharose, cellulose, Sephadex, Spheron, Toyopearl and Trisacryl by methoxycarboxycarbonyl chloride in the presence of pyridine and subsequent hydrazinolysis of the methoxycarbonylated support has been described by Klyashchitsky et al. (1986). A review of hydrazido-derivatized supports in bioaffinity chromatography has been published by O 'Shannessy (1990).

Under suitably mild conditions, oxidation of glycoproteins with periodate results in the generation of reactive aldehydes which can subsequently be bound with nucleophiles such as primary amines or hydrazides. Both primary amines and hydrazides will bind with aldehydes only when they are in the unprotonated form. The very low pK of hydrazides, usually around 2.6, as compared to primary amines, which have pK_s in the range of 9 - 10, allows one to significantly reduce the formation of Schiff bases between protein and oligosaccharide by performing the coupling reaction under mildly acidic

conditions between pH 4.5 - 5.5. Under such conditions the majority of the primary amines of the protein moiety will be protonated and unreactive. Secondly, the product of binding between an aldehyde and a hydrazide is a hydrazone, which is much more stable than a Schiff base and does not require reduction (Fig. 6.7).

Lamed et al. (1973) have described the coupling of several nucleotide di- and triphosphates, after their periodate oxidation to adipic acid dihydrazide, attached to Sepharose 4B after CNBr activation. The coupling was performed under mild conditions. To 1 ml Sepharose hydrazide in 0.1 M sodium acetate (pH 5) was added 4 - 5 μmole of periodate oxidized nucleotide in 2.5 ml of the same buffer. The suspension was stirred for 3 h at 4^0 C. An amount of 7.5 ml of 2 M NaCl solution was added and stirring continued for another 30 min to remove unbound nucleotide from the Sepharose hydrazide. Blank reactions were carried out with unoxidized nucleotides. The absorbance of the supernatant was measured to determine the extent of binding. The amounts of bound nucleotide ranged from 3 to 4.5 μmoles per ml Sepharose with the various nucleotides. Only trace amounts (0.1 - 0.2 μmole /ml gel) of nonoxidized nucleotides were adsorbed on the Sepharose hydrazide. The same paper discusses the advantages of Sepharose hydrazide over aminoethyl cellulose; stable products are obtained without further reduction with sodium borohydride, and unreacted hydrazide groups do not impose ion-exchange properties on the resin at neutral and high pH. In the case of nucleosides, nucleotides and RNA, immobilization onto hydrazido-derivatized matrices would appear to be the method of choice. A schematic drawing of the preparation of the efficient biospecific sorbent for concanavalin A by using oriented immobilized antibodies with adsorbed ovalbumin has been already shown in Fig. 2.6. A biospecific sorbent for the isolation of ovalbumin antibodies from rabbit antiserum was prepared by bioaffinity chromatography on a column of ovalbumin immobilized *via* only one its carbohydrate moieties (Nisbet et al., 1981) after periodate oxidation to a hydrazide derivative of bead cellulose (Turková et al., 1990).

Ovalbumin (60 mg) isolated on the Con A-Spheron column was dissolved in 60 ml of 0.1 M acetate buffer (pH 5.5), 6 ml of 0.1 M $NaIO_4$ were then added and the reaction mixture was stirred in the dark for 20 min at 4^oC. The reaction was stoped with 48 μl of

ethylene glycol and the mixture stirred for a further 5 min. The low - molecular - weight components were removed on a Sephadex G-25 Fine column (30 x 3.5 cm I.D.) equilibrated with 0.1 M acetate buffer containing 0.5 M NaCl (pH 4.8).

Oxidized ovalbumin (25 ml) was added to 5 ml of cellulose activated with adipic acid dihydrazide and equilibrated with 0.1 M acetate buffer containing 0.5 M NaCl (pH 4.8). After 30 h of coupling with stirring at 4°C, sodium cyanoborohydride (35 mg) was added and the reaction allowed to proceed for 6 h at the same temperature. The conjugate obtained was then washed on a sintered glass filter with the above mentioned 0.1 M acetate buffer containing 0.5 M NaCl (pH 4.8) to the point where protein was absent in the eluate. The amount of ovalbumin coupled was 1.8 mg/ml cellulose. As a control, ovalbumin oxidized with $NaIO_4$ was bound under the same conditions to non-activated bead cellulose. The anti-ovalbumin IgG fraction isolated on this sorbent from immune rabbit serum contained only antibodies against protein determinants of ovalbumin. Thus, when these IgG were immobilized through their carbohydrate moieties to cellu-

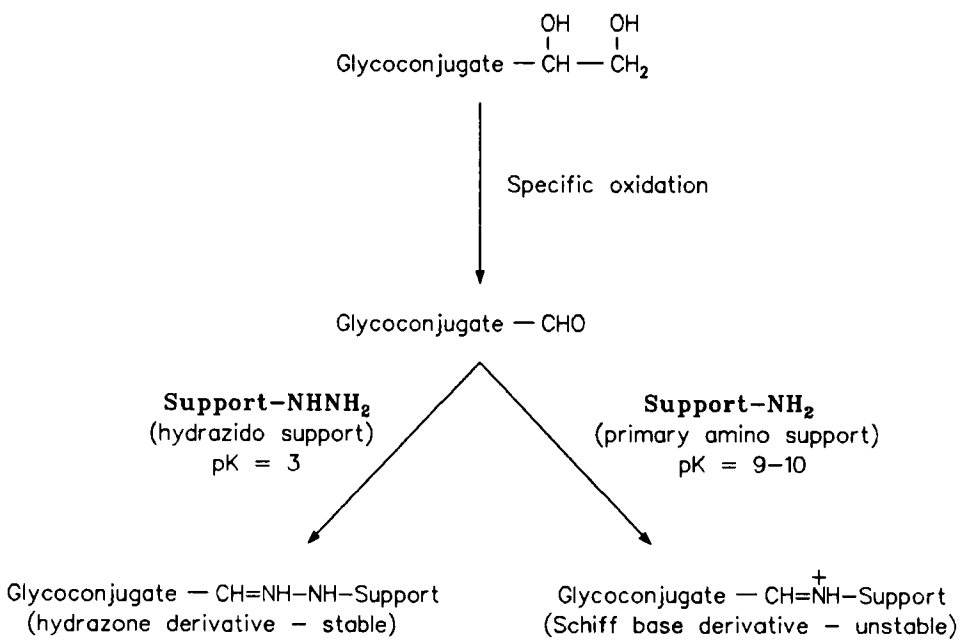


Fig. 6.7. Flow diagram summarizing the chemistry involved in the site-specific immobilization of glycoconjugates via their glycosylation onto hydrazido-derivatized and amino-derivatized matrices.

lose beads it became possible to prepare a biospecific sorbent for concanavalin A by oriented adsorption of ovalbumin. Ovalbumin was specifically adsorbed *via* its protein moiety and its carbohydrate part remained free for interaction with concanavalin A.

The successful immobilization of glucose oxidase by activation of its carbohydrate residues by oxidation with periodic acid has been described by Zaborsky and Ogletree (1974). Chemical oxidation of the oligosaccharides may result in the oxidation of some amino acid residues (such as serine, threonine, proline and methionine), thereby leading to a decrease in enzymatic activity. Pazur et al. (1970) reported that extensive oxidation of the carbohydrate residues in glucoamylase by periodate markedly affected the stability of the enzyme. Avigad et al. (1962) stated that active aldehyde groups can be generated in glycoproteins containing galactose or galactopyranosides by oxidation catalyzed by galactose oxidase. Therefore Petkov et al. (1990) used specific oxidation of D-galactose present in the carbohydrate moiety of glucose oxidase from *Aspergillus niger* by galactose oxidase in the presence of catalase. Galactose oxidase (0.7 ml) and catalase (0.3 ml) were added to a glucose oxidase solution (10 mg glucose oxidase in 3 ml 0.1 M phosphate buffer pH 7.0) and left to react at 20°C for 6 h. The reaction mixture was passed through a Sephadex G 25 Fine column (23.0 x 1.5 cm I.D.) equilibrated with 0.1 M acetate buffer pH 4.8 containing 0.5 M NaCl. Oxidized enzyme was coupled to hydrazide derivatives of O-α-D-galactosyl Separon H 1000 or Sepharose 4B. Both solid supports were modified with adipic acid dihydrazide after their activation with galactose oxidase. Each immobilized preparation of glucose oxidase showed a higher activity than was achieved by other immobilizing procedures.

As in the case of the immobilization of glycoenzymes, the results obtained for the immobilization of antibodies clearly demonstrate that site-directed immobilization *via* the oligosaccharide moieties is superior to amino acid-directed immobilization chemistries (O' Shannessy, 1990). Quash et al. (1978) were the first to describe the immobilization of polyclonal immunoglobulin G (IgG) *via* the oligosaccharide moieties onto hydrazido-derivatized latex particles.

Fleminger et al. (1990a) described the optimization of oriented immobilization of periodate-oxidized monoclonal antibodies on hydrazide derivatives of Eupergit C. They

used carboxypeptidase A (CPA) and horseradish peroxidase (HRP) as antigens and selected monoclonal antibodies (m Abs) that did not inhibit the respective enzymatic activities. Binding of the antigen by the matrix-immobilized antibody may thus be monitored by direct measurement of the enzymatic activity of the matrix-bound immunocomplex. An Eupergit C-hydrazide derivative was prepared by the reaction of Eupergit C beads (containing 0.8 mmol oxirane group/g) with 0.1 or 0.5 M adipic acid dihydrazide (ADH). As shown in Fig. 6.8, the preparation efficiently yielded bound oxidized antibodies when a relatively low concentration of antibodies was applied to the matrix. At higher loads, binding was less efficient, even when the amount of ADH groups on the matrix was increased. The treatment of Eupergit C with 0.1 M ADH and blocking of residual oxirane groups by reaction with β-mercaptoethanol were therefore used for the preparation of Eupergit C-ADH.

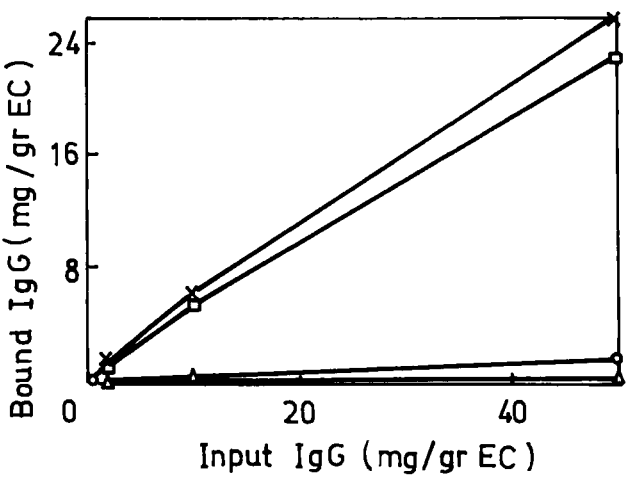


Fig. 6.8. Binding of radiolabelled oxidized ^{125}I IgG to Eupergit C-ADH beads. Eupergit C beads (100 mg) were treated with 1 mL of 0.1M (❑), or 0.5M (x) ADH in 0.2M sodium carbonate buffer, pH 9.0, for 16 h. The beads were then incubated for 4 h with β-mercaptoethanol (0.2M final concn.) and extensively washed with 0.1M sodium acetate buffer, pH 5.5. Oxidized IgG was added to the reaction mixture (5 mg of beads/tube) and incubated for 4 h at room temperature. The amount of the bound IgG was determined by the radioactivity of the beads. Nonspecific adsorption of the bound oxidized IgG was determined by extensively washing the beads with a solution containing 10% SDS in 8M urea and measuring the radioactivity in the supernatant (o)above the beads. In control experiments the binding of nonoxidized IgG to Eupergit C beads treated with 0.1M ADH (Δ) was also determined. Reproduced with permission from G. Fleminger et al., Appl. Biochem. Biotech., 23 (1990) 123 - 137.

Before optimization of the binding of a certain oxidized antibody onto Eupergit C-ADH the sensitivity to periodate oxidation of a series of anti-CPA antibodies, as well as an anti-HRP antibody, was examined by incubation of these antibodies with various concentrations of sodium periodate for different periods of time. All the antibodies tested were relatively stable to periodate oxidation at low concentrations (up to 10 mM), short oxidation periods (1 h), and low temperature (0°C). Increased periodate concentration (100 mM) or a longer oxidation period (16 h) had a marked effect on the activity of some antibodies, but only a minor effect on others. Similarly, increasing the temperature during the oxidation step to 4°C or room temperature resulted in a rapid inactivation of the more sensitive antibodies, whereas the more stable antibodies were less affected. It is thus necessary to examine the sensitivity of all glycoconjugates to periodate oxidation in order to minimize any damage to their activities.

As result of their studies, Fleminger and co-workers adopted the following oxidation and binding procedure: antibodies (0.7 - 3.0 mg) are oxidized by 10 mM periodate in 1.0 mL of 0.1 M sodium acetate buffer, pH 5.5, for 1 h at 0°C. Three drops (approx. 100 μL) of ethylene glycol are then added to destroy excess periodate; the sample is applied to a gel filtration column, and the fraction containing the antibodies is collected. Oxidized antibodies are coupled to Eupergit C-ADH by reaction in the dark for 1 h. The antigen-binding activity of the anti-CPA antibodies was considerably enhanced when coupled by this procedure. With some antibodies, the antigen binding activity increased to a level of close to 2 mol antigen bound/mol of antibody. It is appropriate to note that, although with most antibodies binding of periodate-oxidized antibodies to Eupergit C-ADH apparently yields more active antibody preparations, exceptions may be anticipated. For example, anti-HRP antibodies showed very high antigen binding activity (approx. 1.8 mol antigen /mol of immobilized antibody) when coupled to Eupergit C *via* oxirane groups. When the same antibody was oxidized and coupled to Eupergit C-ADH, its activity decreased to 1.5 mol Ag bound/mol immobilized antibody.

Solomon et al. (1990) consider an enzymatic procedure for the generation of aldehyde groups on the oligosaccharide moieties of antibodies utilizing the neuraminidase-galactose oxidase system to be more specific than chemical oxidation. The data

presented in their paper show that the co-immobilization of neuraminidase and galactose oxidase on Eupergit C-ADH beads provides an economical, efficient and selective system for the enzymic oxidation of monoclonal antibodies without impairing their immunological activity. When immobilizing only minute amounts of neuraminidase (NA) and galactose oxidase (GO) 0.01M ADH proved to be sufficient for the preparation of Eupergit C-ADH. The modified support was thoroughly washed with phosphate-buffered saline (pH 7.4) and excess of oxirane groups was blocked with 0.2M β-mercaptoethanol for 2 days at room temperature. After thorough washing with 0.1M acetate buffer (pH 5.5) Eupergit C-ADH was used for the immobilization of NA and GO in 0.1M acetate buffer (pH 5.5), after their oxidation with 10mM sodium periodate. After coupling of the enzymes to the carrier, the preparations were treated with 0.2M acetaldehyde in 0.1M acetate buffer (pH 5.5) for 2 days to block the residual reactive hydrazide groups. Aldehyde groups formed on anti-CPA monoclonal antibody using different amounts of the immobilized enzymes (a ratio of 1 : 4 units of the enzymic activity NA to GO) are shown in Fig. 6.9. A smaller number of aldehyde groups was formed on the enzymically oxidized antibodies as compared with the chemically oxidized material. Nevertheless, the 2 mol of aldehyde formed per mole of enzymically oxidized antibodies were sufficient to achieve efficient binding of the antibody to the matrix. These antibodies possessed a higher antigen-binding activity than the corresponding chemically oxidized antibodies immobilized on the same matrix. The amount of antigen bound to the corresponding antibody-matrix conjugate was close to the theoretical value of 2 mol of antigen (CPA or HRP) bound per mole of immobilized antibody. The co-immobilization of galactose oxidase and neuraminidase exhibits the well known advantages of immobilized enzymes, such as repeated use of the same enzymes, a good recovery of the antibodies, and the possibility of continuous oxidation of mAB. However, Kelleher et al.(1988) described the possibility that purified galactose oxidase not only converted the C-6 hydroxymethyl group of galactose to aldehyde, but also catalyzed further oxidation to the carboxyl group.

The immobilization of sugars onto hydrazido-supports for the bioaffinity purification of lectins has been described by Ito et al. (1986). In this case use is made of the

linear-cyclic equilibrium of the reducing end sugar which, in the linear form, exists as an aldehyde (O 'Shannessy, 1990). The aldehyde condensed with the hydrazido support and the hydrazone produced was stabilized by performing the reaction in the presence of sodium cyanoborohydride (hydrazine derivative: conjugate-CH_2NHNH - support). Hydrazino carriers and immobilized sugars were prepared by Ito et al. (1986) by simple reactions. The following methods were used: to 1 volume of suction-dried epoxy-activated Sepharose 6B or epoxy-activated Fractogel TSK HW-55 was added a 1.5 volume of hydrazine hydrate containing 2 % $NaBH_4$ and the mixture was then incubated at 40°C for 1.5 h. The carriers were then extensively washed with distilled water. For the coupling of disaccharides, 4 g of suction-dried hydrazinocarrier was suspended in 3 ml of 0.2 M K_2HPO_4 containing 100 mg of lactose or maltose and 50 mg of $NaCNBH_3$. The suspension was incubated at room temperature with shaking. The gel was washed extensively with water and hydrazino groups remaining in the gel were then acetylated

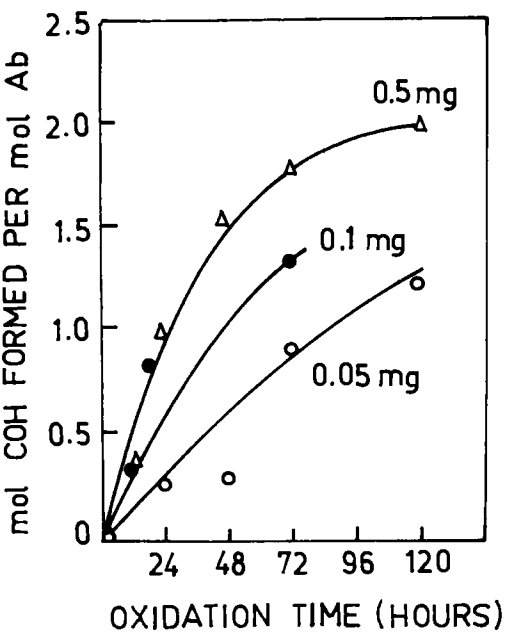


Fig. 6.9. Determination of aldehyde groups formed on anti-CPA monoclonal antibody using the immobilized bifunctional enzymic system. Different amounts of immobilized enzymes (o, 0.05 mg; ●, 0.1 mg; Δ, 0.5 mg) were incubated with 10 μg of antibody. The enzymic activity of alkaline phosphatase-avidin measured as the increase in absorbance at 405 nm was proportional to the number of aldehyde groups formed. Reproduced with permission from B. Solomon et al., J. Chromatgr., 510 (1940) 321-329.

with acetic anhydride. The glycamyl-Sepharose thus obtained showed high adsorption capacities for lectins. Glycamyl-TSK gel G3000 PW obtained with TSK gel G3000 PW, could be successfully used for the high- performance liquid affinity chromatography of lectins.

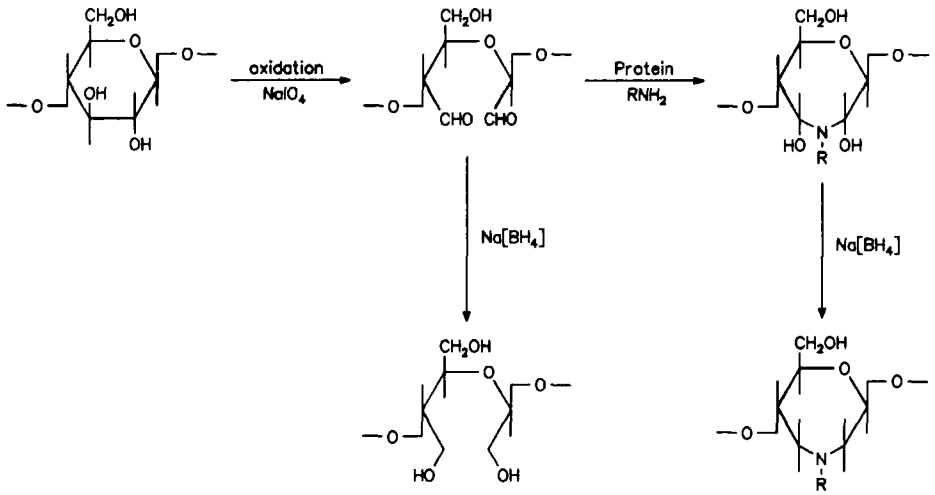
In a similar manner, heparin has been immobilized onto the same hydrazido supports and used in studies on heparin-binding proteins (Sasaki et al., 1987). It is worth noting at this stage that multi-site attachment of ligands to the hydrazido support is thought to stabilize the ligand-matrix complex. Single-site attachment of ligands may result in a less stable bond, depending on proximal functional groups, and reduction of the hydrazone in such situations is recommended.

The contact of nonpolar residues of the surface area of proteins with water is thermodynamically disadvantageous and decrease the stability of the protein in *vitro*. Hydrophilization of nonpolar surface areas is the most simple and reliable approach to the artificial stabilization of proteins and it has been already discussed in Section 4.3.3. Galactosylations of nonpolar amino acids or of surface residues near to nonpolar clusters of trypsin have been used for its stabilization and immobilization through the galactose residues to hydrazide derivatives of cellulose or glass (Turková et al.,1992). Glycosylated albumin was immobilized in a site specific manner onto hydrazide derivatives of cellulose by Gemeiner and Viskupovič (1981).

Ligands containing functional groups other than aldehydes may also be immobilized onto hydrazido-derivatized supports. Examples of this include the immobilization of the tresyl ester of T-2 fungal toxin for the bioaffinity purification of anti-T-2 antibodies (Allam et al., 1987) and the immobilization of proteins and other ligands through carboxylic acid functions using carbodiimide activation of the ligand (Wilchek and Miron, 1974). A number of hydrazido support matrices have been used as intermediates in the synthesis of other "activated" supports, such as acyl azides (Suttnar et al., 1977; Miron and Wilchek, 1981).

6.2.3 Periodate oxidation

A simple method for the binding of proteins to insoluble polysaccharides has been described by Sanderson and Wilson (1971). The reaction sequence for the binding of proteins to polysaccharides is:



The polysaccharide is "activated" by oxidation with 0.01 - 0.5M sodium periodate for 1 h. The aldehyde formed reacts with the protein. For example, the oxidized polysaccharides were washed with water by centrifugation and 10 mg (dry weight) suspended in 1 ml of phosphate-buffered saline (pH 8) containing 10 mg of bovine serum albumin and agitated continuously for 20 h. Subsequent reduction with sodium borohydride (a freshly prepared 1% solution) led to the stabilization of the bonds between the protein and the polysaccharide, and to the reduction of the residual aldehyde groups.

The use of the immobilization *via* reductive alkylation for the coupling of trypsin to periodate-oxidized glucose-Separon and cellulose beads enables a comparison of the methacrylate matrix with natural polysaccharide supports.

Optimum conditions for the coupling of trypsin onto hydroxyalkyl methacrylate gel containing glucose on its surface after the oxidation of the gel with periodate were developed by Vančurová and co-workers (1979). The amount of trypsin coupled onto periodate-oxidized glucose derivative Separon H1000-glc in relation to pH first increa-

ses moderately with increasing pH, but above pH 8 the increase is steep up to pH 10. The amount of the immobilized trypsin and its activity, determined by means of benzoyl-L-arginine p-nitroanilide as substrate, increases with increasing concentration of free trypsin in the reaction mixture during the coupling.

Optimum conditions for the coupling of trypsin onto cellulose in bead form after its oxidation with periodate have been developed by Turková and co-workers (1979). Just as in the case of the preceeding gel, the rate of binding of trypsin increases with increasing pH. However, the solubilization of cellulose derivatives also increases with pH, due to alkaline degradation yielding soluble immobilized trypsin fractions and soluble oxidized oligosaccharides. Just as with glucose-hydroxyalkyl methacrylate gel, the amount of bound trypsin, as well as its activity, increase with increasing concentration of free trypsin in the reaction mixture during the coupling. The Schiff base-type bond between the oxidized glucose matrix and the peptide is very unstable and reduction with sodium borohydride is necessary for stabilization (Koelsch et al., 1984).

In order to compare the coupling of ligands to solid supports *via* different aldehyde groups we determined the amount of Gly-D,L-Phe coupled to periodate-oxidized glucose-Separon and to Separon with attached hexamethylene diamine and activated by glutaraldehyde in relation to pH (Koelsch et al., 1986). From Fig. 6.10 it is evident that the two activated matrices are clearly different. Another big difference between these two types of aldehyde groups was also found in the stability of bonds with glycyl-D, L-phenylalanine. Unlike the situation with oxidized glucose residues, in which case the reduction with $NaBH_4$ is necessary, the bond between glutaraldehyde and dipeptide is very stable. There is no difference between reduced and untreated preparation. In both variants no release of nitrogen was found after 11 weeks at pH 7.0 and 24°C.

6.2.4 Glutaraldehyde activation technique

The glutaraldehyde activation technique can be used for solid supports having amide or primary amine groups. In the latter case, the activated matrix is often coloured. Weston and Avrameas (1971) developed a method for the direct binding of affinants

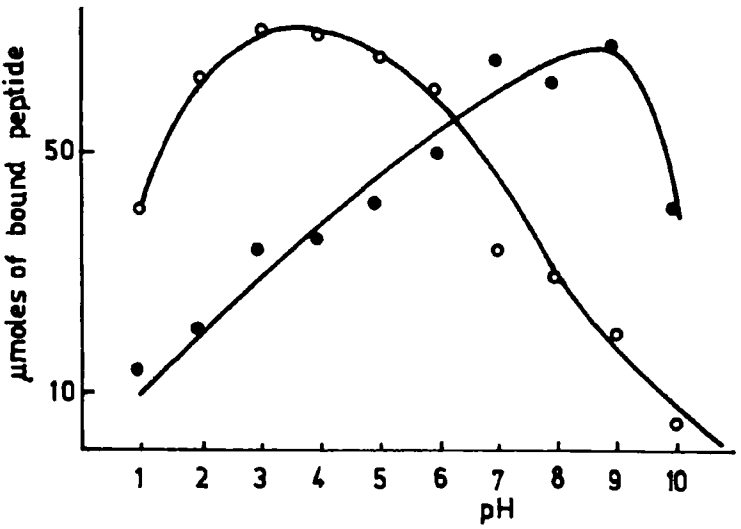


Fig. 6.10. pH dependence of the amount of Gly-D,L-Phe (µmol/g of dry support) bound to Separon H 1000 glc oxidized by $NaIO_4$ (x) and Separon H 1000 E-NH_2 activated by glutaraldehyde (o). Data from R. Koelsch et al., Biotechnol. Lett., 8 (1986) 283-286.

on to polyacrylamide gels using glutaraldehyde which, if present in excess, reacts *via* one of its two aldehyde groups with the free amide group present in the polyacrylamide gel. The remaining free active group then reacts with the amino group of the affinant added during the subsequent binding reaction. A firm bond is thus formed between the support and the affinant.

Bio-Gel P-300 is allowed to swell in water and is washed twice with a fourfold volume of 0.1 M phosphate buffer of pH 6.9. Then 19.4 ml of gel (1 g of dry beads per 45 ml) is mixed with glutaraldehyde solution (4.8 ml; 25 %, v/v) and incubated at 37°C for 17 h. The gel is washed and centrifuged four times with a fourfold volume of 0.1 M phosphate buffer of pH 6.9, then three times with 0.1 M phosphate buffer of pH 7.7. The coupling of the protein is carried out after mixing of 3 ml of gel in 13.5 ml of a buffer of pH 7.7 with 0.3 ml of protein solution (20 mg/ml) at 4°C for 18 h on a shaker. After the reaction the gel is centrifuged and washed. Using this method, 70 mg of acid phosphatase could be coupled per gram of dry gel.

6-Aminohexyl-Sepharose 4B was activated with glutaraldehyde by Cambiaso et al. (1975) and used for immobilization of immunoglobulins G and A, ferritin and albumin.

The aminated gel was first washed with ten volumes of phosphate buffer (pH 8.5) on a sintered-glass filter. To 3 ml of packed gel 7 ml of the same buffer containing one ml of 25 % glutaraldehyde was added under stirring (final concentration: 2.5 %). This represents a 100-fold molar excess of glutaraldehyde over the amino groups of the aminated gel (8 -μmole NH_2/ml packed gel). A yellow-green colour soon developed after this addition. The reaction was allowed to proceed for ten minutes at room temperature ($\pm 22^{\circ}C$), after which the unreacted glutaraldehyde was eliminated by washing with five times 20 ml of buffer. Time was not found to play an important role at this stage. To 3 ml packed activated gel was added, under stirring, 7 ml of the protein solution in the same buffer as used for activation. The reaction was usually allowed to proceed at room temperature for fifteen minutes. After the incubation period, the gel was washed with five times 20 ml of buffer to allow the non-conjugated proteins to diffuse out of the gel beads. A further washing with 10 ml of 3 M ammonium thiocyanate was performed to remove non-covalently bound proteins. The absolute protein content of both washings was estimated and subtracted from the amount of protein applied, in order to obtain the amount coupled per ml of packed gel.

When a moderate excess of protein (about 20%) was applied to the activated gel, it was not necessary to attempt to block possibly unreacted aldehyde groups of the gel. However, when smaller amounts of protein were coupled, the gel was further incubated for 16 hr at $4^{\circ}C$ with 0.2 M glycine buffer pH 8.5 or with 0.2 M ethanolamine at pH 9.0. Such incubations were actually found to block unreacted aldehyde groups.

Yang et al. (1981) used glutaraldehyde to couple acetylcholine receptor to partially hydrolysed nylon tubes. A support based on nonporous silicon dioxide of particle size 0.01 to 0.1 μm, modified by 3-(amino)-propyltriethoxysilane and activated by glutaraldehyde, was employed for the immobilization of concanavalin A, immunoglobulins, basic pancreatic trypsin inhibitor and trypsin by Fusek et al. (1987). Activation of supports by glutaraldehyde for cell immobilization by covalent linkage was used by Jirků and Turková (1987). Suitable supports were glycyl, β-alanyl or ε-aminocaproyl derivatives of Separon with attached 1,6-diaminohexane.

A number of competing mechanisms have been proposed to describe the reaction between the aldehyde and the amino function of the ligand (Ford and Pesce, 1981). The most plausible one is that reported by Monsan et al. (1975) who presented evidence showing that glutaraldehyde polymerizes to an unsaturated aldehyde. The latter subsequently reacts with amines to form α,βunsaturated imines which undergo resonance stabilization with the neighbouring ethylene function. Richards and Knowles (1968), on the other hand, suggested that the amine undergoes addition to the ethylene double bond of the unsaturated aldehyde to form a Michael adduct. None of the mechanisms proposed fully explains all the data on the coupling products of glutaraldehyde and polypeptides. The products actually formed depend strongly on the pH of the reaction and on the initial ratio and character of the reactants. In addition, competing reaction mechanisms lead to different products. But in all cases the resulting bonds can be expected to be chemically very stable. However, Fusek et al. (1988) described comparison of chymotrypsin immobilized to AH-Sepharose by use of glutaraldehyde or noncovalently by biospecific sorption to Sepharose with attached anti-chymotrypsin IgG. In the latter case of immobilization they obtained 10 times more proteolytic activity. The advantage of this oriented immobilization is discussed in Section 10.3.

A number of other bifunctional derivatives, summarized by Lowe and Dean (1974), are listed in Table 6.I.

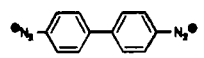
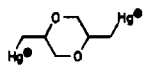
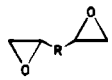
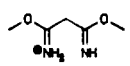
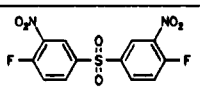
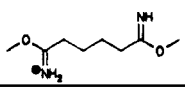
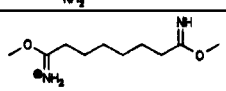
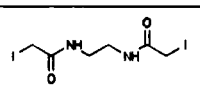
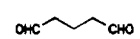
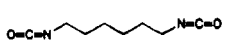
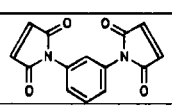
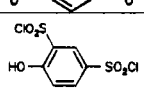
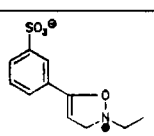
However, when using bifunctional derivatives it should be born in mind that side reactions may occur, such as crosslinking of the support, and permeability may decrease drastically (Mohr and Pommerening, 1985).

6.2.5 Cyanogen bromide activation

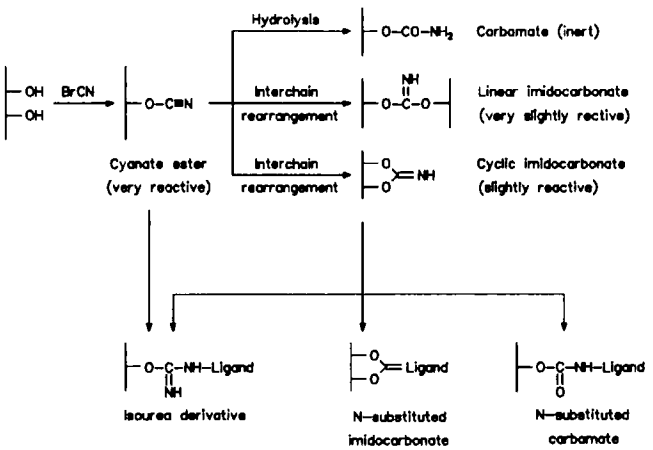
A widely used activation procedure for agarose, dextran, but less commonly of cellulose and hydroxyalkyl methacrylate gel, employs cyanogen bromide. This method was developed by Porath et al. (1967) and Axén et al. (1967). The mechanism of activation by CNBr and the subsequent coupling of the affinant was elucidated by Kohn and Wilcheck (1981):

Table 6.1.

Bifunctional reagents

REAGENT	FORMULA	REACTION
Bisazobenzidine		Phenol. OH–
3,6–Bis(mercurimethyl)–dioxane		HS–
Bisoxiranes		–OH to NH_2
Diethyl malonimidate		$-NH_2$
p,p'–Difluoro–m,m'–di–nitrophenyl sulphone		$-NH_2$ Phenol. –OH
Dimethyl adipimidate		$-NH_2$
Dimethyl suberimidate		$-NH_2$
Divinyl sulphone		–OH
N,N'–Ethylenebisiodo–acetamide		–SH
Glutaraldehyde		$-NH_2$
Hexamethylene diisocyanate		$-NH_2$
N,N'–(1,3–Phenylene)–bismaleimide		–SH
Phenol–2,4–disulphanyl chloride		$-NH_2$
Woodword's K		–COOH to $-NH_2$

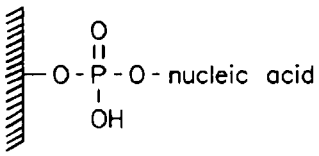
The same authors developed analytical methods for the determination of cyanate esters and imidocarbonates. Cyanate esters are most stable in acid, decreasing in



stability as the pH is raised. Imidocarbonates behave in an opposite fashion: they are most stable in 0.1 M NaOH. During a brief acid hydrolysis of an activated matrix all imidocarbonates will be removed quantitatively, while over 95% of the cyanate esters will remain intact. On the other hand, a brief hydrolysis in 0.1 M NaOH leads to a rapid hydrolysis of all cyanate esters, leaving a substantial amount of imidocarbonates. Based on the assumption that the coupling of an affinant to cyanate esters and to imidocarbonates occurs simultaneously and independently, the total coupling capacity of an activated support can be predicted according to the equation:

Total coupling capacity = 0.80 (cyanate esters) + 0.15 (imidocarbonates)

The initially formed cyanate esters are stable on Sepharose, but rearrange to cyclic imidocarbonates on Sephadex. The coupling of an affinant occurs predominantly *via* the free amino groups. The amine reacts in the unprotonated state. The following alkalinities are appropriate for coupling: aliphatic amines, pH ~ 10; amino acids, pH ~ 9; aromatic amines, pH 7 - 8. Ribonucleotides are fixed at pH 6, probably by an intramolecular rearrangement (Kempf et al., 1978) to give



Alkali-treated lipopolysaccharide from *Brucella abortus* coupled to agarose beads by acetonitrile-activated CNBr resulted in an immunoadsorbent with which a large number of specific antibodies could be purified. This method, described by Stiller and Nielsen (1983), gave alkali-treated lipopolysaccharide binding efficiencies of up to 98 %.

The attachment of amines to cyanogen bromide-activated Sepharose produces N-substituted isourea, which is capable of protonation in the neutral and alkaline region. In the presence of primary amines and ammonia this linkage is unstable (Wilchek et al., 1975). This is the primary flaw of this method when it is used for a single attachment.

Ikuta et al. (1981) described the cytotoxicity of a human α_1-antitrypsin preparation purified using concanavalin A (Con A) bound to CNBr-activated Sepharose. Con A was found in the effluents from the Con A-Sepharose column, even after extensive washing with sodium phosphate buffer. The amount of Con A in the effluents was sufficient to induce blastoid transformation of the lymphocytes, and was cytotoxic against lymphocytes when the effluents were concentrated.

A review of cyano-based activation procedures for polysaccharides was published by Kohn and Wilchek (1984). They classified these procedures into three major categories:

(1) Conventional procedures, which employ CNBr as activating agent and a strong inorganic base as reaction catalyst; (2) cyano-transfer procedures and (3) novel cyanylating agents. Cyanogen bromide activation of agarose at neutral pH was developed by Kohn and Wilchek (1982) using triethylamine as a "cyano-transfer" reagent. This procedure requires only about 5% of the usual amount of CNBr. Activated resins are free of interfering nitrogen derivatives, containing only active cyanate esters. Cyanylating agents used instead of CNBr (p-nitrophenylcyanate by Kohn et al., 1983; 1-cyano-4-dimethylamino pyridinium tetrafluoroborate by Kohn and Wilchek, 1983) yield resins that are identical with (or at least similar to) the commonly employed CNBr-activated resins, thereby avoiding the use of the hazardous CNBr itself. Discussions of the

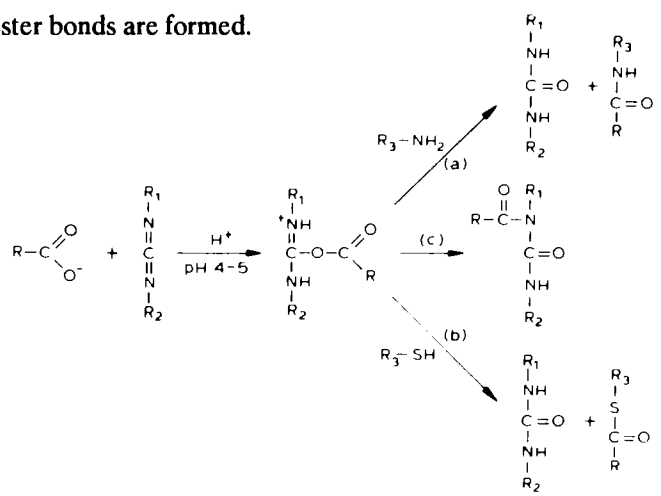
mechanism for CNBr-activation and the difference between a freshly activated and commercially available CNBr-activated Sepharose (which is treated with acid in order to achieve better stabilization of activated resin) have been published by Wilchek and Miron (1986). CNBr-activated Sepharose was treated with 1N hydrochloric acid for 1h in order to form carbonate. This new type of activated Sepharose can be used to couple amino-containing ligands to the resin, yielding columns that consist of stable and uncharged carbonate groups. These activated resins will mainly be useful for coupling of low-molecular-weight ligands such as diamino-hexane or aminocaproic acid, as the coupling to the resin has to be performed at high pH (ca. 9.5), owing to the low activity of the carbonate formed. The derivatized Sepharose can be further used to couple proteins under mild conditions and in high yields.

6.2.6 Coupling with condensation agents

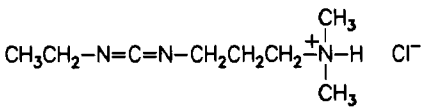
As it is evident from Table 9.1, one of the most frequent combinations of gel and spacer, used for the binding of low-molecular-weight affinity ligands, is Sepharose with attached hexamethylenediamine (trade-name AH-Sepharose) or ε-aminocaproic acid (trade-name CH-Sepharose). In order to bind them with affinants carrying primary aliphatic or aromatic amine or carboxyl groups, the condensation reaction with a carbodiimide-promoted method is used. The scheme of this reaction is as follows

(a) A nucleophilic attack gives an acyl-nucleophile product and the urea of the corresponding carbodiimide.

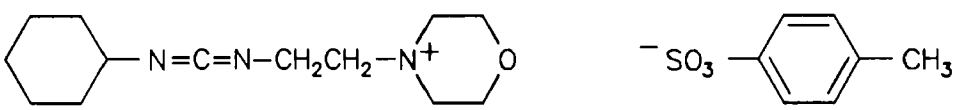
(b) Thiol ester bonds are formed.



(c) The O-acylisourea intermediate is converted into N-acylurea by an intramolecular acyl shift. The binding reaction between the carboxyl group and the nucleophile can be almost quantitative in the presence of excess of carbodiimide and the nucleophilic reagent such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC):



and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulphonate (CMC):



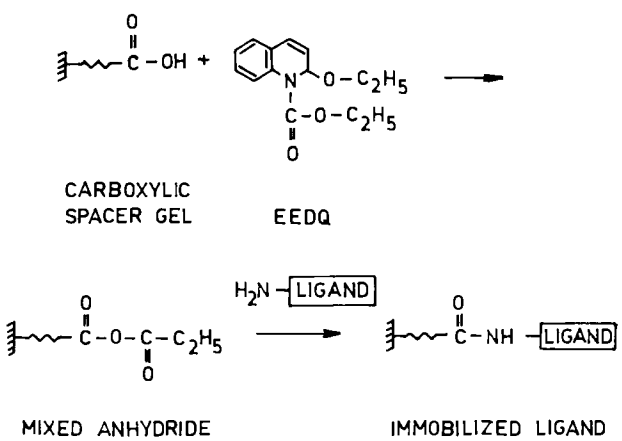
Their main advantage is that their corresponding urea derivatives are soluble in water and they can therefore be easily eliminated from the gel by washing with water. The pH range used for the carbodiimide condensation is 4.7 - 6.5, according to Lowe and Dean (1974), and the reaction time is 1.5 - 72 h at a carbodiimide concentration of 2 - 100 mg/ml. The disadvantage is that carbodiimides are relatively unstable compounds and must be handled with care because of their toxicity.

As an example, the preparation of estradiol-Sepharose (Cuatrecasas, 1970) is described below.

A 300-mg amount of 3-O-succinyl-[^{3}H]estradiol dissolved in 400 ml of packed aminoethyl-Sepharose 4B. The dimethylformamide is needed in order to solubilize the estradiol, and it is not required for affinants that are soluble in water. The suspension is maintained at pH 4.7 with 1 M hydrochloric acid. Then 500 mg (2.6 mmole) of 1-ethyl-3-(dimethylaminopropyl)carbodiimide, dissolved in 3 ml of water, is added to the suspension over 5 min and the reaction is allowed to proceed at room temperature for 20 h. Substituted Sepharose, after being transferred into the column, is washed with

50% aqueous dimethylformamide until the eluate is no longer radioactive. It is recommended that the derivative should be washed with about 10 l of the washing liquid over 3 - 5 days. Using this procedure, about 0.5 μmole of estradiol can be bound per millilitre of packed Sepharose. The same soluble carbodiimide was used for coupling of pepsin to NH_2-Spheron and COOH-Spheron (Turková, 1976) or to wideporous and nonporous ω-amino-alkylderivatized inorganic supports (Ivanov et al., 1990).

Boschetti et al. (1978) published a comparative study of soluble carbodiimide CMC with a condensation agent: N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). They used EEDQ for the preparation of a biospecific adsorbent for chymotrypsin by coupling D-tryptophan methyl ester to ε-aminocaproyl-Ultrogel AcA-34. The scheme of the reaction by use of EEDQ is as follows:

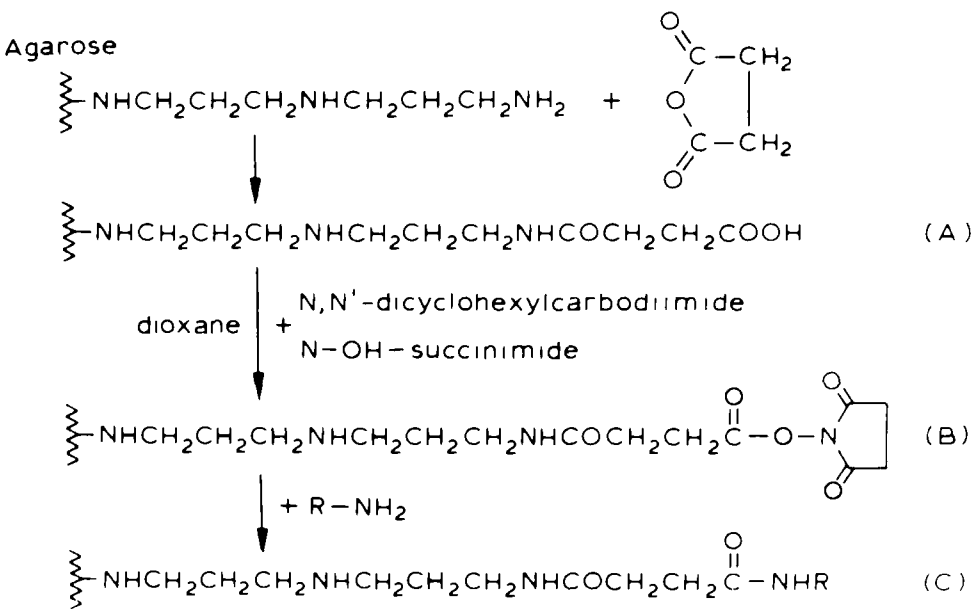


EEDQ catalyzes the reaction between spacer arm and ligand by forming a mixed anhydride on the carboxyl group of the spacer arm. The mixed anhydride then reacts with the complementary amine to form a stable amide bond. The mixed anhydride can also react with other nucleophilic groups such as sulphydryl and hydroxyl groups (Pougeois et al., 1978). EEDQ must be used in a water-ethanol mixture, which is an advantage when working with ligands that are only slightly soluble in water. The total capacity of each gel for chymotrypsin adsorption, after saturation of the gels with enzyme, was 5.2 - 13.7 mg per ml of EEDQ gel and 3.5 - 4.3 mg per ml of CMC gel. These results showed that EEDQ is a suitable condensation agent, being stable,

non-toxic and cheap. Coupling of testosterone hemisuccinate on AH-Sepharose 4B with EEDQ has been studied by Maingault et al. (1984).

6.2.7 Active esters

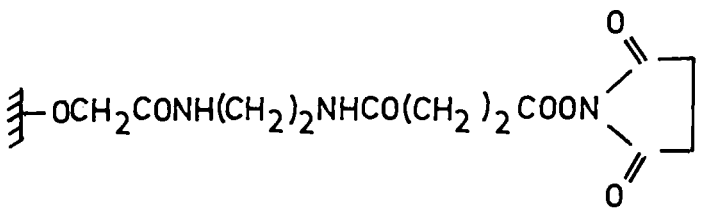
Cuatrecasas and Parikh (1972) described the preparation of N-hydroxysuccinimide (NHS) esters of succinylated aminoalkyl agarose derivatives. These active ester derivatives of agarose, when stored in dioxane, are stable for several months. These derivatives very rapidly form stable amide bonds (at 4°C) with non-protonated forms of primary aliphatic or aromatic amino groups at pH 6 - 9. Among the functional groups of amino acids tested, only sulphydryl groups compete effectively with the amino groups during the binding reaction. The reaction takes place according to the following scheme:



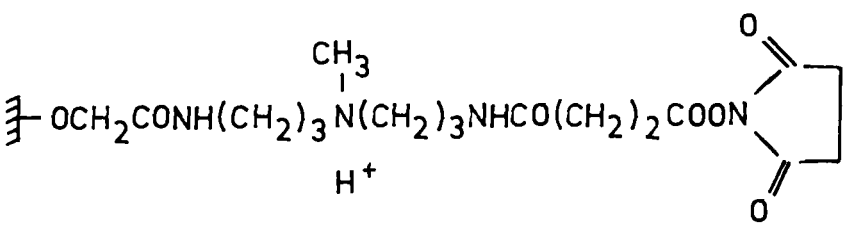
Diaminodipropylaminoagarose is treated with succinic anhydride in saturated sodium borate buffer to obtain the corresponding succinylated derivative (A). The latter is made to react with N,N'-dicyclohexylcarbodiimide and N-hydroxysuccinimide in dioxane to yield the active agarose ester (B). After removing dicyclohexylurea and the unreacted reagents (dioxane and methanol washes), the active ester of agarose is subjected to

reaction in aqueous medium with ligands or proteins to yield stable amide-linked derivatives (C).

Using the esterification of the carboxyl groups of CH-Sepharose 4B (i.e. Sepharose to which ε-aminocaproic acid is bound after activation with cyanogen bromide) with the application of N-hydroxysuccinimide, Pharmacia (Uppsala, Sweden) produces "activated CH-Sepharose 4B". The pH range suitable for binding on this derivative is indicated by Pharmacia to be 5 - 10, with an optimum of pH 8. The advantage of lower pH values consists in the decreased hydrolysis of esters but, on the other hand, the reaction is slower. For binding, buffers that contain amino acids cannot be used (Tris or glycine buffers). Agarose derivatives containing N-hydroxysuccinimide ester have been introduced by Bio-Rad Labs. (Richmond, Calif., U.S.A.) under the name Affi-Gel 10:



and Affi-Gel 15:



As shown in Figure 6.11 Affi-gel 10 couples proteins best at a pH near or below their isoelectric point, and Affi-Gel 15 couples proteins best near or above their isoelectric point. Therefore, when coupling at neutral pH (6.5 - 7.5), Affi-Gel 10 is recommended for proteins with isoelectric points of 6.5 to 11 (neutral or basic proteins) and Affi-Gel 15 is recommended for proteins with isoelectric points below 6.5 (acidic proteins). The difference in coupling efficiency of Affi-Gel 10 and Affi-Gel 15 for acidic and basic proteins can be attributed to interactions between the charge on the protein and charge

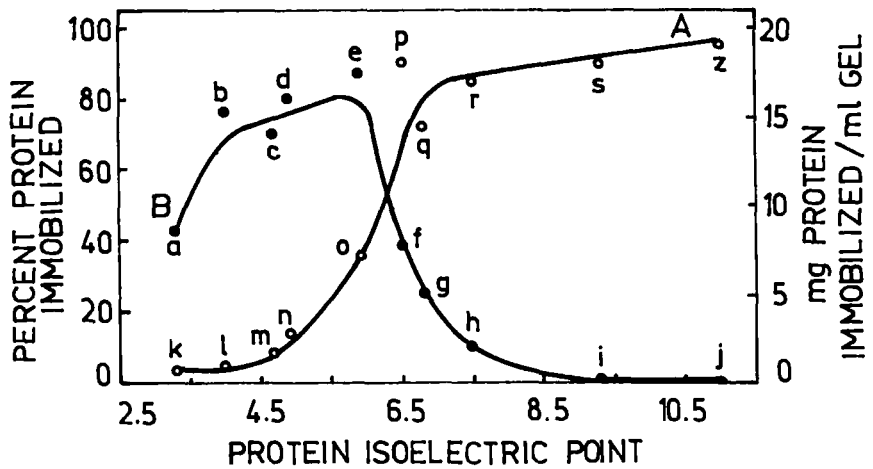


Fig. 6.11. Immobilization of proteins to Affi-Gel 10 and Affi-Gel 15. Protein solutions were gently mixed at 0 - 4°C with 2 ml Affi-Gel 10 or Affi-Gel 15 for 2h. The reaction was terminated by addition of ethanolamine, pH 8.0, to a concentration of 0.10 M, and after 30 min, transfered to a 1 x 10 cm chromatography column. Unreacted protein was eluted with 7 M urea containing 1 M NaCl. Published values for the isoelectric points used to construct this figure were: fetuin (a,k), pH 3.3; human α-antitrypsin (b,l), pH 4.0; human γ-globulin (f, p), pH 5.8 - 7.3; human transferrin (e, o), pH 5.9; ovalbumin (c, m), pH 4.7; bovine serum albumin (d, n), pH 4.9; bovine hemoglobin (g, q); pH 6.8; equine myoglobin (h,r), pH 6.8 - 7.8; cytochrome c (i, s), pH 9.0 - 9.4; and lysozyme (j, z), pH 11.0. (o) Affi-Gel 10; (●) Affi-Gel 15. Reproduced with permission from R. G. Frost et al., Biochim. Biophys. Acta, 670 (1981) 163 - 169.

on the gel. Hydrolysis of some of the active esters during aqueous coupling will impart a slight negative charge to Affi-Gel 10. This negative charge will attract positively charged proteins (proteins buffered at a pH below their isoelectric point) and enhance their coupling efficiency. Conversely, the negative charge will repel negatively charged proteins (proteins buffered at a pH above their isoelectric point) and lower their coupling efficiency. Affi-Gel 15, due to the tertiary amine incorporated into its arm, has a slight overall positive charge, and the effects are reversed. Coupling under anhydrous conditions is the preferred method when this is suitable for the ligand. Since there is no hydrolysis of active esters in the absence of water, the only reaction will be that of the ligand with the gel.

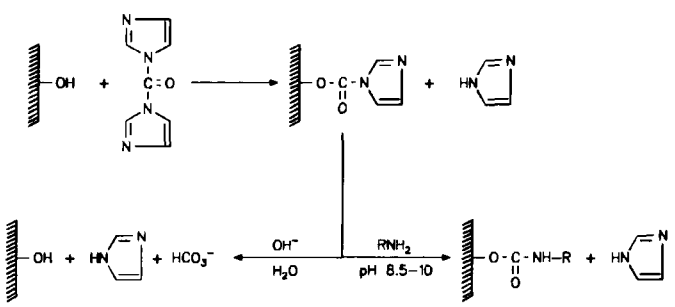
Many applications of Affi-Gel 10 and 15 are shown in Table 9.1. Garcia and Singhal (1979) have shown that the extent of interaction between the bioaffinity matrix - agarose

with attached castor bean lectin and tyrosine transfer RNA is highly dependent upon the length of the spacer arm. Their results indicate that a spacer arm length of 10 Å between the agarose bead (Affi-Gel 10) and the lectin gives the best separation. However, Wilchek and Miron (1987), in their study involving the purification of different lymphokinases by immunoaffinity chromatography, coupled several antibodies to commercially available, spacer-containing polymeric NHS esters (Affi-Gel 10, Affi-Gel 15, activated CH-Sepharose). They found a substantial amount of leakage, which characterized all of the above mentioned resins. To overcome this problem the use of p-nitrophenyl esters was recommended in place of NHS esters (Wilchek and Miron, 1987).

A p-nitrophenol ester of hydroxyalkyl-methacrylate gels (NPAC) was described by Turková (1976) and Čoupek et al. (1977). Procedures for the binding of heterocyclic ligands, such as imidazole were developed by Kühn et al. (1981) on the basis of reactive sulphoester groups. These methods are well suited to the introduction of imidazole ligands *via* the annular nitrogen in position 1.

6.2.8 Activation with carbonylating reagents

Activation of crosslinked agarose with 1,1' -carbonyldiimidazole (CDI) has been described by Bethell et al. (1979, 1981). The reaction scheme for the covalent coupling of ligands to OH containg solid supports is as follows:



The highly activated imidazolylcarbamate supports react with primary amino groups at pH 8.5 - 10.0 and are more stable to hydrolysis in comparison with N-hydro-

xysuccinimide ester-activated supports (Mohr and Pommering, 1985). The CDI-activated agarose was found to have a half-life of more than fourteen weeks when stored in dioxane. A further advantage of this method is that the N-alkylcarbamates obtained are uncharged in the pH range normally used.

The CDI-activation of agarose has been performed by Bethell et al. (1981) for 15 - 30 min in dioxane, acetone, or dimethylformamide at room temperature. Walters (1982) used CDI-activation of dry diol-bonded silica in dioxane for the study of pore-size effects in high-performance bioaffinity chromatography of concanavalin A on columns with immobilized glucosamine. Optimization of protein immobilization on CDI-activated diol-bonded silica has been described by Crowley et al., (1986). In their work acetonitrile was used as the solvent in CDI-activation, since better coupling yields were obtained. It was found that extensive sonication and vacuum-degassing during the activation step significantly increased the amount of ligand coupled. In the coupling step, it was found that a subpopulation of active groups was resistant to hydrolysis and resulted in increased coupling yields of ligand over a six-day reaction time. The coupling and hydrolysis reactions were more rapid in carbonate buffer than in phosphate buffer, but the overall yields were the same. Coupling yields of several proteins (bovine serum albumin, immunoglobulin G, soybean trypsin inhibitor, Protein A, acetylcholinesterase and horse liver alcohol dehydrogenase) were found to be relatively insensitive to pH over the range 4 - 8. In the same year, a paper on the evaluation of CDI-activation and ligand coupling effects with different support materials (Sepharose CL-6B, Fractogel HW-65, Trisacryl GF-200, Li Chrospher Si 500 diol I and II) was published by Hearn (1986). The preparation and properties of a variety of different immobilized-ligand chromatographic supports were described as a prelude to an examination of the importance of matrix and CDI-activation effects on zone broadening or multizoning phenomena in bioaffinity chromatography. Hearn and Davies (1990) used antilysozyme monoclonal antibody attached to CDI-activated Fractogel HW-65F and Trisacryl GF-2000 and lysozyme systems as model studies for the evaluation of factors which affect column performance. The data obtained from the study of the influence of buffer conditions on immobilization efficiency showed that a maximum amount of bovine

γ-globulin was coupled to CDI-activated supports at pH 7.0 by the use of 0.1 M sodium phosphate.

Optimizations of conditions for the coupling of ligands to primary hydroxyl-containing silica for high-performance affinity chromatography were described by Ernst-Cabrera and Wilchek (1987). The most frequently used silica was prepared by silanization with 3-glycidoxypropyltrimethoxysilane under anhydrous conditions, as described by Larsson et al. (1983). Hydrolysis of 3-glycidoxypropyl-silica with acid resulted in diol silica, which was then oxidized to yield aldehyde silica. Subsequent reduction led to the production of hydroxyethyloxypropyl-silica containing primary hydroxyl groups. The degree of activation of this derivative of silica and the coupling capacities generated by various activating agents are given in Table 6.2. Although 1,1' -carbodiimidazole appeared to give high levels of activation, the coupling yield of trypsin was similar to that achieved with p-nitrophenyl chloroformate-activated silica, indicating that the p-nitrophenyl carbonate has a higher activity towards nucleophiles and is less hydrolysable. Coupling of trypsin to the activated carriers was achieved by using of 100 mg of activated silica suspended in 5 ml of 0.1 M phosphate buffer (pH 7) with the addition of 10 mg trypsin. The suspension was then gently shaken or stirred at 4°C for 2 days. The resultant silica was washed with 0.1 M phosphate buffer. In order to determine the optimum coupling conditions Ernst-Cabrera and Wilchek (1987) coupled trypsin and bovine serum albumin (BSA) to p-nitrophenyl chloroformate-activated resins under various pH conditions. The maximum amount of coupled trypsin was obtained between pH 6 and 7; the amount of coupled BSA was relatively constant between pH 4 and 6, with a maximum at pH 6. A significant reduction in coupling capacities was observed at higher pH. These results are in agreement with those reported by Crowley et al. (1986), who showed that the efficient coupling of many proteins on carbodiimidazole-activated resins takes place at pH 4 - 5. Both results clearly indicate that it is preferable not to use basic conditions for coupling ligands to activated carbonates. CDI-activated glass matrices are commercially available from Pierce (Rockford, USA).

Table 6.2.

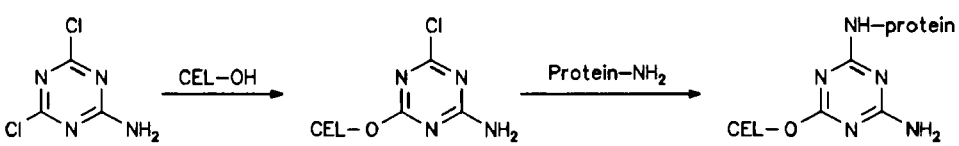
Activation of hydroxyethyloxypropyl-silica of LiChrosorb Si 60 with different reagents: determination of active groups and coupled trypsin

Activating reagent	*Activated groups (μmol/g)*	*Coupled trypsin (mg/g)*
p-Nitrophenyl chloroformate	282	50.5
N-Hydroxysuccinimide chloroformate	320	47.1
1,1,1-Trichloro-2-monochloroethyl-succinimide carbonate	129	33.8
1,1'-Carbodiimidazole	480	51.5
p-Toluenesulphonyl chloride	363	14.6

6.2.9 Triazine method

The covalent linkage of ligands to hydroxyl-containing supports using their activation with cyanuric chloride (2,4,6-trichloro-s-triazine) was developed by Kay and Crook (1967). Kay and Lilly (1970) used the triazine method of protein binding with derivative triazine, in which a chlorine was replaced by a solubilizing NH_2 group. 2-Amino-4,6-dichloro-s-triazine is bound to the hydroxyl group of cellulose and reacts further with the amino group of the protein:

2-Amino-4,6-dichloro-s-triazine was prepared from cyanuric acid and then allowed to react with various polysaccharide carriers, such as cellulose, DEAE-cellulose, CM-



cellulose and also Sephadex and Sepharose. Solution A was prepared by dissolution of 10 g of 2-amino-4,6-dichloro-s-triazine in 250 ml of acetone at 50°C with addition of 250 ml of water at the same temperature. Solution B was a 15% (w/v) aqueous solution of sodium carbonate to which a 0.6-fold volume of 1 M hydrochloric acid was added. Cellulose (20 g) or CM-cellulose was then added to 100 ml of solution A and the mixture stirred at 50°C for 5 min. After addition of 40 ml of solution B, the stirring was continued

at 50°C for a further 5 min. The pH of the suspension was rapidly decreased to below 7 by addition of concentrated hydrochloric acid. After filtration, the modified cellulose was washed with a mixture of acetone and water, pure water and finally 0.1 M phosphate buffer of pH 6.7, in which it was also stored. The binding of approximately 1.5 % chymotrypsin solution in 0.5 M borate buffer of pH 8.75 at 23°C after 4.5 h, gave a product containing 19 mg of enzyme per gram, with an enzyme specific activity retention of 70 %.

Activation of agarose or filter paper with trichloro-s-triazine and coupling of ethylenediamine to triazine-activated agarose or of m-aminophenylboronic acid to triazine-activated paper was described by Longstaff (1985). Macroporous cellulose beads activated with 2,4,6-trichloro-1,3,5 triazine (TCT) were used after the attachment of the cyclic nonapeptide bacitracin as an efficient biospecific adsorbent for the isolation of proteolytic enzyme subtilisin from culture medium of *Bacillus subtilis* (Turková et al., 1987). The same activation of bead cellulose was used for the coupling of adipic acid dihydrazide (Turková et al., 1990). Recently, a new, easily controllable variant of the activation of bead macroporous cellulose with TCT has been developed by Beneš et al. (1991). Activation: 10 g of suction-filtered cellulose (water content 86%) was stirred with 20 ml of acetone for 30 min, 10 ml of aqueous acetone was withdrawn after the stirring had been interrupted, 10 ml of acetone was added, stirring was continued for another 30 min, after which another 10 ml of aqueous acetone was withdrawn. An alkali solution was added (10 % NaOH or 20 % K_2CO_3) and the stirring continued for one hour, the solution was cooled to 0°C and a solution of TCT (1 mole per one equivalent of alkali) in acetone (5.5 ml/g) was added and left to react for 45 min. The product was washed in a column with 40 ml of acetone cooled to 0°C and 40 ml of ice-cold water. Preparation of the hydrazide derivative: to a solution of 233 mg of adipic acid dihydrazide in 17.5 ml 0.05 M borate buffer, pH9, 20 g cellulose activated with TCT was added. The mixture was stirred for 4 h at room temperature and a constant pH of 9, maintained by addition of NaOH. The product was washed in a column with five volumes of distilled water. The final content of dihydrazide was 13.3 μmol/ml. The disadvantage of this method is that the activating reagents are highly toxic.

6.2.10 Reversible covalent immobilization of proteins by thiol-disulphide interaction

Carlsson et al. (1975) employed epoxide-activated agarose as the basis for the preparation of the mercaptohydroxypropyl ether of agarose gel, which they used for covalent immobilization of α-amylase and chymotrypsin by thiol-disulphide interchange. This technique consists of two steps: (a) thiolation of enzymes with methyl 3-mercaptopropiomidate; (b) binding of thiolated enzymes to a mixed disulphide derivative of agarose obtained by reaction of the mercaptohydroxypropyl ether of agarose with 2,2 -dipyridyl disulphide. The immobilized preparations formed possessed high activity. Immobilized α-amylase was used for continuous hydrolysis of starch. When the preparation had lost its enzymatic activity, the inactive protein was reduced off and the gel used for the binding of a new active thiolated α-amylase.

The thiol enrichment of enzyme was carried out in the following manner: 30 mg of enzyme was dissolved in 5 ml of 0.1 M sodium hydrogen carbonate solution, pH 8.2. The solution was deaerated in a nitrogen atmosphere for 15 min and 0.1 - 2 mg of methyl β-mercaptopropioimidate was added. The thiolation was carried out at room temperature under nitrogen for 60 min. Excess of imidate was eliminated by gel filtration on Sephadex G-25, using 0.1 M sodium hydrogen carbonate solution as eluting reagent. In order to prevent the oxidation of thiolated enzymes, dithiothreitol (1 mM final concentration) was added to the solution just prior to the gel filtration.

Activated thiol-Sepharose was prepared according to Brocklehurst et al. (1973). Epoxide-activated agarose (50 g) was washed on a glass filter with 0.5 M phosphate buffer (4.1 g of $NaH_2PO_4.H_2O$ + 2.8 g $Na_2HPO_4 . 2H_2O$ dissolved in 100 ml of distilled water, pH 6.3). Interstitial buffer was removed from the gel by suction, and the gel was resuspended in the same buffer to a final volume of 100 ml. A 2 M solution of sodium thiosulphate (50 ml) was added and the reaction mixture was shaken for 6 h at room temperature. The gel was washed free of sodium thiosulphate with distilled water. The thiosulphate ester gel (50 g) was suspended in 0.1 M sodium hydrogen carbonate solution (1mM EDTA) to a total volume of 100 ml. Dithiothreitol (60 mg) dissolved in 4 ml 1mM EDTA solution was added to the suspension. The reaction time was 30 min

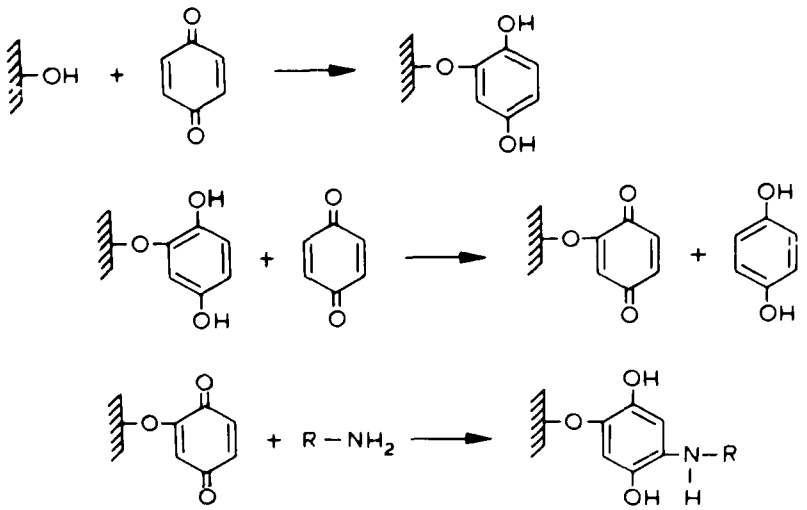
at room temperature. The gel was washed on a glass filter with 0.1 M sodium hydrogen carbonate solution (1 M in sodium chloride and 1mM in EDTA) and finally with 1mM EDTA solution. The thiol-agarose (50 g) was washed on a glass filter with 1mM EDTA solution. The washed gel was rapidly added to 200 ml of 2.2 '-dipyridyl disulphide solution (1.5 mM in 0.1 M sodium hydrogen carbonate solution). The mixture was stirred during the reaction, which was allowed to proceed for 30 min at room temperature. The product was washed with 0.1 M sodium hydrogen carbonate solution, 1M sodium chloride solution and finally with 1mM EDTA solution. The degree of substitution was determined by nitrogen determination according to Kjeldahl. The product, called activated thiol-agarose, is stable to storage.

The binding of the thiolated enzyme was carried out in the following manner: 1 - 20 mg of thiolated enzyme in 10 ml of 0.1 M sodium hydrogen carbonate solution were mixed with 3.0 ml of sedimented activated thiol-agarose (pre-washed with 0.1 M sodium hydrogen carbonate solution) and allowed to react at 23°C for 24 h under constant stirring. The conjugate was washed on a sintered-glass funnel with 100 ml of 0.1 M sodium hydrogen carbonate solution, transferred into a column and washed at the rate of 10 ml/h with the following solutions: (1) 0.1 M sodium hydrogen carbonate solution containing 0.2 M sodium chloride (24 h); (2) 0.1 M sodium acetate solution of pH 5.4, containing 0.2 M sodium chloride (24 h); (3) 0.2 M sodium chloride solution (24 h).

The inactivated enzyme was eliminated from the carrier in the column (0.5 ml) by washing with 50 ml of a 20 mM solution of dithiothreitol in 0.1 M sodium hydrogen carbonate solution at a flow-rate of 20 ml/h. The reduced carrier was washed with 150 ml of 1 M sodium chloride solution and activated by passing 100 ml of a 1.5 mM solution of 2,2 '-dipyridyl disulphide in 0.1 M sodium hydrogen carbonate solution. The activated thiol gel was washed with 100 ml of 1 M sodium chloride solution and 100 ml of 0.1 M sodium hydrogen carbonate solution and re-used for the immobilization of a new thiol system.

6.2.11 Benzoquinone activation

The mechanism of the activation of hydroxyl-containing solid supports by means of benzoquinone and coupling of NH_2-containing compounds was described by Brandt et al. (1975) in terms of the following series of reactions:



Benzoquinone is a very active reagent and, presumably due to the secondary reaction, the immobilized compounds are mostly strongly coloured. Brandt et al. (1975) determined the amount of bovine serum albumin and chymotrypsin coupled to benzoquinone-activated Sepharose 4B in relation to pH during the coupling reaction (Fig. 6.12). The same proteins were immobilized in relation to pH *via* benzoquinone on hydroxyalkyl methacrylate copolymer by Stambolieva and Turková (1980). Section 6.1 has already mentioned that the pH optima of the coupling of different proteins on the epoxy-containing methacrylate copolymer and epoxy-activated agarose are different (Fig. 6.2, A and B). Even the pH optima of the coupling of individual proteins on the different supports shown in these figures differ. By contrast, when serum albumin and chymotrypsin were coupled *via* benzoquinone, the pH optima were the same on methacrylate copolymer (Stambolieva and Turková, 1980) as on Sepharose, as shown in Fig. 6.12.

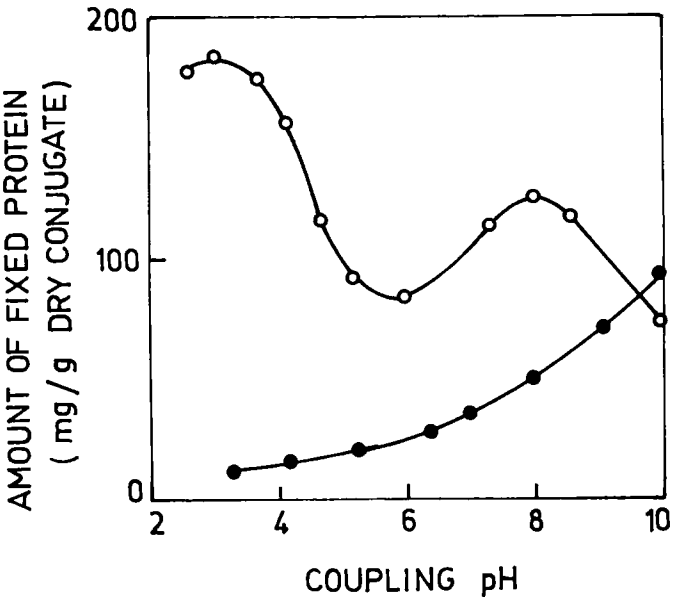
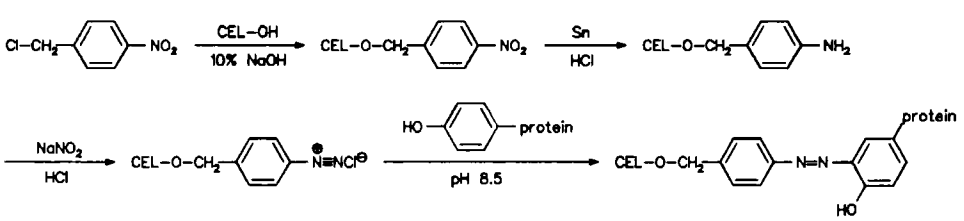


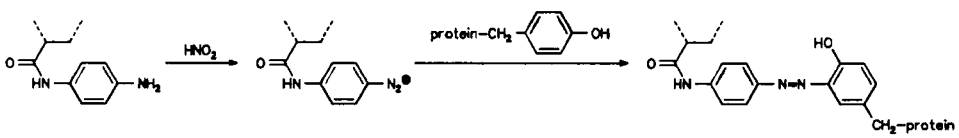
Fig. 6.12. Amount of bovine serum albumin and chymotrypsin fixed to Sepharose 4B gel as a function of pH during the coupling reaction. o, bovine serum albumin; ●, chymotrypsin. Reproduced with permission from J. Brandt et al., Biochim. Biophys. Acta, 386 (1975) 196 - 202.

6.2.12 Diazotization

The first attachment of an affinant to cellulose was carried out by means of diazonium groups (Campbell et al., 1951):



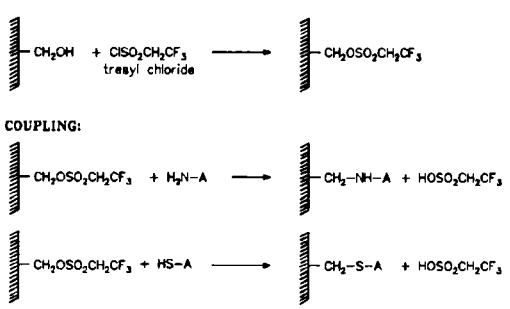
The affinants are bound by their aromatic residues (in the case of proteins mainly by tyrosine and histidine), but also non-specifically and more slowly by their amino groups (Gundlach et al., 1962; Tabachnik and Sobotka, 1960; Paik et al., 1979). Although this method is not frequently used at present, it offers two essential advantages: (a) the bound ligand can easily be split off by complete reduction; (b) this reductive cleavage allows intact protein-inhibitor conjugates to be isolated under mild conditions (Mohr and Pommerening, 1985). The affinity ligands bound by an azo linkage can be detached with sodium dithionite. Polyacrylamide gels containing residues of aromatic amines, when diazotized with nitrous acid, bind affinants mainly through their aromatic residues:



The azo groups are relatively photolabile and unstable in both acidic and alkaline media.

6.2.13 Sulphonyl chloride-containing supports

A simple method for the activation of supports carrying hydroxyl groups, such as agarose, cellulose, diol-silica, glycophase-glass or hydroxyalkyl methacrylate copolymer, with 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride), was introduced by Nilsson and Mosbach (1981):

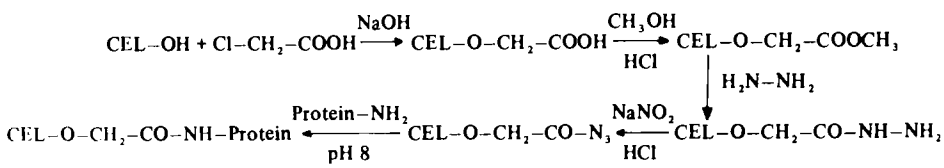


The advantage of tresyl chloride is that it is very reactive and it therefore allows efficient coupling under very mild conditions, and to a number of different supports, including those used for HPLC.

6.2.14 Other methods

The structure of the bifunctional derivative divinyl sulphone is shown in Table 6.1. Its advantages in the production of adsorbents were described by Porath and Sundberg (1972). The activation takes place under fairly mild conditions and in a short time. The coupling can not only be performed not only with amino-group containing molecules, but also with carbohydrates, phenols and alcohols, albeit at a higher pH. As a consequence divinyl sulphone crosslinking considerably improved the flow-through properties of agarose. The product coupled *via* divinyl sulphone is very stable at acidic and neutral pH, but it is labile under alkaline conditions.

The substances with a free amino group can be bound onto cellulose (CEL) by the Curtius azide method, used for the first time by Micheel and Ewers (1949) and later mostly applied using the modification by Hornby et al. (1966).



After the preparation of carboxymethylcellulose azide by Curtius rearrangement, an isocyanate is formed, to which the amino group of the affinant is bound.

The application of agarose activated with 2,4,6-trifluoro-5-chloropyrimidine to the synthesis of a biospecific adsorbent for trypsin was described by Gribnau et al. (1978). The availability of new supports activated with fluoromethylperidinium (FMP) has been published by Ngo (1986, 1988) and reported by Taylor (1991).

6.3 BLOCKING OF UNREACTED GROUPS AND WASHING OUT OF NONCOVALENTLY BOUND LIGANDS

When the attachment of the affinity ligand onto the solid support is terminated, it is necessary that the remaining active groups that are capable of coupling should be eliminated. Some of the complications encountered in the application of bioaffinity methods could be due to the neglect of deactivation (Egly and Porath, 1978). The coupling yield is never quantitative. When large affinants are being attached to the solid support, the concentration of excess reactive groups is particularly large. Further coupling can occur during the chromatographic procedure, which may result in the irreversible adsorption of the substance to be isolated (Eveleigh and Levy, 1977). The impurities coupled to the unreacted groups of a biospecific adsorbent can act as non-specific adsorption centres in the subsequent use of the adsorbent.

The problem of excess reactive groups is encountered in the use of all coupling methods. This problem can be solved by one of two methods.

(1) By coupling a highly penetrable substance with no effect on the adsorption-desorption procedure.

(2) By solvolysis. This method may be used when the affinants are stable in alkaline solution at elevated temperature; these conditions affect the hydrolytic removal of the activated groups. The excess epoxide groups may be hydrolysed to diols by treatment with 0.1 M perchloric acid (Turková et al., 1981) or with 10 mM HCl (Lowe et al., 1981).

In the methods that make use of the amino groups for the coupling of affinants, ethanolamine is most frequently used as an inactivating reagent. For example, according to the recommendation of Pharmacia for the binding to Sepharose by cyanogen-bromide activation, the inactivation of the remaining active groups can be effected simply by reaction with 1 M 2-aminoethanol at pH 9 and room temperature for 2 h, while, after binding to epoxide-activated Sepharose, 4 h are required under the same conditions. The buffer used is the same as during the binding, for example 0.1 M sodium hydrogen carbonate solution containing 0.5 M sodium chloride. Turková et al. (1981) employed the coupling of 2-aminoethanol to hydroxyalkyl methacrylate copolymer containing 1.7

mmol of attached epichlorohydrin per gram of dry Separon to check the completeness of coupling in addition to the determined epoxide groups. Fig. 6.13 shows the coupling of 2-aminoethanol in relation to pH and coupling time. The blocking of epoxide groups proceeded slowly and required a high pH. Practically complete removal of the epoxide groups was obtained by hydrolysis with 0.1 M perchloric acid.

In Eupergit C oxirane acrylic beads Fleminger et al. (1990 b) determined about 1.6 mmol of epoxide groups per gram of matrix by titration of the oxirane groups with thiosulphate. About half of these groups are readily available for reaction with amino or thio groups, whereas the other half is much less reactive and requires a long time (hours to days) to react. β-Mercaptoethanol was found to be an efficient blocker of residual oxirane groups. The comparison of blocking by ethanolamine and β-mercaptoethanol was published by Fleminger et al. (1990 a). Eupergit C beads modified with adipic acid dihydrazide (ADH) treated with 0.2M ethanolamine, pH 9.5, tended to adsorb proteins at pH 5.5, apparently owing to positive charges on the secondary amino groups formed by the reaction. In contrast, when β-mercaptoethanol was used

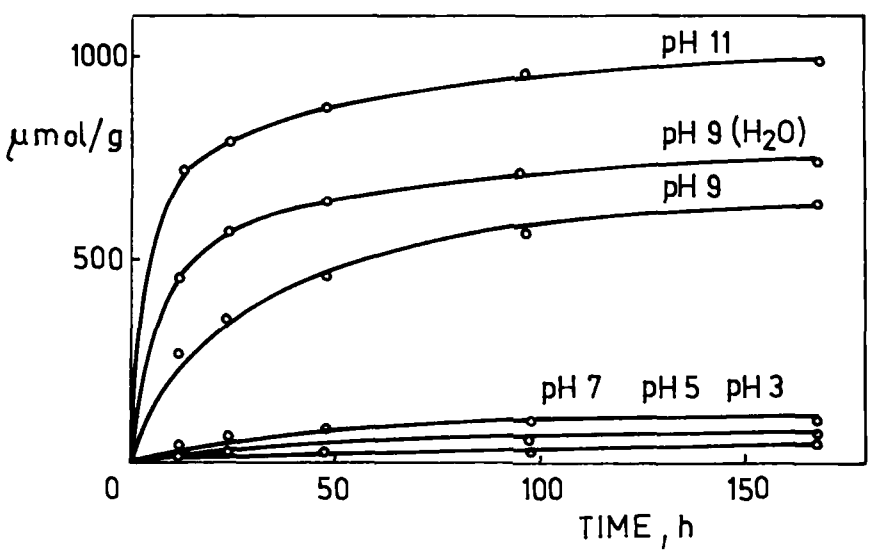


Fig. 6. 13. Coupling of 0.5 M 2-aminoethanol (in μmol/g dry conjugate) bound on Separon H 1 000 E_{max} as a function of coupling time and pH. Britton-Robinson buffers were used for pH 3, 5, 7, 9 and 11, with the exception of pH 9, where an aqueous solution was also used. Coupling performed at room temperature. Data from J. Turková et al., J. Chomatogr., 215 (1981) 165 - 179.

as a blocker, both protein adsorption at pH 5.5, as well as covalent protein binding to residual oxirane groups at pH 7.5, were markedly reduced. Consequently, the ADH-modified beads were routinely treated with 0.2M β-mercaptoethanol, pH 8.0, for 4 h at room temperature before protein binding. The blocking step eliminated most of the residual oxirane groups. The 10 % of the original oxirane groups that still seem to remain active even after the blocking step most probably represent groups that are remote from the outer surface of the beads and are apparently not available for protein binding.

β-Mercaptoethanol was also used to quench remaining activated groups in Sepharose 4B after its activation with CNBr and the coupling of RNA polymerase by Ratner as early as in 1974. For blocking, other low-molecular-weight molecules can also be used, such as glucosamine, glycine, 2-amino-2-hydroxymethyl-propane-1,3-diol, etc. The manufacturer of Sepharose states that, for the elimination of CNBr-activated groups from Sepharose, it is sufficient to suspend the gel in a Tris buffer of pH 8 for 2 h. The effect of the small number of charges introduced by the use of Tris or glycine can be overcome by using relatively high concentrations of salts during affinity chromatography. Almost complete elimination of these active groups can be achieved, however, even by merely standing the gel overnight in a mildly alkaline solution.

In gels that contain active ester groups for spontaneous covalent bonding (for example, activated CH-Sepharose, Affi-Gel 10 or hydroxyalkyl methacrylate gels with a p-nitrophenyl ester group), the unreacted groups are eliminated by addition of 0.1 M Tris buffer of pH 8. After standing for 1 h virtually no groups capable of binding proteins or peptides remain on the gel. In carriers that contain aldehyde groups as active binding groups after periodate oxidation, reduction with sodium borohydride may be used with advantage after the binding. This leads both to the reduction of the remaining aldehyde groups and to the stabilization of the bond between the protein and the solid matrix.

If the affinity ligand is bound onto a spacer, it may occur that a part of the spacer molecules - most commonly containing amino or carboxyl groups at their terminal - will remain unoccupied. The unreacted carboxyl groups on the extending arms were eliminated by Rafestin et al. (1974) by blocking with 0.5 mM Tris [(2-amino-2-hydro-

xymethylpropane-1,3-diol)] and with 1.6 mM N-cyclohexyl-N -[2-(4-morpholinyl)ethyl] carbodiimide metho-p-toluenesulphonate. Whiteley et al. (1974) blocked the carboxyl groups in the following manner: 15 ml of Sepharose, onto which 5-fluoro-2 -deoxy-uridine-5- (p-aminophenyl)phosphate was bound*via* hexamethylenediamine and succinic anhydride, was suspended in 50 ml of a 1:1 (v/v) mixture of dimethylformamide and 0.1 M cacodylate buffer (pH 4.5) containing 1.1 g of glycinamide. A 2-g amount of 1-ethyl-3-(3 -dimethylaminopropyl)carbodiimide hydrochloride dissolved in 10 ml of the same mixture was added to the suspension, which was then stirred at room temperature for 12 h. When the affinity ligand has to be bound to CH- or AH-Sepharose by means of the carbodiimide binding reaction, the producer recommends that, after the binding of the affinant, the binding reaction be continued by carrying out further carbodiimide reactions with glucosamine (Fransson, 1976) or 2-aminoethanol in the case of CH-Sepharose, or with acetic acid as blocking agent for the amino groups of AH-Sepharose. The unsubstituted amino groups can also be eliminated with acetic anhydride (Kanfer et al., 1973). A suspension in 100 ml of water of 60 ml of Sepharose, onto which D-galactono-α-lactone had been bound through benzidine, was sonicated with 2 ml of acetic anhydride for 10 min in a bath-type sonicator.

Another step in the preparation of specific sorbents before the bioaffinity chromatography proper consists in the thorough washing out of all substances that are not covalently bound to the surface of the solid matrix. Most commonly the best results were obtained if the washing is carried out alternately with alkaline and acidic buffers of high ionic strength. To avoid the need for exhaustive and prolonged washing procedures, Santa-Colona et al. (1987) developed a cyclic system of bioaffinity chromatography for the purification of sex steroid binding protein. A charcoal column connected in series to the bioaffinity column allows the removal of any ligand that may be non-covalently bound to the matrix or released during its storage. This system will be particularly useful when difficulties arising from ligand leakage cannot be ignored.

6.4 LEAKAGE OF THE COUPLED AFFINANT

A serious limitation to the use of bioaffinity chromatography in systems with a high affinity (for example, hormone-receptor interactions), and in the isolation of picomole and nanomole amounts of proteins, is due to the relatively facile release of affinity ligands into the solution. These are primarily affinity ligands that are bound monovalently through a spacer on to cyanogen bromide-activated agarose. The mechanism of leakage of affinants bound on cyanogen bromide-activated Sepharose in the presence of compounds that contain nucleophiles has been discussed in Section 6.2.5. The release of ligands from agarose with bound 8-(ε-aminocaproyl-3-aminoethylthio)adenosine 3', 5 'cyclophosphate as a function of time and pH is shown in Fig 6.14 (Tesser et al., 1972).

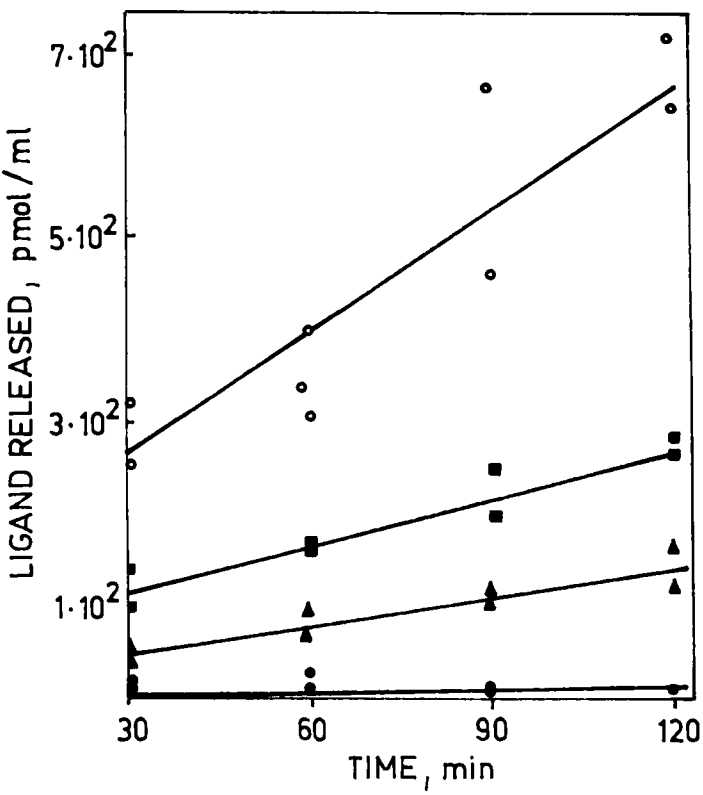


Fig. 6.14. Liberation of ligand from cAMP-spacer-agarose as a function of time and pH. (●) Sodium acetate, pH 5; (▲) imidazole-HCl, pH 6; (■) Tris-HCl, pH 7; (o) Tris-HCl, pH 8; Room temperature. The incubation medium contained 0.005 M of the buffers plus 0.015 M NaCl. Reproduced with permission from G.I. Tesser et al., FEBS Lett., 23 (1972) 56 - 58.

In order to elucidate the leakage of affinity ligands from specific sorbents more closely, Tesser et al. (1974) prepared sorbents by binding adenosine 3',5'-cyclic monophosphate and other substances through spacers of a variety of lengths to agarose, cellulose and crosslinked dextran by means of cyanogen bromide activation, and to polyacrylamide by an amide bond formed with the carboxyl groups of the carrier, according to the method of Inman and Dintzis (1969). From the dependence of the amount of individual detached affinants on pH and time, it followed that the leakage of affinants from the matrices takes place predominantly at the site of the fixation on the surface of the solid carrier, and that it can be enhanced by anchimeric assistance of neighbouring carboxyl and carboxamide groups in polyacrylamide gels, and hydroxyl groups in agarose, cellulose and crosslinked dextrans. The releasing reaction is general and independent of the structure of the bound affinity ligand. The affinants bound to polyacrylamide gels by the R-NH-CO-acrylamide gel bonds are detached more slowly. The leakage of bound ligands can be suppressed if binding through polyvalent spacers is used. A further improvement can be achieved by using binding reactions that afford a stronger binding of the affinant to the carrier, such as the periodate oxidation method, or attachment to an epoxide-activated support. Parikh et al. (1974) discussed the variation of the degree of leakage as a function of the carrier used. In the isolation of estrogen and insulin receptors, it is impossible, for example, to use glass and polyacrylamide carriers, owing to the excessively rapid leakage of affinity ligands.

A mathematical approach to ligand leakage has been described by Gribnau and Tesser (1974) and Lasch and Koelsch (1978). Lasch et al. (1982) not only characterized the ligand leakage, but also studied the heterogeneity of the distribution of protein ligands coupled to Sepharose and Sephadex by electron microscopy and microfluorometry. Depending on molecular size, matrix porosity and reactivity, both an even distribution as well as radial gradients of ligand density have been found. A second type of heterogeneity resides in the diversity of ligand-matrix interactions. Circumstantial evidence from ligand leakage electrophoretic desorption and thermal inactivation studies points to the existence of at least three distinct populations of immobilized proteins.

6.5 GENERAL CONSIDERATIONS IN THE CHOICE OF SORBENTS, COUPLING AND BLOCKING PROCEDURES

When choosing the carrier and the coupling procedure one should not only take account of the properties associated with the nature of bioaffinity chromatography itself, but also their field of use. Kukongviriyapan et al. (1982) described the maximum binding of antibodies against *Naja naja siamensis* toxin 3 (T3) and operational half-life of T3 immobilized at different ligand density in the way described in Table 6.3. The maximum binding capacity of antibody was obtained on immobilized T3 with the lowest half-life (19 days). The lowest antibody binding capacity was obtained on an adsorbent containing T3 coupled to albumin-Sepharose, where the half-life was 108 days. The investigation described was undertaken to study various parameters in the use of bioaffinity chromatography to purify antibodies against cobra postsynaptic toxin from horse refined globulin for therapeutic purposes.

Table 6.3.

Ligand density, maximum antibody binding capacity and operational half-life of various adsorbents

Affinity sorbent[a]	*Toxin immobilized (mg/ml packed gel*[b]*)*	*Maximum binding capacity*[c] *(mg/mg immobilized toxin)*	*Half-life (days)*
Seph-T3	2.60	4.75	43
Seph-Ae-Suc-T3	2.01	6.00	19
Seph-BSA-T3	1.50[d]	2.60	108
Biogel-Ae-Suc-T3	2.01	5.50	25

[a]Abbreviations: Seph, Sepharose 4B; Ae, aminoethyl; Suc, succinyl and T3 *N. n. siamensis* toxin 3.
[b]Volume of gel was measured in 0.15 N NaCl
[c]Protein eluted at pH 2.05
[d]Determined by using ^{125}I-labeled *N. n. siamensis* toxin 3

The choice of coupling procedures and solid supports used depends on the use to which the material will be put: analytical, semipreparation or preparation purposes. It is also necessary to take into consideration whether low-molecular-weight or high-molecular-weight affinity ligands are being used. It should also be born in mind that the toxicity of such reagents as hydrazine, cyanogen bromide, trichloro-s-triazine and

divinylsulphone is high; that of epichlorohydrin, bisepoxiranes, glutaraldehyde, carbonyldiimidazole, benzoquinone, diazonium and tresyl chloride is moderate; only periodate is non-toxic.

In recent times the use of high-performance liquid bioaffinity chromatography (HPLBAC) has been a considerable increase. Its advantages are its high resolution, short analysis or preparation times, reproducibility, sample load capacity and biocompatibility. In bioaffinity chromatography it is desirable that both the effect of the carrier and that of the attachment be excluded. On the other hand, it is the immobilization that permits a study of the effect of the microenvironment. Very often a new environment favourably affects the properties of the immobilized substance. For the selection of the method of linkage, the primary consideration is which groups of the affinity ligand can be used for the linkage to a solid matrix without affecting its biospecific binding site. If several groups are available, it is advisable to choose the most selective method, because a linkage specifically through one particular functional group is desirable. The attachment should not introduce non-specifically sorbing groups into the specific sorbent. From this point of view, it is better to link the spacer to an affinity ligand, and only when it has been modified in this manner to attach it to a solid support. The linkage between the surface of a solid support and an affinity ligand should not introduce non-specifically sorbing groups either, and it should be stable during adsorption, desorption and regeneration. In the choice of a method, the dependence of the stability of the affinity ligand on pH should also be born in mind.

For the binding of proteins, the most commonly used groups are the N-terminal α-amino group and ε-amino group of lysine, and also the C terminal carboxyl group and the carboxyl groups of glutamic and aspartic acids. Phenolic hydroxyl groups of tyrosine, imidazole anion of histidine or the -SH groups of cysteine residues, may also participate in the binding. With carbohydrates and their derivatives, the groups that often take part in the binding are the hydroxyl and the amino groups; in nucleic acids they are the phosphate groups; the sugar hydroxyl groups; and amino or enolate groups in the bases. If high-molecular-weight compounds, which possess more groups for binding, are bound, they are attached at several places. As a consequence, the risk

arising from the detachment of the bound molecules decreases considerably, but, on the other hand, there is a certain risk that the multiple binding will introduce a deformation of the native structure of the immobilized molecule and will thus also change its properties.

An important factor is a rapid increase in the production of commercially available activated solid supports and many biospecific adsorbents. Scherago (1990) in his "Guide to Biotechnology Products and Instruments" in the Supplement of the journal "Science", included the companies producing compounds for bioaffinity chromatography: activated supports, ready-to-use immobilized supports, sample preparation cartridges, spacer and linker arms. Recently Taylor (1991) has published tables which list a number of commercially-available activated supports for protein and cell immobilization and the companies marketing these supports. The informations from both authors is presented in Tables 6.4. and 6.5. The sources of commercially produced activated supports and biospecific adsorbents described in Table 9.1., 2. and 3. are to be found in Table 9.4. Taylor's (1991) summarization of ligands bound and reaction conditions for the major types of commercially available, preactivated supports forms the basis for Table 6.6. However, some reaction conditions are not quite correct.

Table 6.4.

Examples of commercially available supports (See Table 6.5. for full names and addresses of supliers).

Base support	*Supplier*
Epoxy-Activated	
Agarose beads with a 12-atom spacer arm (Sepharose 6B)	Pharmacia LKB
Cellulose cartridge (MEMSEP)	Domnick Hunter
Crosslinked, regenerated cellulose with a 6-atom spacer (Indion)	Phoenix
Microporous hydrophilic nylon sheets and	

membranes (Immunodyne)	Pall
Macroporous beads of copolymerized methacryl-amide and N-methylenebismethacrylamide (Eupergit)	Rohm Pharma
Macroporous beads of copolymerized 2-hydroxyethylmethacrylate and ethylene dimethacrylate (Separon HEMA or Spheron)	Tessek Lachema
Macroporous vinyl acetate-divinylethylene urea beads (VA-Epoxy BIOSYNTH)	Crescent
Macroporous silica beads coated with a hydrophilic polymer (Hydropore-EP)	Rainin
Silica beads (Durasphere AS)	Alltech
Macroporous silica beads (Bakerbond)	Baker
Macroporous silica particles (Ultraffinity-EP)	Beckman
Macroporous polymer-coated silica beads with 7 to 12-atom spacer arms (NuGel-AF)	Separation Industries
Macroporous irregular silica particles	Serva

Hydrazide (and Azide)-Activated

Agarose beads (Affi-Gel HZ)	BioRad
Agarose beads (adipic acid hydrazide) with a 9 atom spacer arm (Sepharose 4B)	Pharmacia LKB
Agarose beads (ImmunoPure)	Pierce
Proprietary polymeric beads (AvidGel F)	BioProbe
Nonporous polystyrene beads	Pierce
Macroporous silica beads activated with diazofluoroborate (Bakerbond)	Baker
Macroporous irregular silica particles	Serva

Glutaraldehyde-Activated

Agarose beads (ActiBind ALD) with 5 to 12-atom spacer arms	ICN
4% Crosslinked agarose beads (Amino Link)	Pierce
Crosslinked agarose beads (ACTIGEL A)	Sterogene
Cellulose beads with a 10 atom spacer arm (Matrex Cellufine)	Amicon
Cellulosic filter membranes (Nalgene U12 and U38)	Nalge
Cellulosic/acyllic polymer membrane (Zetaffinity)	CUNO
2% Polyacrylamide/2% agarose beads with (Act-Magnogel) or without (Act-Ultrogel) Fe_3O_4 particles	IBF
Monodisperse polyacrolein microspheres (Polybead)	Polysciences
Cellulosic-polymer membranes	Memtek
Hydrophilic, microporous PVC sheets, filters and cartridges impregnated with silica and treated with polyethyleneimine (Acti-Disk)	FMC
Macroporous silica beads (Bakerbond)	Baker
Macroporous silica irregular particles (HiPac)	ChromatoChem
Macroporous polymer-coated silica beads with 7 to 12-atom spacer arms (NuGel-AF)	Separation Industries
Macroporous irregular silica particles	Serva
Macroporous polyethyleneimine impregnated alumina particles (Ketomax, Aldomax)	UOP

CNBr-Activated

Agarose beads (Sepharose 4B)	Pharmacia LKB
Macroporous irregular silica particles	Serva

N-Hydroxysuccinimide Activated	
Agarose beads with 10- or 15-atom spacer arms (Affi-Gel 10 and 15)	BioRad
Agarose beads with a 6-atom spacer arm (CH-Sepharose 4B)	Pharmacia LKB
Proprietary polymer macroporous beads with a 10-atom spacer arm (Affi-Prep 10)	BioRad
Macroporous irregular silica particles	Serva
Carbonyldiimidazole-Activated	
6% Crosslinked agarose beads (Reacti-Gel 6X)	Pierce
Beads of polymerized N-acryloyl-2-amino-hydroxymethyl-1,3-propane diol (Reacti-Gel GF-2000)	Pierce
Proprietary polymer beads (Reacti-Gel HW-65F)	Pierce
Macroporous irregular silica particles	Serva
Divinylsulphone-Activated	
Agarose beads (MINI LEAK)	Kem-En-Tec
Cellulose cartridge (Memsep)	Domnick Hunter
Macroporous beads of copolymerized 2-hydro-xyethylmethacrylate and ethylene dimethyl-acrylate (Separon HEMA)	Tessek
Acrylate-coated microporous polyvinylidene difluoride discs and sheets (Immobilon)	Milipore
Tresyl-Activated	
Agarose beads (Sepharose 4B)	Pharmacia LKB
Macroporous silica beads (Selecti Sphere-10)	Pierce

Fluoromethylperidinium-Activated	
Crosslinked agarose beads (Avid Gel Ax)	BioProbe
Polyhydroxylalkylamidoacrylate beads (Avid Gel T)	BioProbe
Proprietary polymeric beads (Avid Gel F)	BioProbe
Biologically Activated	
Avidin-coated glass beads for immobilization of biotin-labelled glycoproteins (BioSpheres)	Kontes
Nonactivated, Functional Group Supports	
Agarose beads with 9 to 12-atom spacer arms and $-NH_2$, -COOH and -SH end groups (Affi-Gels 102, 202, and 401)	BioRad
Agarose beads with hexamethylendiamine (Sepharose 4B) or ε-aminocaproic acid (AH- or CH-Sepharose)	Pharmacia LKB
Macroporous kiselguhr (diatomite) silica(6% crosslinked agarose beads with available hydroxyl groups (Macrosorb KAX)	Sterling
Carboxy- or amino-terminal cellulose beads with 5 to 9-atom spaacer arms (Matrex Cellufine Amino or Carboxyl)	Amicon
Aminophenylthioether- and aminobenzyloxymethyl-terminal cellulose paper for activation by diazotization (Transa-Bind)	S & S
Amino- and carboxyl-terminal polyacrylamide beads (Bio-Gel)	BioRad
5% Crosslinked high porosity (90 to 97%)	

granules of polystyrene, polychloromethyl-styrene or sulphonated polystyrene (POLYHIPE)	National Starch and Chemical
Nonporous polystyrene beads with available $-NH_2$ groups	Pierce
Monodisperse latex beads with available amino, carboxyl, hydroxyl, sulphate, and amidino groups, with/without spacer arms (Microspheres, CML particles, Polybead)	IDC, Bangs, Polysciences
Proprietary polymer beads with -COOH and $-NH_2$ terminal groups on 16 to 20-atom spacer arms (Avid Gel F)	BioProbe
Amino- and hydroxyl-terminal cellulose/acrylic polymer membranes with 5- or 18-atom spacer arms (Zetaffinity)	CUNO
Macroporous bead cellulose Perloza MT	North Bohemian Chemical Works
Derivatives of Perloza MT	Spolchemie
Aminopropyl-, succinylaminopropyl-, hexamethyl-enediamine-, and diol-terminal macroporous silica beads (Bakerbond)	Baker
Glycerol-coated, controlled pore glass beads with available hydroxyl groups (Glycophase)	Pierce
Aminopropyl- and alkylamine-terminal silylated controlled-pore glass beads	Pierce
Amino-, carboxyl-, and hydroxyl-terminal macro-porous polymer-coated glass beads with 10- or 12-atom spacer arms (NuGel-AF)	Separation Industries

Aminohexyl-, carboxymethyl-, and mercaptopropyl-terminal macroporous irregular silica particles	Serva

Table 6.5.

Representative companies providing activated supports and biospecific adsorbents

Accurate Chem. and Sci.Corp., Westbury, NY 11590, USA
Adv. Magnetic, Inc., Cambridge, MA 02138, USA
Adv. Separation Technols., Whippany, NJ 07981, USA
Alltech Associates, Inc., Deerfield, IL 60015, USA
Amicon Division, Danvers, MA 01923, USA
Analychem Corp. Ltd., Markham, ON L3R 2Z8, Canada
Appl. Protein Technols., Inc., Cambridge, MA 02139, USA
Asahi Chem. Ind. Co., Ltd., Kawasaki, Japan
J.T.Baker, Inc., Phillipsburg, NJ 08865, USA
Bangs Laboratories, Inc., Carmel, IN 46032, USA
Beckman Instruments, Inc., San Ramon, CA 94583, USA
Bellco Glass, Inc., Vineland, NJ 08360, USA
Biomat Corp., Belmont, MA 02178, USA
BioProbe International, Inc., Tustin, CA 92680, USA
Bio-Rad Laboratories, Richmond, CA 94804, USA
CAMX Sci. Corp., Rochester, NY 14603, USA
ChromatoChem, Missoula, MT 59801, USA
Collaborative Res., Inc., Bedford, MA 01730, USA
Crescent Chemical Co., Inc., Hauppauge, NY 11788, USA
CUNO, Inc., Meriden, CT 06450, USA
Daicel Chem. Ind., Ltd., Himeji 671-12, Japan
Domnick Hunter Filters, Ltd., Durham, UK
Dynal AS, 0212 Oslo 1, Norway
Elcatech, Inc., Winston-Salem, NC 27101, USA

Fisher Sci. Co., Pittsburg, PA 15219, USA

FMC Bioproducts, Rockland, ME 04841, USA

Genex, Gaithersburg, MD 20877, USA

Hyclone Laboratories Inc., Logan, UT 84321, USA

IBF (Industrie Biologique Francaise), Villeneuve la Garenne, France

ICN Biomedicals, Inc., Cleveland, OH 44128, USA

Inovar Inc., Gaithersburg, MD 20879, USA

Interfacial Dynamics Corp. (IDC), Portland, OR 97207, USA

Isolab, Akron, OH 44321, USA

Jackson Immuno Research Labs., Inc., West Grove, PA 19390, USA

Kendrick Labs., Inc., Madison, WI 53713-2410, USA

Kem-En-Tec Biotechnology Corp., Hellerup, Denmark

Kontes Biotechnology, Vineland, NJ 08360, USA

Kurita Water Industries, Ltd., Kanagawa, Japan

Lachema, Brno CS 621 33, Czechoslovakia

Life Technologies, Inc., Grand Island, NY 14072-0068, USA

Manville Filtration and Minerals, Denver, CO 80217, USA

Membrex, Inc., Garfield, NJ 07026, USA

Memtek Corp., Billerica, MA 01821, USA

Millipore Corp., Bedford, MA 01730, USA

Nalge Company, Rochester, NY 14602, USA

The Nest Group, Southboro, MA 01772, USA

Nord Bohemian Chemical Works, Lovosice, CS 41017, Czechoslovakia

NY Gene Corp., Yonkers, NY 10701, USA

Organon Technika-Cappel, West Chester, PA 19380-9902, USA

OROS Instrs. Ltd., Cambridge, MA 02142, USA

Pall BioSupport Division, Glenn Cove, NY 11542, USA

Pharmacia LKB Biotechnology AB, Uppsala, S-75182, Sweden

Phenomenex, Inc., Torrance, CA 90501, USA

Phoenix Chemicals Ltd., New Zealand

Pierce Chemical, Rockford, IL 61105, USA

Polymer Inc., Amherst, MA 01002, USA

Polysciences, Inc., Warrington, PA 18976, USA

Protan Laboratories Inc., Richmond, WA 98073, USA

Rainin Instrument Co., Inc., Woburn, MA 01801, USA

Repligen Corp., Cambridge, MA 02139, USA

Richard Scientific, Inc., Novato, CA 94948, USA

Rockland, Inc., Gilbertsville, PA 19525, USA

Rohm Pharma GmbH, Darmstadt, FRG

Sankyo Co., Ltd., Tokyo, Japan

Schleicher and Schuell, Inc., Zürich, Switzerland

Sci.Can.Diags. Ltd., Edmonton, AB T5L 4S9, Canada

Separation Industries, Metuchen, NJ 08840, USA

Sepracor Inc., Marlborough, MA 01752, USA

Seva Biochemicals, Westbury, NY 11590, USA

Serva, Heidelberg, FRG

Shionogi Co., Ltd., Osaka, Japan

Sigma Chem. Co., St. Louis, MO 63178, USA

Spectrum Medical Inds., Inc., Los Angeles, CA 90069, USA

Spolchemie, Ústí nad Labem, CS 40032, Czechoslovakia

Sterling Organics Ltd., Newcastle-upon-Tyne, UK

Sterogene Biochemicals, San Gabriel, CA 91775, USA

Supelco Inc., Bellefonte, PA 16823-0048, USA

Tanabe Seiyaku Co., Ltd., Osaka, Japan

Tessek, Ltd., Prague 1, CS 110 00, Czechoslovakia

Thomson Instr. Co., Newark, DE 19711, USA

Toso Haas, Philadelphia, PA 19105, USA

Toyo Soda Mfg. Co., Ltd., Tokyo, Japan

Un. States Biochem. Corp., Cleveland, OH 44122, USA

UOP, Inc., San Diego, CA 92121, USA

VWR Scientific Corp., Philadelphia, PA 19101-3645, USA

Waitaki Intl. Biosciences, Toronto, Canada

Waters Chromatography Div., Millipore Corp., Milford, MA 01757, USA

Wescan Instrs., Inc., Deerfield, IL 60015, USA

Whatman, Maidstone, Great Britain

Zymed Labs., Inc., South San Francisco, CA 94080, USA

Section 6.1 discusses the effect of the nature of proteins and solid supports. Fig. 6.4 shows the dependence of the aminoacylase activity and its binding on the nature of salt added during the immobilization reaction, Fig. 6.5 shows the dependence of immobilized aminoacylase on the salt and protein concentration in the medium during immobilization. Our experiments with a number of solid supports and with various methods of immobilization have shown that neither a universal immobilization method nor a universal support exist, and that the conditions must be developed in each case individually having regard to the application required. The use of oriented immobilization *via* suitable biospecific interaction or coupling of glycoproteins, such as enzymes and antibodies *via* their carbohydrate moieties to porous or nonporous supports, can provide us not only with biologically active adsorbents suitable for various applications, but also with models to be used to advantage in studies on chemical processes in the living cell.

Table 6.6.

Summary of ligands bound and reaction. Conditions for the major types of commercially available, preactivated supports.

Active group	*Ligands bound*	*Typical reaction conditions*
Epoxy	$-NH_2$	pH 5-12, 4-72h, 4-60°C
	-OH,	
	-SH,	
Hydrazide	$-NH_2$	pH 6-9, 2-12h, 4-25°C
	-CHO,	
Aldehyde (Glutaraldehyde)	$-NH_2$	pH 3-10, 1-12h, 4-25°C
CNBr	$-NH_2$	pH 7-10, 2-18h, 4-25°C
Aldehyde (periodate oxidation)	$-NH_2$	pH 7-10, 1-12h, 4-25°C
N-Hydroxysuccinimide	$-NH_2$	pH 5-10, 1-4h, 4-25°C
Carbonyl diimidazole	$-NH_2$	pH 8-10, 18-24h, 4-25°C
Divinylsuphone	$-NH_2$	pH 6-11, 2-24h, 4-25°C
	-OH,	
	-SH,	
Tresyl	$-NH_2$	pH 7-9, 2-16h, 4-25°C
Fluoromethylperidinium	$-NH_2$	pH 7-9, 2-8h, 4-37°C
	-SH,	

REFERENCES

Allam, K., Ehrat, M., Cecchini, D., Warden, B.A. and Giese, R. W., Anal. Biochem., 162 (1987) 171-177.

Andersson, K., Bywater, R., Crnogorčevic, G., Mc Kenzie, R. and Ottosson, T., Reactive Polymers, 1 (1983) 273-279.

Avigad, G., Amaral, D., Asensio, C. and Horecker, B.L., J. Biol. Chem., 237 (1962) 2736-2743.

Axén, R., Porath, J. and Ernback, S., Nature (London) , 214 (1967) 1302-1304.

Beneš, M.J., Adámková, K. and Turková, J., J. Bioactiv. Compatible Polymers, 6 (1991) 406-413.

Bethell, G.S., Ayers, J. S., Hancock, W.S. and Hearn, M.T.W., J. Biol. Chem., 254 (1979) 2572-2574.

Bethell, G.S., Ayers, J.S., Hearn, M.T.W. and Hancock, W.S., J. Chromatogr., 219 (1981) 353-372.

Boschetti, E., Corgier, M. and Garelle, R., Biochimie, 60 (1978) 425-427.

Brandt, J., Andersson, L.O. and Porath, J., Biochim. Biophys. Acta, 386 (1975) 196-202.

Brocklehurst, K., Carlsson, J., Kierstan, M.P.J. and Crook, E.M., Biochem. J., 133 (1973) 573-584.

Cambiasso, C.L., Goffinet, A., Vaerman, J.P. and Hereman, J.F., Immunochem., 12 (1975) 273-278.

Campbell, D.H., Luescher, E.L. and Lerman, L.S., Proc. Nat. Acad. Sci. U. S., 37 (1951) 575-578.

Carlsson, J., Axén, R. and Unge, T., Eur. J. Biochem., 59 (1975) 567-572.

Čoupek, J., Labský, J., Kálal, J., Turková, J. and Valentová, O., Biochim. Biophys. Acta, 481 (1977) 289-296.

Crowley, S.C., Chan, K.C. and Walters, R.R., J. Chromatogr., 359 (1986) 359-368.

Csiky, I. and Hansson. L., J. Liq. Chromatogr., 9 (1986) 875-886.

Cuatrecasas, P., J. Biol. Chem., 245 (1970) 3059-3065.

Cuatrecasas, P. and Parikh, I., Biochemistry, 11 (1972) 2291-2299.

Dean, P.D.G., Johnson, W.S. and Middle, F.A. (Editors) , IRL Press, Oxford, 1985, pp. 1-215.

Domen, P.L., Nevens, J.R., Krishna Mallia, A., Hermanson, G.T. and Klenk, D.C., J. Chromatogr., 510 (1990) 293-302.

Egly, J.M. and Porath, J., in O. Hoffmann-Ostenhof, M. Breitenbach, F. Koller, D. Kroft and O. Schneider (Editors), Affinity Chromatography, Biospecific Sorption, Pergamon Press, Oxford, 1978, pp. 5-22.

Ernst-Cabrera, K. and Wilchek, M., J. Chromatogr., 397 (1987) 187-196.

Ernst-Cabrera, K. and Wilchek, M., Trends Anal. Chem., 7 (1988) 58-63.

Eveleigh, J.W. and Levy, D.E., J. Solid-Phase Biochem., 2 (1977) 45-78.

Fleminger, G., Hadas, E., Wolf, T. and Solomon, B., Appl. Biochem. Biotech., 23 (1990a) 123-137.

Fleminger, G., Wolf, T., Hadas, E. and Solomon, B., J. Chromatogr., 510 (1990b) 311-319.

Ford, D.J. and Pesce, A.J., in E. Ishikawa, T. Kawai and K. Mityai (Editors), Enzyme Immunoassay, Igaku-Shoin Ltd., Tokyo-New York, 1981, pp. 54-66.

Fransson, L.Å., Biochim. Biophys. Acta, 437 (1976) 106-115.

Frost, R.G., Monthony, J.F., Engelhorn, S.C. and Siebert, C.J., Biochim. Biophys. Acta, 670 (1981) 163-169.

Fusek, M., Čapka, M. and Turková, J., Biotechnol. Lett., 9 (1987) 561-566.

Fusek, M., Turková, J., Šťovíčková, J. and Franěk, F., Biotechnol. Lett., 10 (1988) 85-90.

Gahmberg, C.G. and Andersson, L.C., J. Biol. Chem., 252 (1977) 5888-5894.

Gahmberg, C.G. and Hakomori, S., J. Biol. Chem., 248 (1973) 4311-4317.

Gahmberg, C.G., Myllyla, G., Leikola, J., Pirkola, A. and Nordling, S., J. Biol. Chem., 251 (1976) 6108-6116.

Garcia, C.M. and Singhal, R.P., Biochem. Biophys. Res. Commun., 86 (1979) 697-703.

Gelsema, W.J., de Ligny, C.L., Roozen, A.M.P. and Wilms, G.P., J. Chromatogr., 209 (1981) 363-368.

Gemeiner, P. and Viskupič, E., J. Biochem. Biophys. Method., 4 (1981) 309-319.

Gribnau, T.C.J., van Eekelen, C.A.G., Tesser, G.I. and Nivard, R.J.F., J. Solid-Phase Biochem., 3 (1978) 271-289.

Gribnau, T.C.J. and Tesser, G.I., Experientia, 30 (1974) 1228-1230.

Gundlach, G., Kohne, C. and Turba, F., Biochem. Z., 336 (1962) 215-228.

Henriksen, A.Z. and Maeland, J.A., Acta Path. Microbiol. Immunol. Scand Sect. B, 94 (1986) 257-263.

Hearn, M.T.W., J. Chromatogr., 376 (1986) 245-257.

Hearn, M.T.W. and Davies, J.R., J. Chromatogr., 512 (1990) 23-39.

Hornby, W.E., Lilly, M.D. and Crook, E.M., Biochem. J., 98 (1966) 420-425.

Ikuta, T., Okubo, H., Ishibashi, H., Shibata, K., Nagano, M. and Kudo, J., Biochem. Int., 2 (1981) 619-628.

Inman, J.K. and Dintzis, H.M., Biochemistry, 8 (1969) 4074-4082.

Ito, Y., Yamasaki, Y., Seno, N. and Matsumoto, I., J. Biochem. Tokyo, 99 (1986) 1267-1272.

Ivanov, A.E., Turková, J., Čapka, M. and Zubov, V. P., Biocatalysis, 3 (1990) 235-241.

Jirků, V. and Turková, J., Methods Enzymol., 135 (1987) 341-357.

Kanamori, A., Seno, N. and Matsumoto, I., J. Chromatogr., 363 (1986) 231-242.

Kanfer, J.N., Petrovich, G. and Mumford, R.A., Anal. Biochem., 55 (1973) 301-305.

Kay, G. and Crook, E.M., Nature (London) , 216 (1967) 514-515.

Kay, G. and Lilly, M.D., Biochim. Biophys. Acta, 198 (1970) 276-285.

Kelleher, F.M., Dubbs, S.B. and Bhavanandan, V.P., Arch. Biochem. Biophys., 263 (1988) 349-354.

Kempf, J., Pfleger, N. and Egly, J.M., J. Chromatogr., 147 (1978) 195-204.

Kennedy, J.F. and Cabral, J.M.S., in W.H. Scouten (Editor) , Solid Phase Biochemistry, Wiley, New York, 1983, pp. 253-391.

Klyashchitsky, B.A., Mezhova, I.V., Pozdnev, V.F., Slavík, E.P. and Shvets, V.I., Bioorg. Khim., 12 (1986) 141-148.

Koelsch, R., Fusek, M., Hostomská, Z., Lasch, J. and Turková, J., Biotechnol. Lett., 8 (1986) 283-286.

Koelsch, R., Lasch, J., Bláha, K. and Turková, J., Enzyme Microb. Technol., 6 (1984) 31-34.

Kohn, J., Lenger, R. and Wilchek, M., Appl. Biochem. Biotechnol., 8 (1983) 227-235.

Kohn, J. and Wilchek, M., Anal. Biochem., 115 (1981) 375-382.

Kohn, J. and Wilchek, M., Biochem. Biophys. Res. Commun., 107 (1982) 878-884.

Kohn, J. and Wilchek, M., FEBS Lett., 154 (1983) 209-210.

Kohn, J. and Wilchek, M., Appl. Biochem. Biotech., 9 (1984) 285-305.

Krämer, D.M., Lehmann, K., Pennewiss, H. and Plainer, H., Enzyme Eng., 4 (1978) 153-154.

Kühn, M., Pommerening, K., Mohr, P., Beneš, M. and Štamberg, J., Angew. Makromol. Chem., 97 (1981) 161-169.

Kukongviriyapan, V., Poopyruchpong, N. and Ratanabanangkoon, K., J. Immunol. Methods, 49 (1982) 97-104.

Lamed, R., Levin, Y. and Wilchek, M., Biochim. Biophys. Acta, 304 (1973) 231-235.

Larsson, P.O., Glad, M., Hansson, L., Mansson, M.O., Ohlson, S. and Mosbach, K., Adv. Chromatogr., 21 (1983) 41-85.

Lasch, J. and Koelsch, R., Eur. J. Biochem., 82 (1978) 181-186.

Lasch, J., Koelsch, R., Weigel, S., Bláha, K. and Turková, J., in T.C.J. Gribnau, J. Visser and R.J.F. Nivard (Editors) , Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982, pp. 245-254.

Lewis, C. and Scouten, W.H., in W.H. Scouten (Editor) , Solid Phase Biochemistry, Wiley, New York, 1983, pp. 665-678.

Longstaff, C., in P.D.G. Dean, W. S. Johnson and F.A. Middle (Editors) , Affinity Chromatography, a Practical Approach, IRL Press, Oxford, Washington DC, 1985, pp. 37-38.

Lowe, C.R. and Dean, P.D.G., Affinity Chromatography, Wiley, New York, London, 1974, pp. 1-272.

Lowe, C.R., Glad, M., Larsson, P.O., Ohlson, S., Small, D.A.P., Atkinson, T. and Mosbach, K., J. Chromatogr., 215 (1981) 303-316.

Maingault, P., Hoinard, C., Crouzat-Reynes, G. and Guilloteau, D., J. Chromatogr., 314 (1984) 445-449.

Matsumoto, I., Seno, N., Golovtchenko-Matsumoto, A.M. and Osawa, T., J. Biochem. Tokyo, 87 (1980) 535-540.

Micheel, F. and Ewers, J., Makromol. Chem., 3 (1949) 200-209.

Miron, T. and Wilchek, M., J. Chromatogr., 215 (1981) 55-63.

Mohr, P. and Pommerening, K., Affinity Chromatography: Practical and Theorethical Aspects, Marcel Dekker Inc., New York, Basel, 1985, pp. 41-64.

Monsan, P., Puzo, G. and Mazarquil, H., Biochimie, 57 (1975) 1281-1292.

Ngo, T.T., Bio/Technology, 4 (1986) 134-137.

Ngo, T.T., Makromol. Chem., Macromol. Symp., 17 (1988) 229-239.

Nilsson, K. and Mosbach, K., Biochem. Biophys. Res. Commun., 102 (1981) 449-457.

Nisbet, A.D., Saudry, R.H., Moir, A.J.G., Fothergill, L.A. and Fothergill, J.E., Eur. J. Biochem., 115 (1981) 335-345.

O'Shannessy, D.J., J. Chromatogr., 510 (1990) 13-21.

Paik, C.H., Eckelman, W.C. and Reba, R.C., Bioorg. Chem., 8 (1979) 25-34.

Parikh, I., Sica, V., Nola, E., Puca, G.A. and Cuatrecasas, P., Methods Enzymol., 34 (1974) 670-688.

Pazur, J.H., Knull, H.R. and Simpson, D.L., Biochem. Biophys. Res. Commun., 40 (1970) 110-116.

Petkov, L., Sajdok, J., Rae, K., Šůchová, M., Káš, J. and Turková, J., Biotechnol. Techniques, 4 (1990) 25-30.

Porath, J., Axén, R. and Ernback, S., Nature (London) , 215 (1967) 1491-1492.

Porath, J. and Sundberg, L., Nature New Biol., 238 (1972) 261-262.

Potuzak, H. and Dean, P.D.G., Nucl. Acid. Res., 5 (1978) 297-303.

Pougeois, R., Satre, M. and Vignais, P.V., Biochemistry, 17 (1978) 3018-3023.

Quash, G., Roch, A.M., Niveleau, A., Grange, J., Keolouangkhot, T. and Huppert, J., J. Immunol. Methods, 22 (1978) 165-174.

Rafestin, M.E., Obrenovitch, A., Oblin, O. and Monsigny, M., FEBS Lett., 40 (1974) 62-66.

Ratner, D., J. Mol. Biol., 88 (1974) 373-383.

Richards, F.M. and Knowles, J.R., J. Mol. Biol., 37 (1968) 231-233.

Santa-Coloma, T.A., Garraffo, H.M., Gros, E.G. and Charreau, E.H., J. Chromatogr., 415 (1987) 297-304.

Sasaki, H., Hayashi, A., Kitagaki-Ogawa, H., Matsumoto, I. and Seno, N., J. Chromatogr., 400 (1987) 123-132.

Scherago, E.J., Science Supplement, 247 (1990) , pp. 6-40.

Sii, D. and Sadana, A., J. Biotechnol., 19 (1991) 83-98.

Small, D.A.P., Atkinson, T. and Lowe, C.R., J. Chromatogr., 216 (1981) 175-190.

Smalla, K., Turková, J., Čoupek, J. and Hermann, P., Biotechnol. Appl. Biochem., 10 (1988) 21-31.

Solomon, B., Koppel, R., Schwartz, F. and Fleminger, G., J. Chromatogr., 510 (1990) 321-329.

Stambolieva, N. and Turková, J., Collect. Czech. Chem. Commun., 45 (1980) 1137-1143.

Staudenbauer, W.L. and Orr, E., Nucleic Acid. Res., 9 (1981) 3589-3603.

Stiller, J.M. and Nielsen, K.H., J. Clin. Microbiol., 17 (1983) 323-326.

Sundberg, L. and Porath, J., J. Chromatogr., 90 (1974) 87-98.

Suttnar, J., Hrkal, Z. and Vodrážka, Z., J. Chromatogr., 131 (1977) 453-457.

Švec, F., Hradil, J., Čoupek, J. and Kálal, J., Angew. Makromol. Chem., 48 (1975) 135-143.

Tabachnick, M. and Sobotka, H., J. Biol. Chem., 235 (1960) 1051-1054.

Taylor, R.F., in R.F. Taylor (Editor) , Protein Immobilization, Fundamentals and Applications, Marcel Dekker, New York, 1991, pp. 139-160.

Tesser, G.I., Fisch, H.U. and Schwyzer, R., FEBS Lett., 23 (1972) 56-58.

Tesser, G.I., Fisch, H.U. and Schwyzer, R., Helv. Chim. Acta, 57 (1974) 1718-1730.

Turková, J., Methods Enzymol., 44 (1976) 66-83.

Turková, J., in L. Vitale and V. Simeon (Editors) , Pergamon Press, Oxford, 1980, pp. 65-76.

Turková, J., Beneš, M.J., Kühn, M., Stepanov, V. M. and Lyublinskaya, L.A., in J. Chaloupka and V. Krumphanzl (Editors) , Extracellular Enzymes of Microorganisms, Plenum Press, New York, London, 1987, pp. 99-103.

Turková, J., Bláha, K., Horáček, J., Vajčner, J., Frydrychová, A. and Čoupek, J., J. Chromatogr., 215 (1981) 165-179.

Turková, J., Bláha, K., Malaníková, M., Vančurová, D., Švec, F. and Kálal, J., Biochim. Biophys. Acta, 524 (1978) 162-169.

Turková, J., Petkov, L., Sajdok, J., Káš, J. and Beneš, M. J., J. Chromatogr., 500 (1990) 585-593.

Turková, J., Vajčner, J., Vančurová, D. and Štamberg, J., Collect. Czech. Chem. Commun., 44 (1979) 3411-3417.

Turková, J., Vohník, S., Helusová, Š., Beneš, M.J. and Tichá, M., J. Cromatogr., 597 (1992) 19-27.

Ustav, M.B., Remme, J.L., Lind, A. J. and Villems, R.L.E., Bioorg. Khim., 5 (1979) 365-369.

Uy, R. and Wold, F., Anal. Biochem., 81 (1977) 98-107.

Vančurová, D., Turková, J., Čoupek, J. and Frydrychová, A., Collect. Czech. Chem. Commun., 44 (1979) 3405-3410.

Walters, R.R., J. Chromatogr., 249 (1982) 19-28.

Weston, P.D. and Avrameas, S., Biochem. Biophys. Res. Commun., 45 (1971) 1574-1580.

Wilchek, M., Advan. Exp. Med. Biol., 42 (1974) 15-31.

Wilchek, M. and Miron, T., Mol. Cell. Biochem., 4 (1974) 181-187.

Wilchek, M. and Miron, T., J. Chromatogr., 357 (1986) 315-317.

Wilchek, M. and Miron, T., Biochemistry, 26 (1987) 2155-2161.

Wilchek, M., Oka, T. and Topper, Y. J., Proc. Nat. Acad. Sci. U. S., 72 (1975) 1055-1058.

Whiteley, J.M., Jerkunica, I. and Deits, T., Biochemistry, 13 (1974) 2044-2050.

Yang, B.H., Sundaram. P.V. and Maelicke, A., Biochem. J., 199 (1981) 317-322.

Zaborsky, O.R. and Ogletree, J., Biochem. Biophys. Res. Commun., 61 (1974) 210-216.

Zemanová, I., Turková, J., Čapka, M., Nakhapetyan, L.A., Švec, F. and Kálal, J., Enzyme Microb. Technol., 3 (1981) 229-232.

This Page Intentionally Left Blank

Chapter 7

Characterization of supports and immobilized affinity ligands

In order to master the bioaffinity chromatography method successfully, it is important to know the characteristics of both the solid supports employed and the immobilized affinity ligands. One of the main characteristics that determines the suitability of solid supports for bioaffinity chromatography is their sorption properties. Sections 4.6 and 7.1 give procedures for the determination of non-specific sorption. For the linkage of affinants proper, the amount of activatable or active groups capable of binding the molecules of the affinity ligand is important. Section 7.2 presents methods for the determination of carboxyl, amino and hydrazide groups, including the colour test with sodium 2,4, 6-trinitrobenzenesulphonate. Further methods for the determination of oxirane groups, p-nitrophenol esters and vinyl and sulphydryl groups are also given. The number of groups that can be utilized for immobilization is often determined on the basis of the attachment of low molecular - weight substances, for example glycine or glycylleucine for epoxy-activated gels. The amount of substance attached can then be determined by some of the methods described in Section 7.3. From Table 5.1, giving the amounts of glycine and chymotrypsin attached on various types of Spheron, it is evident how important it is for coupling that the size of immobilized molecules, the porosity and the surface of the solid support are taken into consideration.

The most commonly used methods for the determination of the content of immobilized affinity ligands are listed in Section 7.3. Among them there are spectroscopic methods, acid-base titrations, the determination of affinants after their release by acid, alkaline or enzymatic hydrolysis, determination on the basis of elemental analysis, and also by the measurement of radioactivity. Examples of procedures used for the observation of conformational changes are given in Section 7.4 and the determination of the distribution of the proteins bound on the surface of the solid support in Section 7.5.

A thorough characterization of an immobilized affinity ligand is necessary mainly when bioaffinity chromatography and the immobilized enzymes are used for the study of questions such as the interaction of biologically active compounds with affinity ligands

and the effect of various micro-environments on the stability and the biological properties of biomolecules.

7.1 METHODS FOR THE DETERMINATION OF NON-SPECIFIC SORPTION

Non-specific sorption is a complicating factor in bioaffinity chromatography. This has been stressed briefly several times in the present text, and it is discussed in detail in Section 4.6. Control of non-specific binding is shown in Fig. 2.6 (A, B, C). Fig. 2.6 (B) illustrates the high-performance liquid bioaffinity chromatography of pepsin on a column packed with Separon H 1000E with bound ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester. Comparative measurements were performed with pepsin on the same column packed with unmodified sorbent (A). Fig. 2.6 (C) shows the baseline characteristics of the high-performance bioaffinity chromatographic system. Two types of groups that cause sorption - sulphate monoester and carboxyl groups, for example in agarose - have been mentioned by Porath et al. (1971).

7.1.1 Determination of adsorption capacity

A solution of cytochrome C (0.1%) in 0.01 M ammonium acetate solution is applied at pH 4.1 on to a gel column (3 x 0.5 cm) equilibrated with the same buffer until the column is saturated, followed by washing until no protein appears in the eluate. The adsorbed cytochrome is then displaced with 0.15 M ammonium acetate solution of pH 4.1, and the amount of cytochrome desorbed is determined on the basis of the absorbance at 280 nm. After each determination the gel should be washed with 0.5 M sodium hydroxide solution and then with distilled water until neutrality is achieved. The washed gel is then freeze-dried and weighed.

In synthetic polymers, for example hydroxyalkyl methacrylate gels of the Spheron type, the reason for non-specificity may lie in the residues of uneluted organic solvents that were used during the preparation of the polymer. The adsorption capacity can also be controlled by dividing a solution of serum albumin dissolved in 0.05 M sodium acetate into two halves, one half being applied to the column prepared from the tested gel

equilibrated with the same buffer. After passage through the column, the fractions containing the protein are combined and made up to a certain volume with 0.05 M sodium acetate solution. The second half, serving as a control, is made up to the same volume. Agreement between the absorbances (at 280 nm), of the two solutions shows that no non-specific sorption has taken place on the gel. With Spheron, if the two solutions do not show the same absorbance, the gel must be washed with organic solvents, dilute acid and then thoroughly with distilled water. Gels that display non-specific sorption should not be used for bioaffinity chromatography.

7.1.2 Determination of residual negatively charged groups (according to Porath et al., 1975)

About 5 g wet gel is supported on a glass filter and washed first with water and then with 25 ml of 1 M hydrochloric acid. The gel is then washed with distilled water until neutrality is achieved and until the test for chlorides (with silver nitrate) is negative. The weighed gel is then suspended in 5 ml of 2 M potassium chloride solution and titrated with 0.01 M sodium hydroxide solution.

7.2 DETERMINATION OF ACTIVATABLE AND ACTIVE GROUPS

7.2.1 Determination of carboxyl, hydrazide and amino groups on the basis of acid-base titration (Inman, 1974)

7.2.1.1 Dry weight determination

For reasons of better reproducibility it is best to refer the amount of functional groups to the dry weight. Inman recommended transferring the titrated sample into a weighed glass filter, washing it first under suction with 0.2 M hydrochloric acid (for carboxyl groups) to render the groups eletrically neutral. Thorough washing with distilled water eliminates all of the electrolyte, while water is eliminated by washing with methanol. The material is further dried in a vacuum over anhydrous calcium chloride. The sample should be weighed immediately after taking it out of the desiccator. For example,

vacuum-dried polyacrylamide absorbs moisture from the air and increases its weight by about 2 % per hour.

7.2.1.2 Determination of carboxyl groups

The washed gel sample is suspended in 0.2 M sodium chloride solution, keeping its volume below twice the bed volume. Using dilute sodium hydroxide solution, the pH of the suspension is adjusted to 6.5 - 7.5. A standard solution of hydrochloric acid is then added while stirring with a magnetic stirrer until the pH is 2.6. The content of the carboxyl groups in the samples is determined on the basis of the milliequivalents of HCl added, after subtraction of the milliequivalents of free H^+ (0.0025V). The quantity of carboxyl groups is finally referred to the dry weight.

7.2.1.3 Determination of hydrazide groups

The acylhydrazide groups have pK_a values close to 2.6 and direct titration is therefore very difficult. The groups are best converted quantitatively into the succinylhydrazide form by using excess succinic hydrazide (0.6 g per 25 ml of suspension) at pH 4.0. The derivatized gel is then washed with 0.2 M sodium chloride solution and the carboxyl group content is determined as in Section 7.2.1.2. The hydrazide group density, D, obtained from the subsequently determined dry weight, relates to the succinylhydrazide derivative with its incremental mass of succinyl groups. The group density in terms of millimoles of hydrazide per gram of original polyacrylamide is $D' = D/(1 - 0.115D)$.

7.2.1.4 Determination of aliphatic amino groups

The titrated gel is washed and suspended in 0.2 *M* potassium chloride solution, taking care that the volume of the solution does not exceed twice the bed volume. The pH of the suspension is adjusted to pH 11.0 with dilute potassium hydroxide solution. Using a standard solution of hydrochloric acid (v ml) of molarity M, the suspension is titrated, bringing the pH to 7 (or pH 6 for α-amino groups). The volume of the suspension (V ml) is then measured. The content of amino groups is calculated from the milliequivalents of HCl consumed (vM) after subtraction of the milliequivalents of free OH^-

titrated [0.001 (V - v)]. Amino groups cannot be titrated by this method if diacylhydrazide has been used as the spacer. Kornbluth et al. (1974) used titration for the determination of the amount of hexamethylenediamine bound to Sepharose. However, in this instance they titrated the gel to a thymolphthalein end-point with 0.1 M sodium hydroxide solution.

7.2.2 Spectrophotometric method for the quantitative determination of solid phase supported amino groups (Ngo, 1986 a)

The method involves allowing the solid support to react with an excess of activated acylating agent N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and an efficient acylation catalyst, 4-dimethylaminopyridine. Then, after thoroughly removing the unreacted SPDP, the solid support is treated with an excess of dithiothreitol to quantitatively release pyridine-2-thione from the solid support to the solution. After an appropriate dilution, the released pyridine-2-thione which has a strong absorbance at 343 nm, is quantitated by reading its absorbance in a spectrophotometer at 343 nm.

7.2.3 Determination of the content of free carboxyl groups (according to Goldstein, 1973)

Glycine ethyl ester hydrochloride (325 mg) is dissolved in 2.5 ml of dimethyl sulphoxide and an equivalent amount of triethylamine is added to liberate the ester in the free-base form. After stirring the reaction mixture for 1 h over ice it is filtered to eliminate the triethylammonium chloride precipitate. The solution is added to a test tube containing the carboxyl derivative of the gel (50 mg). A solution of 325 mg of dicyclohexylcarbodiimide in 3 ml of dimethyl sulphoxide is then added to the magnetically stirred suspension of the gel and the reaction is allowed to proceed at room temperature for 16 h. The gel is then separated by centrifugation, re-suspended in 5 ml of dimethyl sulphoxide and again centrifuged. The washing with dimethyl sulphoxide is repeated three times and is followed by a triple wash with acetone. After drying for 24h over phosphorus pentoxide under vacuum, followed by acid hydrolysis with 6 M

hydrochloric acid for 48 h at 110°C, the amount of glycine in the gel is determined with an amino acid analyser.

7.2.4 Colorimetric determination of the coupling capacity of solid-supported carboxyl groups (Ngo, 1986 b)

The carboxyl groups of a solid support were coupled to cystamine at pH 4 - 4.5, using a water soluble carbodiimide, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide, as the condensing reagent. The solid-phase coupled disulphides were then reduced to sulphydryl groups by treating the solid phase with dithiothreitol. For every single carboxyl group coupled with cystamine, one solid phase sulphydryl group is introduced. After removing all of the reducing reagents by extensive washing, the sulphydryl content, which is equivalent to the carboxyl content of the gel, is quantitated by using 5,5 -dithio bis-(2-nitrobenzoic acid): Ellman 's reagent.

7.2.5 Determination of free amino groups in polymers on the basis of the condensation reaction with 2-hydroxy-1-naphthaldehyde (Esko et al., 1968)

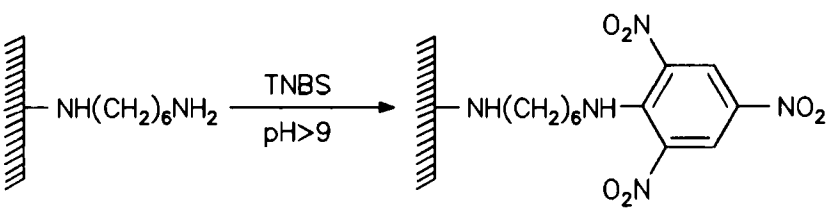
A stable aldimine (Schiff's base) is formed by the condensation of 2-hydroxy-1-naphthaldehyde with the free amino groups in the polymer. After displacement of the chromophore from the polymer with an amine, for example benzylamine, the amount of the soluble aldimine thus formed is determined. Esko et al. (1968) recommended the following procedure. A 10-mg amount of polymer is allowed to react in absolute ethanol with a large excess (50 - 100 fold) of 2-hydroxy-1-naphthaldehyde at room temperature for 12 h. After careful washing of the polymer with dichloromethane, 2 ml of 0.4 M benzylamine in dichloromethane is added and the mixture is allowed to react for 30 min. The amount of 2-hydroxy-1-naphthylidenebenzylamine is determined spectrophotometrically from the absorbance measured at 420 nm. Excess of benzylamine has virtually no effect on the absorbance.

7.2.6 Procedure for azide assay (Brenna et al., 1975)

Freshly prepared azido derivatives are washed 2 - 3 times with ice-cold water in order to remove the acid, then 5 - 10 ml of the washed gel are suspended in 20 ml of 0.1 M sodium hydroxide solution and stirred at room temperature for 2 h. A 0.1-ml volume of clear supernatant is then added to 4.9 ml of 0.1 M ammonium iron(III) sulphate solution. The absorbance at 458.1 nm, which is measured against a blank [4.9 ml of 0.1 M ammonium iron(III) sulphate solution plus 0.1 ml of 0.1 M sodium hydroxide solution], should be read within the first 5 min because hydrazoic acid is slowly released in acidic medium.

7.2.7 The sodium 2,4,6-trinitrobenzenesulphonate colour test

Inman and Dintzis (1969) described a simple qualitative colour test with which the course of the substitution in gels can be followed. The derivatized gel (0.2 - 0.5 ml in distilled water) is added to approximately 1 ml of saturated sodium borate solution, followed by three drops of 3 % aqueous sodium 2,4,6-trinitrobenzenesulphonate (TNBS) solution:



The colour is allowed to develop for 2 h at room temperature. Table 7.1 indicates the colours obtained with individual products. The progress of substitution of the amino derivatives by carboxylic ligands, or of hydrazide derivatives by affinity ligands that contain amino groups, can be determined on the basis of the relative colour intensity of the gel. The test can be made quantitative by washing the gels to remove picric acid and the unreacted TNBS and solubilizing by warming with 50 % acetic acid. The content of amino groups of gel can be determined from the absorbance at 340 nm.

Table 7.1.

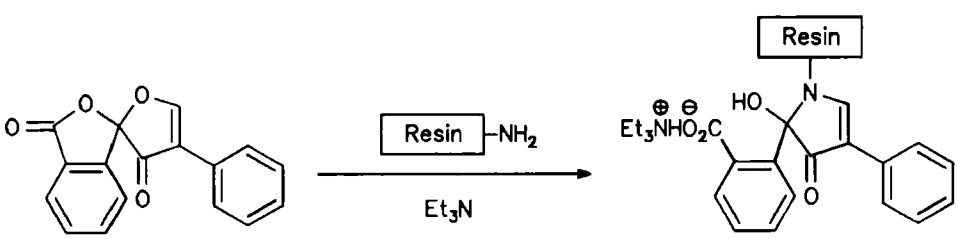
Colours produced by the sodium 2,4,6-trinitrobenzene sulphonate test

Derivative	*Colour*
Unsubstituted agarose or polyacrylamide	Pale yellow
Carboxylic and bromacetyl	Yellow
Primary aliphatic amines	Orange
Primary aromatic amines	Red-Orange
Unsubstituted hydrazides	Deep red

Antoni et al. (1983) used TNBS for the estimation of amino groups on insoluble matrix beads. The method is based on the reaction of Sepharose 4B with excess TNBS and subsequent quantitative determination of unreacted TNBS by reaction with glycine. The method is simple and reproducible and does not require the solubilization of the matrix.

7.2.8 Fluorescamine test for the rapid detection of trace amounts of amino groups (Felix and Jimenez, 1973).

Fluorescamine is a very powerful fluorogenic reagent which is capable of reacting almost instantaneously with primary amino compounds, giving rise to fluorophors with extinction and emission maxima at 390 and 475 nm, respectively. The reagent is capable of detecting picomole amounts of primary amines and it reacts with uncoupled amino groups of the solid support under mild conditions to form highly fluorescent derivatives:



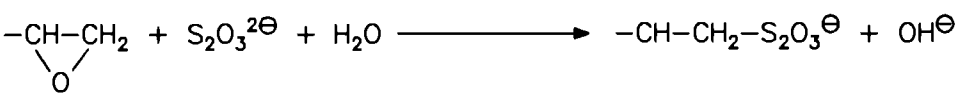
A 5 - 10 mg amount of the gel to be tested is transferred to a glass filter and washed with dichloromethane, ethanol, dichloromethane and chloroform (three times for each wash). The gel is then treated with 10% trimethylamine in chloroform followed by

washing with chloroform, ethanol and chloroform once again (four times for each wash, 1 ml per treatment). After filtering off under suction, the gel is saturated with several drops of 10% triethylamine in chloroform. Fluorescamine solution (10 mg/ml) is then added. Triethylamine must be present in the gel before the addition of fluorescamine in order to prevent the conversion of the fluorophor-containing gel into the non-fluorescent γ-lactone. After standing for 10 min at room temperature, the gel is filtered off under suction and washed with chloroform, methanol and chloroform.

The funnel containing the gel is viewed under long-wave ultraviolet light (366 nm) and its fluorescence is compared visually with that of a blank. As the coupling approaches completion, the intensity of the fluorescence decreases and approaches that of the blank. A fluorescamine solution, if stored at 4°C, gives reproducible results on storage for up to 1 month.

7.2.9 Determination of oxirane groups (Sundberg and Porath, 1974)

The reaction between the oxirane ring and sodium thiosulphate is used for the determination of the amount of oxirane groups in solution and in the agarose gel:



The release of OH^- is controlled by titration with 0.1 M hydrochloric acid using a pH-stat. For the determination of oxirane groups in solution, Sundberg and Porath (1974) recommended the following procedure. The oxirane-containing solution (50 μl) is added to 1.5 ml of 1.3 M sodium thiosulphate solution and the pH is kept constant by addition of hydrochloric acid until the reaction is complete. The amount of oxirane present in the solution is then calculated from the amount of hydrochloric acid needed in order to maintain neutrality.

For the determination of oxirane groups in agarose gel, the same authors recommend the following procedure. Wet agarose gel (0.5 g) is added to 1.5 ml of 1.3 M sodium thiosulphate solution of pH 7.0 and the oxirane content of the gel is determined by titration with hydrochloric acid. The agarose gel is suction-dried under vacuum on a glass filter funnel for 5 min and weighed.

7.2.10 Determination of aldehyde groups on the antibody molecule (Solomon et al., 1990)

Aldehyde groups on oxidized antibodies have been determined using the avidin-biocytin hydrazide system followed by a modified dot blotting method. A 3 μl volume of oxidized antibody is applied to nitrocellulose discs, dried and incubated with 10 μg/ml of biocytin hydrazide for 1 h. After repeated washing with phosphate-buffered saline (pH 7.4) and blocking with bovine serum albumin for 1 h, avidin-alkaline phosphatase conjugate is added and the mixture incubated for 30 min at 37°C. After repeated washing the amount of alkaline phosphatase bound to the blot is determined by hydrolysis of the soluble substrate p-nitrophenyl phosphate. The amount of alkaline phosphatase bound is directly proportional to the amount of aldehyde groups on the oxidized antibodies. A calibration curve for lactoperoxidase-biotin containing a known number of biotin molecules per molecule of protein is used.

7.2.11 Determination of the capacity of p-nitrophenol ester derivatives of hydroxyalkyl methacrylate (NPAC) gels (Turková, 1976)

The amount of active groups is determined on the basis of the spectrophotometric measurement of the amount of p-nitrophenol released. A 100-mg sample of NPAC gel is suspended in 10 ml of dilute ammonia (1:1) and the suspension is allowed to stand for 1 - 4 h with occasional stirring. After the addition of 90 ml of distilled water, the absorbance at 400 nm is measured and the amount of p-nitrophenol released is read from a calibration graph.

7.2.12 Determination of the degree of substitution of benzylated dibromopropanol crosslinked Sepharose (Låås, 1975)

On the basis of exhaustive sulphation of benzylated and non-benzylated Sepharose, the degree of substitution can be calculated by determining the difference in sulphur contents. Pyridine (75 ml) is added dropwise to 10 ml of sulphur trioxide in a round-bottomed flask fitted with a stirrer and a reflux condenser and chilled with ice. A sample of five grams of gel is thoroughly washed with pyridine and allowed to react with 7.5 ml of the suspension of sulphur trioxide-pyridine complex in stoppered tubes for 24 h at 40°C. The sulphated gels are then washed on a glass filter with about 200 ml each of ethanol, water, 1 M acetic acid containing 2 M sodium chloride, 1 M sodium carbonate containing 2 M sodium chloride and finally water. The gels are lyophilized and analysed for sulphur.

7.2.13 Determination of vinyl groups (Porath et al., 1975)

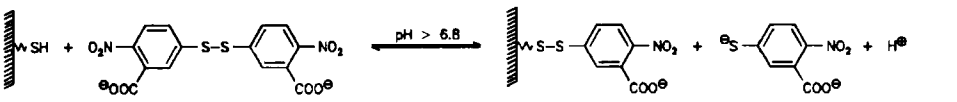
The reaction

$$R\text{-}SO_2\text{-}CH=CH_2 + S_2O_3^{2-} + H_2O \rightarrow R\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}S_2O_3^{2-} + OH^-$$

is used for this determination. A 2-ml volume of 3 M sodium thiosulphate solution is added to 2 ml of crosslinked gel in water. The hydroxyl ions that are formed during the reaction are titrated with 0.1 M hydrochloric acid; the pH of 1.5 M sodium thiosulphate (5.5) is considered to be the end-point of the titration. The reaction rate is very slow, taking about 10 - 15 h for completion.

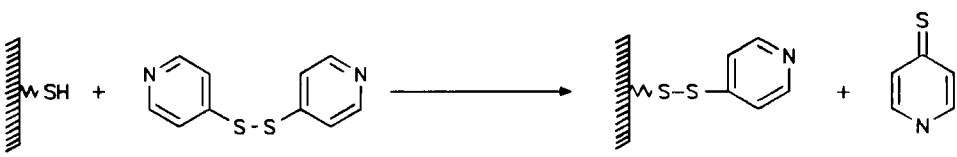
7.2.14 Determination of sulphydryl groups (Lowe and Dean, 1974)

Gel-bound sulphydryl groups can be determined with Elman' s reagent [5,5' -dithio bis-(2-nitrobenzoic acid)], which liberates one mole of strongly coloured 5-sulphido-2-nitrobenzoate anion for each mole of thiol group (see next page):



The amount of coloured anion is determined on the basis of the absorbance measured at 412 nm. This method was used by Cuatrecasas (1970) for the determination of -SH groups in Sepharose derivatives, and was also used for the determination of dihydrolipoic acid covalently attached to Sepharose (Lowe and Dean, 1974).

4,4'-Dipyridyl disulphide (Grassetti and Murray, 1967) and 2,2'-dipyridyl disulphide (Svenson and Carlsson, 1975) give similar results:



4,4'-Dipyridyl disulphite 4-Thioxo-1,4-dihydropyridine

$\lambda_{max} = 324$ nm, $\varepsilon = 1.98.10^4$ l/mole/cm

Sepharose-bound thiols can also be determined on basis of their capability to take up [^{14}C] iodoacetamide on reaction with 0.01 M iodoacetamide in 0.1 M sodium hydrogen carbonate solution (pH 8.0) at room temperature for 15 min (Cuatrecasas, 1970).

7.2.15 Determination of soluble and immobilized N-hydroxysuccinimide esters (Miron and Wilchek, 1982)

A quantitative spectrophotometric assay for N-hydroxysuccinimide and its esters is based on the formation of a chromophore upon titration with mild base. The maximum absorbance for the chromophore is found at 260 nm, the extinction coefficient being 9700 l/mole/cm. The method can be used for measuring the active esters on carriers and their interaction with amines during peptide synthesis.

7.3 METHODS FOR THE DETERMINATION OF IMMOBILIZED AFFINITY LIGANDS

The effect of the concentration of immobilized affinity ligands was discussed in detail in Section 4.4. From that discussion it is evident that, after the attachment of affinity ligands to an inert carrier, it is very useful to determine the proportion of the molecules that are covalently bound to the matrices before the sorption. A gel prepared for sorption should not contain groups that are capable of coupling (blocking of the residual active groups is described in Section 6.3) and it should be washed thoroughly. The preparation of gels is discussed from the practical point of view in Section 6, as there are methods by which one can check that the molecules of affinity ligand covalently bound to the solid support are the only ones present in the gel.

7.3.1 Difference analysis

The amount of the affinity ligand coupled to a gel can be determined on the basis of the difference between the total amount of the affinity ligand added to the coupling mixture and that recovered after exhaustive washing. Although this method is sometimes used, the results obtained are inaccurate, especially when only a small proportion of the affinity ligand is covalently attached, or when the affinity ligand is sparingly soluble and requires large volumes for unbound material to be washed out. However, the method is suitable for an approximate evaluation of the amount attached. An example is the determination of the amount of bound DNA on the basis of the difference between the absorbances of the input solution and final wash solution at 260 nm (Schabort, 1972).

7.3.2 Spectroscopic methods

Direct spectrophotometry of derivatized gels can be employed for the determination of immobilized affinity ligands that absorb at wavelengths above 260 nm. The gel is suspended in optically pure polyacrylamide, ethylene glycol or glycerol in cells of 1 mm path length, and the values are read against the underivatized gel in a double-beam spectrophotometer. Lowe et al. (1973) used this method for the determination of the

concentration of immobilized NAD^+ on the basis of the absorbance at 206 nm. The amount of immobilized NADH was determined on the basis of the absorbance at 340 nm and of immobilized FAD on the basis of the absorbance at 450 nm (Lowe and Dean, 1974). Immobilized NADH, FAD and pyridoxamine are fluorescent when irradiated with light of an appropriate wavelength (Collier and Kohlhaw, 1971; Lowe and Dean, 1974).

When determining the amount of leucine aminopeptidase and trypsin covalently bound to Sepharose, Koelsch et al. (1975), compared five methods for the determination of bound proteins: (1) on the basis of the protein balance before and after binding; (2) from the amino acid analysis after acid hydrolysis; (3) by a modification of Lowry 's method (1951); (4) spectrophotometrically; and (5) by fluorimetric analysis. The advantage of the last two methods, which they describe in detail, is that they do not cause destruction of the gel. Together with Lowry's method, these determinations of bound proteins are characterized by simplicity, sensitivity and high reproducibility.

Several methods have been proposed for the solubilization of derivatized agaroses which permit the quantitative spectrophotometry of immobilized affinity ligands (Lowe and Dean, 1974). Agarose gels can be dissolved in hot water; underivatized agarose can be dissolved at 60°C or higher. The subsequent cooling of highly concentrated aqueous solutions of agarose leads to turbidity in the solutions, while more dilute solutions become viscous but remain transparent. These effects are independent of pH over the whole range. In contrast, derivatizable gels do not dissolve, even with heating at 75°C with 8 M urea or 6 M guanidine hydrochloride solution. They can be solubilized by heating with 0.1 M hydrochloric acid or sodium hydroxide solution at 75°C or, most commonly, with 50% acetic acid. No visible precipitate is formed when solubilized, even when the pH is adjusted to neutrality.

The spectra of solubilized agarose are shown in Fig. 7.1 (Failla and Santi, 1973). Agarose, when dissolved in water, is transparent at wavelengths above 350 nm, but it absorbs slightly between 240 and 350 nm. This absorbance is directly proportional to the concentration of agarose and therefore it can easily be eliminated by using a suitable blank. After treatment with 0.1 M hydrochloric acid (at 75°C for 2 h) agarose displays

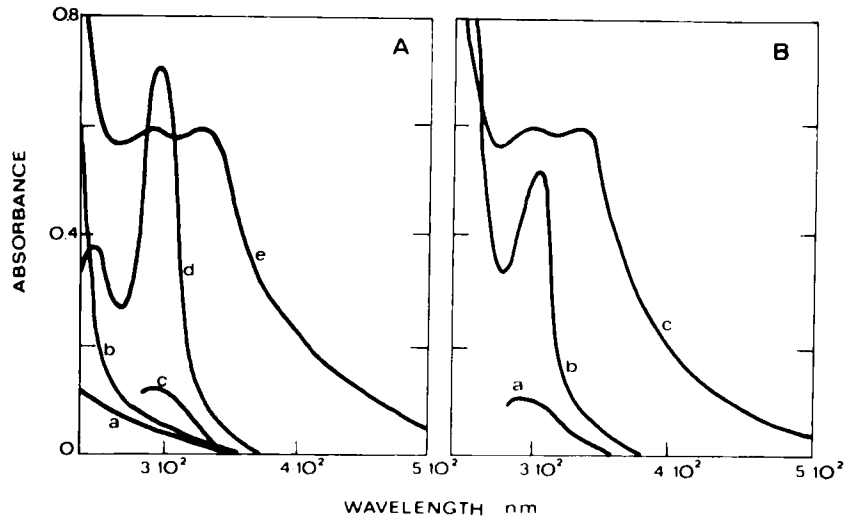


Fig. 7.1. Spectra of solubilized agarose. A suspension containing the equivalent of 0.2 ml of the agarose in 5.0 ml of the indicated solvent was dissolved by heating at 75°C for 2 h. (A) H_2O (a); 0.1 N NaOH-0.1% $NaBH_4$ (b); 50% HOAc (c); 0.1 N HCl (d); 0.1-1 N NaOH (diluted to 10.0 ml with 1 N NaOH) (e). (B) 50% HOAc (a); 0.1 N HCl (b); 0.1-1 N NaOH (diluted to 10 ml with 1 N NaOH prior to recording spectrum) (c). Reproduced with permission from D. Failla and D. V. Santi, Anal. Biochem., 52 (1973) 363-368.

a significant absorption peak at 280 nm, probably produced by the acid-catalysed formation of furfuraldehyde. In contrast, the action of 0.1 - 1 M sodium hydroxide solution, under the same conditions, brings about partial caramelization of the gel, with a broad absorption in the UV-visible spectrum and formation of amber-coloured solutions. This effect can be prevented by adding 0.1% of sodium borohydride prior to heating. In this instance the spectra obtained are similar to those obtained in water.

In Section 7.2.7 it has already been mentioned that the solubilization technique can be used in connection with the TNBS test for the quantitative determination of gels containing amino groups.

Stepanov et al. (1975) solubilized Sepharose with covalently bound mono-N-DNP-hexamethylenediamine with 66% trifluoroacetic acid at 100°C. The solution obtained, when diluted with 20% trifluoroacetic acid, had a maximum at 360 nm in its UV spectrum, which could be used for the calculation of the content of DNP-amine (E^{360}_M = 15,000). Borchardt et al. (1975) hydrolysed the modified gel with 2M sodium

hydroxide solution at 100°C before the spectrophotometric determination of 3,4-dimethoxy-5-hydroxyphenylethylamine.

Larsson et al. (1983) have described a spectroscopic method for measuring the total amount of affinity ligand immobilized to silica matrices after the solubilization of the silica matrix by heating in 1 M NaOH for 3 min at 60°C. Spectra of the dissolved silica affinity matrix *versus* underivatized matrix, after neutralization, have been used to estimate the amount of chromophoric molecule immobilized. In addition, it has been suggested that the amount of ligand bound to silica can be determined from the ultraviolet (UV) absorbance of a suspension of the functionalized matrix in saturated sucrose, which suppresses light scattering due to the similarity of its refractive index to that of silica. The viscosity and density of the suspension prevent settling of the silica while measuring the absorbance.

The use of ^{1}H-NMR spectroscopy for the determination of the degree of substitution in the series of agarose gels substituted with hydrophobic groups has been described by Rosengren et al. (1975). Agarose derivatives were washed carefully and shrunk with acetone on a glass filter. A 100-g sample of the suction-dried gel was transferred into a 50-ml flask, 20 ml of 85% formic acid was added and the mixture was heated under a reflux condenser on a water bath for 1 h. If the polysaccharide carrier did not dissolve during this period, the time of hydrolysis was prolonged. After hydrolysis, the mixture was evaporated to dryness and, after the addition of 2 ml of $^{2}H_2O$, the mixture was again dried. The addition of heavy water and the evaporation were repeated and the remaining residue was dissolved in 1 ml of hexadeuteriodimethyl sulphoxide and used for NMR spectroscopy.

The quantitation of immobilized immunoglobulin G has been determined ellipsometrically by Jönsson et al. (1988). The principle and advantages of this method have already been discussed in Section 5.5.2.

7.3.3 Determination by means of acid-base titration

Polar affinity ligands can be determined by titration of the derivatized gel with an acid or a base. Hixson and Nishikawa (1973) determined titrimetrically the amount of

p-aminobenzamidine bound by the water-soluble carbodiimide to succinylated hexamethylenediamine-Sepharose together with the remaining unsubstituted carboxyl groups. The Sepharose derivative containing carboxylate ions was washed with a 50-fold volume of 0.5 M sodium chloride solution and the volume of the gel was determined by allowing it to settle for 1 h or by gently centrifuging the beads in a calibrated cone. The gel was titrated from pH 7 to pH 3. The whole procedure was repeated after the attachment of p-aminobenzamidine, and the concentration of attached affinant was derived from the difference between the titres. The concentration of N^6-(6-aminohexyl)-AMP immobilized on Sepharose has also been determined by direct titration (Craven et al., 1974).

7.3.4 Determination of immobilized proteins, peptides, amino acids, nucleotides, carbohydrates and other substances after liberation by acid, alkaline or enzymatic hydrolysis

7.3.4.1 Determination of immobilized amino acids, peptides and proteins

The method most often employed for this determination is quantitative amino acid analysis according to Spackman et al. (1958), after acid hydrolysis of the immobilized affinant. The procedure described by Axén and Ernback (1971) is most often used. For agarose with attached enzymes, the gels are freed from buffers on a glass filter (G3) by washing with distilled water and then with a mixture of acetone and water, with increasing concentrations of acetone. Finally, the conjugates are washed with acetone, which is eliminated under vacuum, and the preparations are dried under vacuum over phosphorus pentoxide for 48 h. The weighed amount of the modified gel is hydrolysed with 6 M hydrochloric acid at 110°C for 24 h in a closed, evacuated vessel. Amino acid analysis of the hydrolysate is performed with an amino acid analyser and the protein content was calculated from the content of aspartic and glutamic acid, alanine and leucine. A high content of carbohydrates leads to a dark coloration of the hydrolysate, which, however, does not interfere in the analysis.

Koelsch et al. (1975) pre-purified the dark-coloured hydrolysate, which results from the acid hydrolysis of the polysaccharide carrier on the ion-exchange column. The hydrolysate is prepared using norleucine as an internal reference. After elimination of HCl on a rotatory evaporator, the samples are dissolved in 2 ml of 0.02 N hydrochloric acid and applied either to a 7 x 1 cm column of Amberlite IR 120-X8 and eluted with 25 ml of 1 M ammonia solution, or to a 7 x 1 cm column of Dowex 50W-X4 and eluted with 25 ml of 10% (v/v) pyridine-water. Holleman and Weis (1976) also determined ε-aminocaproic acid eluted shortly before lysine on an amino acid analyser after acid hydrolysis.

A simple, reliable, and sensitive colorimetric procedure for the determination of proteins bound has been described by Marciani et al. (1983). The procedure utilizes the capacity of diethyldithiocarbamate to react with cupric ions, resulting in a complex having a dark yellow colour. The extent of reduction in the colour, due to chelation of Cu^{2+} by the immobilized proteins, indicates the amount of protein. The formation of Cu^{2+}-protein complex proceeds stepwise until enough excess of Cu^{2+} is present to form the final complex. The reliability of the procedure requires that all the protein species, samples and standards, are in the final Cu^{2+}-protein complex form. The method is accurate and simple.

7.3.4.2 Determination of nucleotides

The quantity of nucleotides bound to Sepharose has been determined by calculation of the amount of the soluble product formed after alkaline or nuclease hydrolysis (Hayashi, 1973). The material absorbing in the ultraviolet region, formed in the supernatant during hydrolysis, was determined on the basis of the absorbance, and the hydrolysis was stopped when the absorbance no longer increased. During alkaline hydrolysis, 1 ml of the derivatized gel is mixed with 1 ml of 0.6 M sodium hydroxide solution and the mixture is incubated at 37°C. During enzymatic hydrolysis with T2 ribonuclease, 0.5 ml of the derivatized gel is mixed with 0.5 ml of 0.1 M sodium acetate buffer of pH 4.6, containing one unit of T2 ribonuclease. Incubation is again carried out at 37°C. Enzymatic hydrolysis with phosphodiesterase is carried out in 0.1 M Tris-hydrochloric

acid buffer of pH 8.5, containing 0.01 M magnesium chloride. One milligram of snake venom phosphodiesterase is used per 0.5 ml of the derivatized gel. Phosphodiesterase and alkaline phosphatase have been used simultaneously for the hydrolysis of Sepharose with attached p-aminophenyl-ATP or dATP by Berglund and Eckstein (1972), who determined the release of the nucleotide in the hydrolysate. Similarly, after enzymatic hydrolysis with alkaline phosphatase, the amount of the immobilized AMP was determined on the basis of the phosphate analysis of the terminal phosphate split off (Craven et al., 1974).

On the basis of the total determination of phosphorus (King, 1932), Moudgil and Toft (1975) determined the amount of ATP bound to Sepharose. The sample was first oxidized with a suspension of 7% perchloric acid in 4.5 M sulphuric acid and then incubated at 240°C for 60 min.

7.3.4.3 Determination of carbohydrate

For the determination of the carbohydrate content of copolymers, Hořejší and Kocourek (1973) described the following procedure. A 0.1-g amount of dry pulverized gel is hydrolysed under reflux in boiling 2.5 M sulphuric acid for 6 h. After cooling, the content of carbohydrates in the supernatant is determined by the phenol-sulphuric acid method according to Dubois et al. (1956). Galactosyl pyrophosphate bound to Sepharose has been determined on the basis of the analysis of phosphates after hydrolysis with 0.5 M hydrochloric acid at 100°C for 1 h (Barker et al., 1972). Similarly, immobilized N-acetylglucosamine has been determined on the basis of the liberated glucosamine determined after acid hydrolysis with 6M hydrochloric acid at 110°C (Baker et al., 1972).

After hydrolysis of a lyophilized sample of heparin-Sepharose, the content of heparin was determined, for example, on the basis of the analysis of sulphates (Sternbach et al., 1975).

7.3.4.4 Determination of immobilized dye

The amount of immobilized dye can be determined by brief acid hydrolysis followed by a spectrophotometric measurement (Stellwagen, 1990). A portion of the immobilized

dye is added to 0.6 ml of 5 M HCl and maintained at 37°C for 5 min. Then 2.4 ml of 2.5 M phosphate buffer, pH 7.5, is added and the absorbance of the solution measured at the λ_{max} for dye. Immobilized dye concentration is commonly reported as milligrams of dye per gram wet weight of matrix. The spectral parameters for several reactive dyes have been reported and are listed in Table 7.2. The visible absorbance maximum and the extinction coefficient for other dyes need to be determined by the investigator using the same dye preparation employed in the immobilization reaction.

Table 7.2.

Reactive dye parameters

Group	Generic name	Visible spectrum		Molecular weight[a]
		λ_{max}	E (mM^{-1} cm^{-1})	
1	Yellow 3	385	8.9	578
2	Blue 4	610	10.5	636
3	Blue 2	610	13.6	773
	Red 120	522	30.0	1336
4	Blue 19	590	5.9	566
	Brown 10	530	15.0	588
5	Green 5	675	57.4	1760
	Green 19	630	20.8	------
	Red 58	546	21.3	801

[a] For the acid form

It should be noted that less than 5% of an immobilized dye can retain protein, an observation in keeping with affinity chromatography in general. Nonetheless, this concentration of immobilized dye can facilitate interaction of a retained protein with several immobilized dyes simultaneously. This may result from occupation of more than one of the functional sites on a protein having multiple subunits or from weak interactions at other protein sites. The occurrence of such multiple interactions will increase the concentration of the competitive biomolecules necessary for elution of the desired protein.

7.3.5 Determination of the amount of bound affinant on the basis of elemental analysis

The amount of immobilized nucleoside is most commonly expressed on the basis of the determination of the total content of phosphorus (Lamed et al. 1973) according to King (1932). The amount of sulphanilamide bound to Sepharose can be determined on the basis of the determination of sulphur (Falkbring et al., 1972).

The determination of immobilized affinants on the basis of the determination of nitrogen sometimes leads to erroneous conclusions. As it has already been discussed in Section 6.2, nitrogen is introduced into the gel during cyanogen bromide activation, the amount depending on the temperature used during activation (Nishikawa and Bailon, 1975). The amount of immobilized protein, especially antibodies, is sometimes calculated from the amount of nitrogen determined by the Kjeldahl method (Matheka and Mussgay, 1969).

7.3.6 Determination of labelled affinity ligands

Radioactive affinants have been used in many instances for binding to solid supports. The amount of the bound affinity ligand can easily be determined on the basis of the radioactivity measured. For example, Green and Toms (1973) determined the amount of biotin bound to Sepharose on the basis of the measured radioactivity. Suspension samples (0.1 ml) were taken with a siliconized 0.25-ml tuberculin syringe or an Eppendorf pipette and transferred into the centre of 4-cm^2 planchets. A 0.05-ml volume of 1% sodium dodecyl sulphate solution was added to each sample. The suspensions were dried and their radioactivity was counted. Control experiments with a known amount of [^{14}C] biotin bound to avidin-Sepharose have shown that a self-absorption correction as high as 11% is necessary.

7.3.7 Determination of immobilized diaminodipropylamine by ninhydrin colorimetry (Holleman and Weis, 1976)

Aminoalkylagarose (0.1 ml) is diluted to 2 ml with water and 5 ml of ninhydrin solution is added (4 mg of ninhydrin is dissolved in 150 ml of methyl Cellosolve and 50 ml of 4 N

acetate buffer of pH 5.5, and 80 mg of tin dichloride is added under a constant stream of nitrogen). The mixture is heated on a boiling-water bath for 20 min and, after dilution with 7 volumes of 50% ethanol, agarose is eliminated by centrifugation at 2000 g for 10 min. The absorbance of the supernatant is read at 750 nm against a blank consisting of a solution prepared from non-derivatized agarose treated in an identical manner. A correction must be made when reading the values from the standard graph obtained with the use of 3,3'-diaminodipropylamine, taking into consideration that 3, 3'-diaminodipropylamine carries two primary amino groups when free, but probably only a single amino group when coupled to agarose.

7.3.8 Determination of immobilized proteins on the basis of tryptophan content (Eskamani et al., 1974)

Eskamani et al. (1974) have adapted a method for the determination of tryptophan, which is based on the reaction with ninhydrin in an acid (Gaitonde and Dovey, 1970), to the determination of proteins bound to solid supports. To 1 ml of water or dilute buffer (0.02 M) containing the free or the immobilized protein they added in each instance 1 ml of ninhydrin reagent (250 mg of ninhydrin in a mixture of 6 ml of 90% formic acid and 4 ml of concentrated hydrochloric acid). After mixing, the capped test tubes were incubated in a water bath at 100°C for 20 min and, after cooling, 1-ml aliquots were diluted with 9 ml of 95% ethanol. The absorbance of the solution obtained was read at 400 nm against a blank prepared from the same amount of solid suport without the enzyme. Since collagen does not contain tryptophan, this method could be used for the determination of the content of proteins covalently bound to collagen. The authors therefore determined the calibration graph for lactase and glucoamylase in the presence of collagen (Fig. 7.2).

7.4 STUDY OF CONFORMATIONAL CHANGES OF IMMOBILIZED PROTEINS

The changes in the behaviour of affinity ligands after their binding to a solid carrier are mostly attributed to the modification by the binding, to the effect of the newly formed

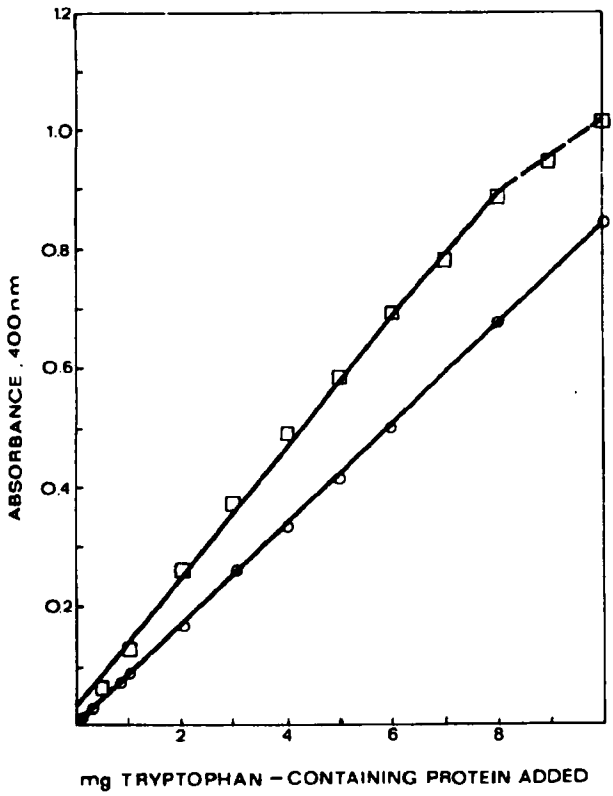


Fig. 7.2. Determination of tryptophan-containing protein in the presence of collagen. A standard solution of lactase was prepared by dissolving 100 mg of lyophilized β-galactosidase in 10 ml of 0.02 M phosphate buffer, pH 7.0. A standard solution of glucoamylase was similarly prepared in 0.02 M acetate buffer, pH 4.0. Aliquots were diluted to 1.0 ml with the same buffer, and 21.0 mg of collagen membrane was added to each. A 1-ml volume of ninhydrin reagent was added to these tubes and to a blank with collagen, and solutions were incubated 20 min in a 100°C bath. After cooling, a 1-ml aliquot was diluted with 9 ml of 95% ethanol, and the absorbance of the samples was determined at 400 nm, using the blank with collagen as a reference. o, Lactase; □, glucoamylase. The lines were determined by linear least-squares analysis of the data points (omitting the two highest levels of glucoamylase). Reproduced with permission from E. Eskamani et al., Anal. Biochem., 57 (1974) 421-428.

micro-environment, limitation of diffusion, etc. Less often a change in conformation is assumed, in spite of the fact that, in comparison with native protein, the changes in the biological activity towards thermal inactivation or denaturation agents show clear changes in conformation or in the ease of submitting to these conformational changes as a consequence of the binding of the protein to the carrier. Among the various

physico-chemical methods developed for the study of the conformation of proteins in solution, the fluorescence technique seems to be most easily adapted for conformational studies of immobilized proteins. Both the intensity and the spectrum of the emitted fluorescent light depend on the environment of fluorescent groups, and changes in the environment are reflected in corresponding changes in the intensity and in the spectrum of the emitted light. However, strong light scattering due to the binding matrix may cause difficulties in the measurement of the fluorescence. Fortunately, the concentrations of the protein absorption bands are generally high. The light absorption thus competes effectively with the light scattering. The excited light is absorbed by a very thin layer on the face of a bed of protein - matrix conjugate and the fluorescence light can be readily separated from the scattered light. Gabel et al. (1971) have described a cell that allowed them to study the conformational changes of the immobilized trypsin and chymotrypsin caused by urea, heat or the presence of specific ligands, using the fluorescence method. Since the use of this cell is not particularly suitable, Barel and Roosens (1974) constructed a very simple cylindrical fluorescence cell, which is shown schematically in Fig. 7.3. The cell consists of a stainless steel top end piece (A) with Teflon tubing and a fitting (B), a Teflon adapter (C), fitting into a cylindrical micro-cell (D), and a stainless steel bottom end piece (E), with Teflon tubing and a stainless steel screw adapter (F). The micro-cell (quartz, 5.00 mm O.D and 3.00 mm I.D.) has inserted a tightly fitting porous Teflon disk (G). An appropriate holder for this cell was made of aluminium and

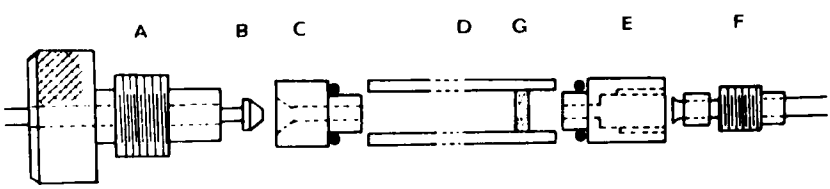


Fig. 7.3. Diagram of fluorescence cell. Reproduced with permission from A.O. Barel and H.Roosens, Anal.Biochem., 60 (1974) 505-511.

fits into the ordinary cell compartment of the fluorimeter. It was necessary to remove the existing screw-button fixing the cell compartment to the apparatus; this was replaced with a bored screw through which the Teflon outlet tubing can pass. The advantages of using the cylindrical cell (even through the scattering may be greater than for rectangular cells) are that the ends of a cylindrical cell are easy to seal with O-rings, allowing its use as a chromatographic column, and making them easy to clean for re-use; futhermore, the amount of material required for the cylindrical system is half that required by a commercially available rectangular cell (50 μl compared to 100 μl).

Barel and Roosents (1974) used an Aminco Bowman SPF spectrofluorimeter equipped with an RCA R 4465 phototube, an off-axis ellipsoidal condensing system and a Houston 2000 X-Y recorder. Correction of the emission spectra was found to be unnecessary since the spectral response of the detection system (i.e. monochromator plus photomultiplier) remains reasonably constant ($\pm$ 10%) from 300 to 500 nm. It was necessary to narrow the exciting beam with slits of 1-mm width in order to decrease the scattering off the round cell. With the combination of slits used, the excitation and emission bandwidths were 10 nm. All experiments were carried out at room temperature.

In order to obtain reproducible fluorescence spectra of insoluble proteins it was necessary to start with the completely dismantled cell. The micro-cell (D and G) (see Fig. 7.3) is filled with a maximum of 100 μl (50 μl is usually sufficient) of the protein agarose gel using a Pasteur pipette and the gel is immediately layered with 100 μl of the appropriate eluting buffer. The Teflon adapter (C) is fitted into the micro-cell and the cell holder (H) and secured in place with the top end-piece (A). The eluting buffer is then passed downwards through the gel with the aid of a peristaltic pump. A flow-rate varying from 0.2 to 0.5 ml/min is used. All of the spectra are recorded after the gel has been washed for 10-15 min with the buffer. The protein-Sepharose 4 B gels are illuminated only during the time necessary to record the emission spectra, in order to minimize photoinactivation. By following this procedure, the protein-gel aliquot can be used repeatedly and it has been shown that photoinactivation of the protein sample is

negligible. It is possible to easily record the fluorescence spectra of about 0.2-300 μg of protein covalently linked to Sepharose 4B.

It is worth noting that the same cell device can be used for classical fluorimetry on protein samples in solution. In this instance the protein solution is introduced upwards into the flow cell. While maintaining the overall cell geometry constant,this procedure allows the comparison of the fluorescence spectra of proteins in solution and on an insoluble matrix. α-Lactalbumin solutions are usually studied at concentrations ranging from 0.05 to 0.15 mg/ml in 0.05 M buffer at pH 7.5.

This procedure was used by Barel and Prieels (1975) for the study of conformational changes of various α-lactamines bound to agarose carriers.

Berliner et al. (1973) have studied conformational changes of trypsin spin-labelled with 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate by means of ESR spectroscopy. Benko et al. (1975) studied the effect of binding of methaemoproteins on to latex particles, with respect to the conformation of bound proteins, using the longitudinal magnetic relaxation rates of solvent protons.

7.5 STUDIES OF THE DISTRIBUTION OF PROTEINS BOUND TO SOLID SUPPORTS

Since, in nature, many biologically active substances exist bound to a membrane or in the form of other native complex structures, well characterized solid supports with bonded biologically active molecules also represent suitable models for their study. For this theoretical reason, but also for practical reasons, it is advantageous to know the spatial distribution of covalently bound biologically active molecules.

The method frequently employed is fluorescence microscopy after labelling with fluorescein isothiocyanate to render the protein molecules visible (Fey and Jost, 1973; Lasch et al., 1972; Stage and Mannik, 1974). In order to make leucine-aminopeptidase bound to Sepharose or Sephadex visible, Lasch et al. (1972) used two procedures, one of which consisted in binding the fluorochrome fluorescein isothiocyanate covalently to the enzyme before its binding to a solid support, and the other in the labelling of the antibody corresponding to the enzyme with the fluorescein derivative.

The labelling of the enzyme with the fluorochrome fluorescein isothiocyanate was carried out by dialysing 2 ml of 13.8 μM enzyme solution with vigorous stirring against 257 μM fluorescein isothiocyanate solution at 4°C for about 8 h. Both solutions were made up in 0.1 M sodium hydrogen carbonate solution. The labelling was terminated by the application of the mixture on to a Sephadex G-25 column. The fluorescein-labelled enzyme passed through the column unretarded and nearly undiluted, while fluorescein isothiocyanate was strongly adsorbed on to the gel. It could be partly eluted with 0.1 M sodium hydrogen carbonate solution, using five times the column's volume. The labelling of the leucine-aminopeptidase antiserum took place in the same manner. The binding of fluorescein-labelled enzyme to cyanogen bromide-activated Sepharose or Sephadex took place in the same manner as with non-labelled enzyme. The fixation of the fluorescein labelled antibody on to the matrix-bound enzyme was carried out in 0.1 M sodium hydrogen carbonate solution. Undiluted labelled antiserum was mixed with the same volume of a suspension of the enzyme bound to a solid support and then allowed to stand in a thermostat at 37°C for 1 h and at 4°C for a further 12 h. After transferring the material on to a glass filter, non-specifically sorbed components were eliminated by intensive washing with 0.1 M sodium hydrogen carbonate solution. In order to determine the distribution of the protein within the support beads, thin sections of the carrier with the attached enzyme had to be prepared, taking care that the structure of the matrix was not damaged. This can be achieved by embedding the support beads in 20% (w/v) gelatin. The gel particles were soaked in the gelatin solution for 5 h at 37°C and then poured into a suitable small container. After standing for several hours in a refrigerator, the gelatin blocks were frozen and cut with a microtome into 10-μm sections. These sections were spread on microscope slides and protected from drying by covering them with thin glass covers. Extinction and emission spectra of fluorescein isothiocyanate, fluorescein-labelled aminopeptidase, both free and bound to an agarose or dextran carrier, were recorded with a fluorescence spectrophotometer.

In studies where the fluorescein technique was used for detection, a uniform distribution of the proteins in the gel has been demonstrated. On the other hand, David et al. (1974), who followed the distribution on the basis of the radiography of ^{125}I-

labelled peroxidase bound to Sepharose beads. Lasch et al. (1975), however, found that the differences are not caused by the detection method used, but by the different methods of preparation of the immobilized protein. Using electron microscopy of ferritin bound to Sepharose, they demonstrated that the application of a very effective method of binding efficiency, higher than 90%, brought about the formation of a shell-like distribution of the covalently bound ferritin, whereas, when the efficiency of the binding was lower than 50%, a uniform distribution of the bound ferritin was again obtained.

REFERENCES

Antoni, G., Presentini, R. and Neri, P., Anal. Biochem., 129 (1983) 60 - 63.

Axén, R. and Ernback, S., Eur. J. Biochem., 18 (1971) 351 - 360.

Barel, A. O. and Prieels, J. P., Eur. J. Biochem., 50 (1975) 463-473.

Barel, A. O. and Roosens, H., Anal. Biochem., 60 (1974) 505-511.

Barker, R., Olsen, K. W. Shaper, J. H. and Hill, R. L., J. Biol. Chem., 247 (1972) 7135 - 7147.

Benko, B., Vuk-Pavlovič, S., Deželič, G. and Maričic, S. J., Colloid Interface Sci., 52 (1975) 444 - 451.

Berlund, O. and Eckstein, F., Eur. J. Biochem., 28 (1972) 492 - 496.

Berliner, L. J., Miller, S. T., Uy, R. and Royer, G. P., Biochim. Biophys. Acta, 315 (1973) 195 - 199.

Borchardt, R. T., Cheng, C. and Thakker, D. R., Biochem, Biophys. Res. Commun., 63 (1975) 69 - 77.

Brenna, O., Pace, M. and Pietta, P. G., Anal. Chem., 47 (1975) 329 - 331.

Collier, R. and Kohlhaw, G., Anal. Biochem., 42 (1971) 48 - 53.

Craven, D. B., Harvey, M. J., Lowe, C. R. and Dean, P. D. G., Eur. J. Biochem., 41 (1974) 329 - 333.

Cuatracasas, P., J. Biol. Chem., 245 (1970) 3059 - 3065.

David, G. S., Chino, T. H. and Resifeld, R. A., FEBS Lett., 43 (1974) 264 - 266.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., Anal. Chem., 28 (1956) 350 - 356.

Eskamani, A., Chase, T., Jr., Freudenberger, J. and Gilbert, S. G., Anal. Biochem., 57 (1974) 421 - 428.

Esko, K., Karlsson, S. and Porath, J., Acta Chem. Scand., 22 (1968) 3342 - 3344.

Failla, D. and Santi, D. V., Anal. Biochem., 52 (1973) 363 - 368.

Falkbring, S. O., Göthe, P. O., Nyman, P. O., Sundberg, L. and Porath, J., FEBS Lett., 24 (1972) 229 - 235.

Felix, A. M. and Jimenez, M. H., Anal. Biochem., 52 (1973) 377 - 381.

Fey, H. and Jost, R., Experientia, 29 (1973) 1314 - 1316.

Gabel, D., Steinberg, I. Z. and Katchalski, E., Biochemistry, 10 (1971) 4661 - 4668.

Gaitonde, M. K. and Dovey, T., Biochem. J., 117 (1970) 907 - 911.

Goldstein, L., Biochim. Biophys. Acta, 315 (1973) 1 - 17.

Grasseti, D. R. and Murray, J. F., Jr., Arch. Biochem. Biophys., 119 (1976) 41 - 49.

Green, N. M. and Toms, E. J., Biochem. J., 133 (1973) 687 - 700.

Havekes, L., Bückman, F. and Visser, J., Biochim. Biophys. Acta, 334 (1974) 272 - 286.

Hayashi, H., J.Biochem., Tokyo, 74 (1973) 203 - 208.

Hixson, H. F., Jr., and Nishikawa, A. H., Arch. Biochem. Biophys., 154 (1973) 501 - 509.

Holleman, W. H. and Weiss, L. J., J. Biol. Chem., 251 (1976) 1663 - 1669.

Hořejší, V. and Kocourek, J., Biochim. Biophys. Acta 297 (1973) 346 - 351.

Inman, J. K., Methods Enzymol., 34 (1974) 30 - 58.

Inman, J. K. and Dintzis, H. M., Biochem., 8 (1969) 4074 - 4082.

Jönsson, U., Malmqvist, M., Olofsson, G. and Rönnberg, I., Methods Enzymol., 137 (1988) 381 - 388.

King, E. G., Biochem. J., 26 (1932) 292 - 297.

Koelsch, R., Lasch, J., Marquardt, I. and Hanson, H., Anal. Biochem., 66 (1975) 556 - 567.

Kornbluth, R. A., Ostro, M. J., Rittmann, L. S. and Fondy, T. P., FEBS Lett., 39 (1974) 190 - 194.

Låås, T., J. Chromatogr., 111 (1975) 373 - 387.

Lamed, R., Levin, Y. and Wilchek, M., Biochim. Biophys. Acta, 304 (1973) 231 - 235.

Larsson, P. O., Glad, M., Hansson, L., Mansson, M. O., Ohlson, S. and Mosbach, K., Adv. Chromatogr., 21 (1983) 41 - 85.

Lasch, J., Iwig, M. and Hanson, H., Eur. J. Biochem., 27 (1972) 431-435.

Lasch, J., Iwig, M., Koelsch, R., David, H. and Marx, I., Eur. J. Biochem., 60 (1975) 163 - 167.

Lowe, C. R. and Dean, P. D. G., Affinity Chromatography, Wiley, New York, London, 1974, pp. 1 - 272.

Lowe, C. R., Harvey, M. J., Craven, D. B. and Dean, P. D. G., Biochem. J., 133 (1973) 499 - 506.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193 (1951) 265 - 275.

Marciani, D. J., Wilkie, S. D. and Schwartz, C. L., Anal. Biochem., 128 (1983) 130 - 137.

Matheka, H. D. and Mussgay, M., Arch. Gesamte Virusforsch., 27 (1969) 13 - 22.

Miron, T. and Wilchek, M., Anal. Biochem. 126 (1982) 433 - 435.

Moudgil, V. K. and Toft, D. O., Proc. Natl. Acad. Sci. U.S., 72 (1975) 901 - 905.

Ngo, T. T., J. Biochem. Biophys. Methods, 12 (1986 a) 349 - 354.

Ngo, T. T., Apl. Biochem. Biotech. 13 (1986 b) 207 - 219.

Nishikawa, A. H. and Bailon, P., Anal Biochem., 64 (1975) 268 - 275.

Porath, J., Janson, J. C. and Låås, T., J. Chromatogr., 60 (1971) 167 - 177.

Porath, J., Låås, T. and Janson, J. C., J. Chromatogr., 103 (1975) 49 - 62.

Rosengren, J., Pahlman, S., Glad, M. and Hjertén, S., Biochim. Biophys., Acta, 412 (1975) 51 - 61.

Schabort, J. C. , J. Chromatogr., 73 (1972) 253 - 256.

Solomon, B.,Koppel, R., Schwartz, F. and Fleminger, G., J. Chromatogr., 510 (1990) 321 - 329.

Spackman, D. H., Stein, W. H. and Moore, S., Anal. Chem., 30 (1958) 1190 - 1206.

Stage, D. E. and Mannnik, M., Biochim. Biophys. Acta, 343 (1974) 382 - 391.

Stellwagen, E., Methods Enzymol., 182 (1990) 343 - 357.

Stepanov, V. M., Lavrenova, G. I., Borovikova, V. P. and Balandina, G. N., J. Chromatogr., 104 (1975) 373 - 377.

Sternbach, H., Engelharadt, R. and Lezius, A. G., Eur. J. Biochem., 60 (1975) 51 - 55.

Sundberg, L. and Porath, J., J. Chromatogr., 90 (1974) 87 - 98.

Svenson, A. and Carlsson, J., Biochim. Biophys. Acta, 400 (1975) 433 - 438.

Svensson, B., Biochim. Biophys. Acta, 429 (1976) 954 - 963.

Turková, J., Methods Enzymol., 44 (1976) 66 - 83.

Chapter 8

General considerations on sorption, elution and non-specific binding

8.1 PRACTICAL ASPECTS OF BIOAFFINITY TECHNIQUES

The properties of suitable biospecific adsorbents have already been discussed in preceeding chapters. The strength of interaction between the covalently bound ligand and its partner, and the steric accessibility of the complementary binding sites, play an essential role in the study of optimum conditions for sorption and elution of substances which are to be isolated or determined.

Since ionic bonds or hydrogen bonds, hydrophobic interactions and Van der Waals-London forces may participate to varying degrees in the binding of the complementary sites of the affinity ligand and of the isolated substances, the optimum conditions for sorption and desorption will vary in particular cases. In general, the starting conditions for sorption should be selected so that they cause maximum sorption of the isolated substance. The choice of the starting buffer is entirely dependent on the optimum conditions of complex formation between the affinant and the isolated substances and, in addition to temperature, pH and ionic strength, it also depends on the content of metal ions and other specific factors.

For sorption, it is best to dissolve a sample of the substance to be isolated in the starting buffer and, if necessary, to change the composition of any salts present in the sample by dialysis or gel filtration. If a substance that forms a strong complex with the immobilized affinity ligand under the given conditions is isolated by column chromatography, the volume of the sample introduced onto the column is irrelevant. However, if substances of low affinity towards the attached affinant are isolated by bioaffinity chromatography, the volume of the sample applied should not exceed 5% of the hold-up volume in order to prevent elution of the isolated substance along with non-adsorbed material. If a substance with a low affinity for the bound affinant is isolated, it often elutes from the column, even without a change of buffer. In this case the isolated substance is obtained in dilute form. An example may be a comparison of

the chromatography course of chymotrypsin on Sepharose with both coupled ε-aminocaproyl-D-tryptophan methyl ester and with directly attached D-tryptophan methyl ester, shown in Fig. 4.5. For the elution of chymotrypsin from the solid support on which the inhibitor is bound via ε-aminocaproic acid as spacer, a change in pH is necessary for displacement, and the chymotrypsin fraction is eluted as a sharp peak. If the inhibitor is coupled directly on a solid support, its steric accessibility is decreased and chymotrypsin is only retarded. The enzyme is eluted in a much greater volume at the same pH, closely after the inactive material.

If a small amount of substance is isolated from a crude mixture by means of an immobilized affinity ligand having high affinity, the batchwise arrangement can be employed to advantage, sometimes combined with elution after a transfer of the material into a column. An example is the isolation of thyroxine-binding globulins by means of bioaffinity chromatography on Sepharose with attached thyroxine. In order to obtain a higher yield, Pensky and Marshall (1974) carried out the first part of the sorption in a batchwise arrangement by stirring normal human serum with insolubilized affinant overnight. After washing with 0.1 M sodium hydrogen carbonate solution, they transferred the insoluble carrier, with together with the adsorbed isolated substances, into a column from which two thyroxine-binding globulin fractions were then eluted with 0.002 M potassium hydroxide solution. This combination of the batchwise and the column arrangement is often encountered in bioaffinity chromatography. It is not only the time necessary for the contact of substances to be isolated with the attached affinity ligand that is thus prolonged; the time necessary for a correct orientation of binding sites is also longer. In the column arrangement, the prolongation of the time of contact of the substance to be isolated with immobilized affinant is achieved by stopping the flow through the column after sample application for a certain time. Manen and Russell (1974) stated that adenosyl-L-methionine decarboxylase on Sepharose with attached p-chloromercuribenzoate was stopped for 2 h; the enzyme was eluted after several fractions. A sufficiently low flow rate through the column of a specific sorbent is of prime importance in the isolation of high molecular weight substances, especially if they occur in high concentrations.

The batchwise arrangement of affinity chromatography is particularly suitable for large-scale isolations if the systems involved have a sufficiently high affinity. It is often appropriate to insert an initial purification step before the specific sorption, in order to dissociate and separate the components of naturally occurring complexes that contain the required substances. This step may consist, for example, of precipitation, extraction or adsorption on an ion exchanger. Frequently, affinity sorption is carried out directly from crude extracts, sera or exudates. Porath (1972) recommended enclosing the gel particles of specific sorbents in containers with semipermeable walls, permitting the free passage of dissolved substances and the retention of large particles. Nylon net bags can be used, for example. If several bags containing various specific sorbents are immersed in the medium, several substances can be isolated simultaneously, or else unwanted substances which may decrease the yields of the isolated substances , such as proteolytic enzymes, can be eliminated from the solution.

The great improvement of bioaffinity chromatography connected with the change over from the soft gel supports to small and hard particles used in high-performance bioaffinity chromatography (HPLBAC) has been already discussed in Chapter 1 and Section 2.2. Mechanically stable, high-porous or nonporous rigid particles of a small and uniform size provide high speed and resolution. The rapid separation obtained in HPLBAC makes this technique especially valuable for analytical assays where time plays an important role. The analysis of drugs and metabolites in serum or plasma can easily be performed within minutes with high recovery rates (Ernst-Cabrera and Wilchek, 1988). A comprehensive review on HPLBAC as a new tool in biotechnology has been published by Ohlson et al. (1989); it contains a list of many suppliers of commercially available HPLBAC matrices and biospecific adsorbents. A biospecific adsorbent prepared from nonporous quartz fibre is shown in Fig. 4.2. It was used for large-scale purification of ox heart lactate dehydrogenase.

The development of cell separation by bioaffinity chromatography has been severely hampered by difficulties in recovering cells from affinity supports. Progress was made by Ugelstad et al. (1988) who used monosize magnetic particles in selective cell separation. Their paper reports, for example, the successful clinical applications of

immunomagnetic beads for depletion of tumour cells or T lymphocytes from bone marrow. Superparamagnetic polystyrene Dynabeads (DYNAL A.S, Oslo, Norway), consisting of a maghemite (Fe_2O_3)-containing core covered with a polymer are at present commercially available. They have a smooth surface that is easily coated with antibodies or other selecting molecules. Combined with a magnet, Dynabeads make a unique tool in positive or negative separation.

The proteolytic enzyme urokinase has therapeutic potential as a plasminogen activator that promotes dissolution of thrombin *in vivo*. Male et al. (1990) described a simple method for purifying urokinase from human urine. They modified a single-step method for urokinase purification by immunoaffinity chromatography by bioaffinity ultrafiltration. They synthesized a water-soluble, ligand-bound polymer by copolymerizing N-acryloyl-m-aminobenzamidine and acrylamide in the absence of oxygen. The bioaffinity ultrafiltration process the described yielded urokinase which exhibited a specific activity close to that of the highest commercial grade. The bioaffinity polymer was synthesized using commercially available material such as acrylamide, aminobenzamidine, etc. Because this type of macroligand is less sensitive to factors such as shear force, temperature and pH, it is easy to handle and should have a long operating life. According to the authors, in contrast to their isolation, immunoaffinity chromatography can be very costly, laborious and time consuming. The monoclonal antibodies are expensive, difficult to prepare, susceptible to degradation and have only a limited operating life. In recent time ligands bound on a membrane, evolved from micro- and ultrafiltration, have provided new biospecific adsorbents for rapid purification.

8.2 SORPTION CONDITIONS

8.2.1 Concentration of adsorbed molecules, equilibration time, geometry of column, and flow rate

The concentration of the solution of the substance to be isolated acts in various ways if the process represents affinity separation in column or batchwise arrangement. Another important factor is the level of affinity of the interacting complementary sites

of the isolated substance and the affinant. The equilibration time and the flow rate are related to this factor.

In column bioaffinity chromatography, a sufficiently strong interaction of the isolated substances with the immobilized affinity ligand causes the substance to be concentrated and slows its migration down the column. This process depends on the concentration of ligand and is almost independent of the starting concentration of the free macromolecule.

Fig. 8.1 shows the application of 50 mg of crude porcine pepsin dissolved in 10, 100, or 1000 ml to a column of ε-aminocaproyl-L-Phe-D-Phe-OMe-Separon (Turková et al., 1981). The increasing dilution of the enzyme solution causes the peak of the first fraction of inert proteins to undergo spreading (it completely disappears at the lowest concentration). By contrast, the peak of the pepsin fraction (eluted by a desorbing buffer of high ionic strength) is sharp in all three cases and is in no way affected by the dilution. However, since nonderivatized Separon was found to be suitable for hydrophobic chromatography (Štrop and Čechová, 1981), it was determined, using unmodified Separon by the first chromatography operation in Fig. 8.1 (A), that under the conditions of bioaffinity chromatography whole proteolytic activity emerged in front of the equilibrium buffer and no material was eluted by the desorption buffer.

The concentration of a substance to be isolated in the sorption solution affects the rate of attainment of equilibrium during the batchwise process. Fig. 8.2 shows an orienting experiment carried out by Porath and Sundberg (Porath and Kristiensen, 1974). One-gram portions of soybean trypsin inhibitor-Sepharose were suspended in solutions containing equal amounts of trypsin, but at different concentrations, and the residual activity of trypsin was then measured at various time intervals. On the basis of the curves obtained, the authors assumed that for a suspension containing 1% of adsorbing gel particles of diameter 50-100 μm in the solution containing the isolated substance in the molecular-weight range 10,000-100,000 Daltons a contact time of 20-30 min is necessary if the gel particles are composed of agarose with a matrix density of 6% or less. Proteins with high molecular volumes, or particles larger than 10^6 Daltons, will require a much longer time for diffusion to the binding sites of the affinity ligand,

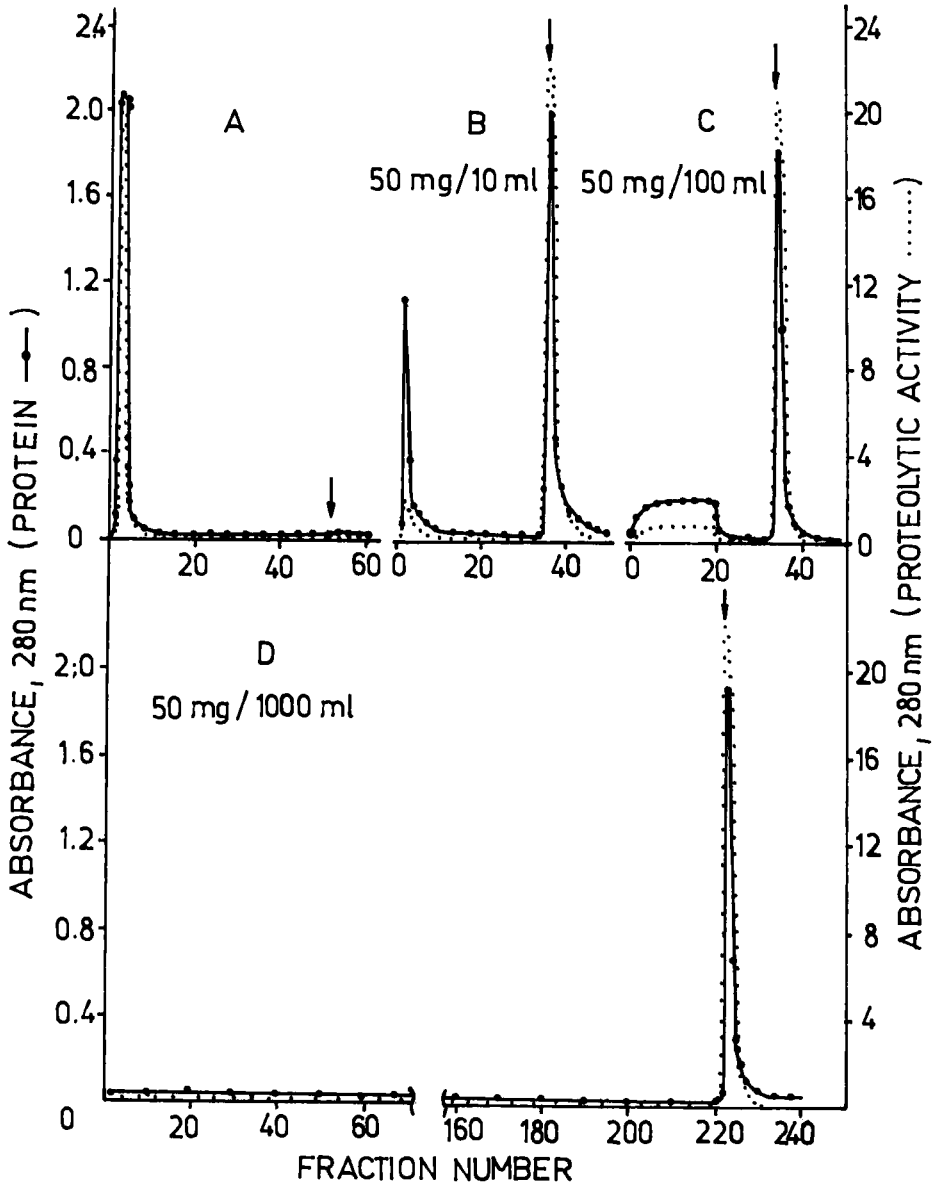


Fig. 8.1 Chromatography of crude pepsin on unmodified Separon H 1000 (A) and Separon H 1000 E_{med} with bound ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (B,C,D). Sample, 50 mg of crude pepsin (A,B in 10 ml, C in 100 ml, D in 1000 ml of 0.1 M sodium acetate buffer, pH 4.5) washed first with the same buffer, starting from the spot denoted with an arrow, washed with the same buffer containing 1 M sodium chloride. Fractions, 5 ml in 4-min intervals; room temperature. Data from J. Turková et al., J. Chromatogr., 215 (1981) 165-179.

even when a gel of high permeability is used. For extra-high molecular weight substances, it is more advantageous to avoid gel permeation and rather to use adsorption on the beads coated with substances that interact specifically with the particles.

In column chromatography, too, the diffusion rate of the molecules into the gel does not depend on the pore size only; it also depends on the relationship between the molecular size and the pore volume. When a concentrated solution of large macromolecules is applied onto the gel, some molecules will diffuse into the accessible pores, but molecules coming in later will find many pores occupied and their probability of diffusion into the occupied pores will be reduced, depending on the reduction in the accessible pore volume. This leads to exclusion from the pores, which is the origin of immobilized ligands whose accessibility is changed. The second reason for the appearance of the isolated protein in the void volume of the eluate at high flow rates applied to highly overloaded columns is the steric hindrance to subsequent molecules by the molecules of the protein that have already been adsorbed. A globular protein of medium

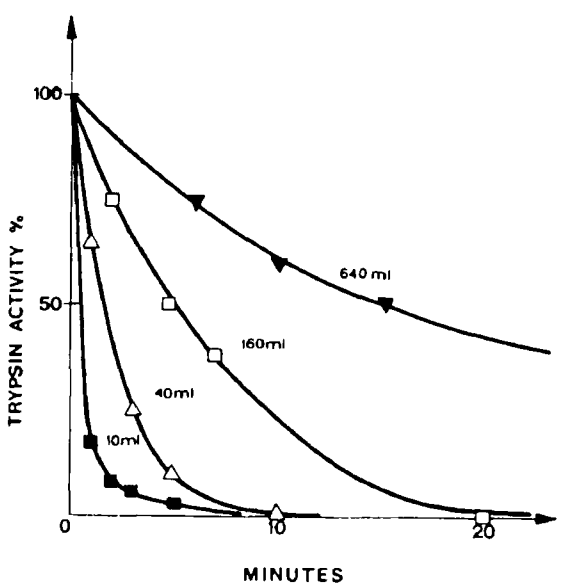


Fig. 8.2. Rate of adsorption of trypsin onto soybean trypsin inhibitor-agarose particles in suspensions of different concentrations. Reproduced with permission from J. Porath and T. Kristiansen, in H. Neurath and R. L. Hill (Editors), The Proteins, Academic Press, New York, 3rd ed., 1975, pp. 95-178.

size, such as a hemoglobin, covers an area of about 2500 Å (Lowe and Dean, 1974), which itself produces considerable steric hindrance.

Since the interaction of macromolecules with an immobilized affinant is a time-dependent process, it is affected by the flow rate and the equilibration time. In many instances adsorption equilibrium is attained very slowly. For the interaction of a macromolecule with an immobilized affinant, mere collision of appropriate molecules does not suffice, because a correct orientation of binding sites, or their conformational adaptation, is also necessary. Table 8.1 shows the influence of the equilibration time on the efficiency of a columnn of N^6-(6-aminohexyl)-5 '-AMP-Sepharose (Lowe et al., 1974a). When glycerokinase and lactate dehydrogenase are equilibrated with the adsorbent, both the column efficiency, expressed as the height equivalent to a theoretical plate (HETP), and the strength of the enzymatic interaction (β) increased with time up to the 67th hour. In general, the lower the HETP value the greater is the effect of the adsorbent (Lowe and Dean, 1974). With glycerokinase, the percentage of the bound enzyme also increased.

Fig. 8.3A shows the effect of lactate dehydrogenase and glycerokinase concentrations on the capacity of N^6-(6-aminohexyl)-5'-AMP-Sepharose in the batchwise arrangement (Lowe et al., 1974a). The percentage of the bound enzyme increases with enzyme concentration, which is mainly operative at concentrations of up to 2 units per millilitre. Although the affinity (β) of both enzymes differs considerably (in a 40 : 1 ratio), the relationship between the percentage of the bound enzyme and its concentration is virtually identical.

The effect of the incubation time on the capacity of N^6-(6-aminohexyl)-5' -AMP-Sepharose to bind lactate dehydrogenase in the batchwise arrangement (Lowe et al., 1974a) is illustrated in Fig 8.3B. The greatest increase in the binding of the enzyme on the immobilized nucleotide occurs in the initial phase of the process, which then continues more slowly up to 100% binding, which is achieved after 16h, while the half-time is about 20 min.

Ferre et al. (1986) studied the influence of the shape of the column of Sepharose 4B with attached guanosine triphosphate through adipic acid dihydrazide (GTP-Sepha-

Table 8.1.

Effect of equilibration time on the efficiency of a column of N^6-(6-aminohexyl)-5' -AMP-Sepharose

The enzyme sample (100 μl), containing enzyme (5 U) and bovine serum albumin (1.5 mg), was applied and washed into a column (34 x 5 mm) of N^6-(6-aminohexyl)-5 '-AMP-Sepharose (0.95 g moist weight, 0.125 μmole/ml of adenosine 5'-monophosphate). HETP = column length/16 $(V_e/w)^2$, where V_e is the elution volume, measured from the start of the KCl gradient to the centre of the enzyme peak, and w is the peak width, determined at the base of the enzyme peak. Binding (β) represents the concentration of KCl required to elute the enzyme.

Equilibration time (h)	*Amount bound (%)*	*Binding (β) (mM KCl)*	*Peak width (ml)*	*HETP (mm)*
	Glycerokinase:			
1	26	20	7.0	0.42
20	51	50	6.0	0.25
67	59	150	5.6	0.17
	Lactate dehydrogenase (pig heart muscle):			
1	100	360	18.5	1.50
20	100	355	11.2	0.52
67	100	490	13.8	0.76

rose) on the recovery of enzyme activity of quanosine triphosphate cyclohydrolase I from *Escherichia coli*. The GTP-Sepharose was used to prepare three columns, each of 3-ml bed volume but with heights and diameters as follows: short (1.7 x 1.5 cm I.D.), medium (3.8 x 1.0 cm I.D.) and long (7.8 x 0.7 cm I.D). The amount of GTP cyclohydrolase that failed to bind to each of the three columns was the same for each column. However, the percentage of enzyme recovered from the shortest column was 6.7 times that recovered from the longest, as shown in Table 8.2. The early results of the studies of the performance of bioaffinity chromatography columns (Katoh et al., 1978) obtained by measuring the rates of adsorption and elution of trypsin in a Sepharose 4B-arginine peptides column showed that chromatography columns of large diameter are suitable for industrial applications. Katoh et al. concluded that, with the adsorbents they used, the rate-determining steps of adsorption and elution of trypsin are the mass transfer within the particles and through the external liquid film. In large-scale columns,

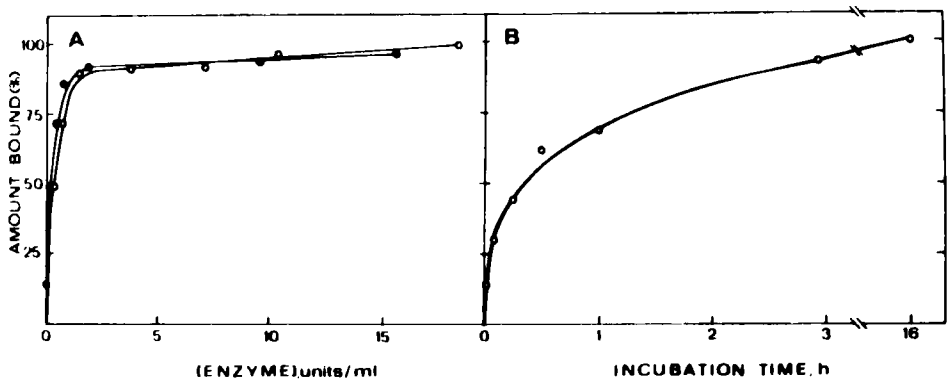


Fig. 8.3. Effect of enzyme concentration (A) and incubation time (B) on the capacity of N^6-(6-aminohexyl)-5'-AMP-Sepharose for lactate dehydrogenase (o) and glycerokinase (●) under batchwise conditions. The enzyme, diluted with equilibration buffer (10 mM KH_2PO_4-KOH, pH 7.5) to the appropriate concentration (A) or 10 U of lactate dehydrogenase suspended in 100 ml of equilibrated buffer (B), was incubated with N^6-(6-aminohexyl)-5'-AMP-Sepharose (0.5 g moist weight, 1.5 μmole/ml of 5 '-AMP) on a Coulter mixer at 4.5°C. After 30 min (A) or at suitable time intervals (B), the incubation was stopped by allowing the adsorbent to settle (15 min) and the supernatant volume was then removed. Using this procedure, with a 5-min incubation period, the adsorbent was washed three times with 5 ml of equilibration buffer prior to re-suspension in 2.5 ml of equilibration buffer and packing in a column (50 x 5 mm). The column was eluted with 11.0 ml of equilibration buffer and then with a linear gradient of KCl (0 to 1.0 M, 20 ml total volume). Enzymes were assayd in the washes and column effluent. Reproduced with permission from C.R. Lowe et al., Eur. J. Biochem., 41 (1974a) 341-345.

Table 8.2.

Comparison of the efficiencies of differently shaped GTP-Sepharose columns in the recovery of the enzyme

The values are the percentage of the amount of GTP cyclohydrolase applied to the column as measured by enzyme activity. The values were obtained from chromatography experiments. The flow-rate was 1 ml/min for sample addition, washing and elution.

Shape of GTP-Sepharose column	*Enzyme activity (%)*	
	Flow-through	*Eluted with GTP*
Short	8	20
Medium	8	10
Long	8	3

however, compaction of the adsorbent bed limits the maximum superficial liquid velocity and tends to cause nonuniform flow of liquid through the bed (Sada et al., 1982).

In the case where a high-flow bioaffinity chromatography system was required for the purification of the enzyme acetylcholinesterase (AChE), a comparison was made between affinity supports prepared with a soft agarose gel and a rigid polymer (Fig. 8.4). In this example, a ligand which binds AChE, trimethyl(p-aminophenyl)ammonium chloride (TAPA), was immobilized onto the carboxyl forms of Sepharose and Separon HEMA using carbodiimide coupling (Taylor and Marenchic, 1984). The pressure stability of the HEMA support (up to 5000 psi) means that, it can be packed into an HPLC column and eluted at flows up to 4ml/min. The Sepharose support could not be run under pressure without compression, and the maximum (gravity) flow through the column was approximately 0.5 ml/min. As a result of the higher flow rate and rigidity of the Separon HEMA support, purification of the enzyme was accomplished in 30-40 min in comparison to over 200 min using the Sepharose column. This example illustrates the advantages of new, stable polymeric supports for the rapid purification of biomolecules.

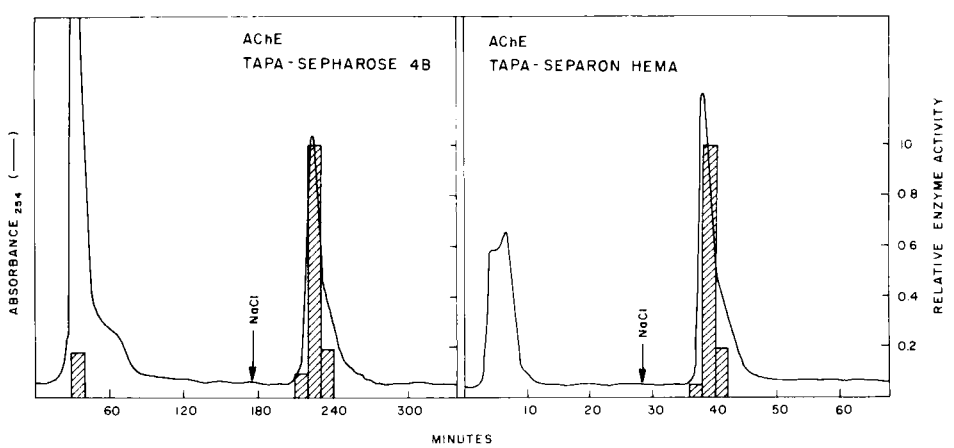


Figure 8.4. Purification of acetylcholinesterase (AChE) on a soft agarose (Sepharose 4B) or a pressure stable polymeric (Separon HEMA) bioaffinity chromatography support. Enzyme was eluted from the columns by applying buffer containing 1 M NaCl. Reproduced with permission from R. F. Taylor and I. G. Marenchic, J. Chromatogr., 317 (1984) 193-200.

A paper describing the effect of flow rate on the isolation of polyadenylated RNA using oligo (dT)-cellulose columns was published by Nadin-Davis and Menzl (1985). Muller et al. (1986) studied the influence of kinetic effects in high-performance bioaffinity chromatography of human thrombin on cross-linked polystyrenes modified with L-arginyl methyl ester. The influence of the flow rate and gradient slope on protein elution clearly demonstrates that kinetic effects are essential in the interaction process. The amount of thrombin adsorbed on the stationary phase increased when the flow rate was decreased from 2 to 0.2 ml/min.

8.2.2 Effect of temperature

Adsorption of a dissolved substance from the mobile phase onto the stationary phase is generally exothermic, and thus according to Le Châtelier' s principle, elevated temperatures will move the equilibrium in the direction of heat absorption. Under chromatographic conditions, an increase in temperature shifts the equilibrium to a higher relative concentration in the mobile phase, and a higher temperature usually leads to more rapid migration through the chromatographic bed. In general, the more exothermic the adsorption of a certain enzyme, the more sensitive it will be to temperature (Harvey et al., 1974).

Fig. 8.5 shows the effect of different temperatures on the binding (β) of the thermophilic enzymes alcohol dehydrogenase and phosphofructokinase from *Bacillus stearothermophilus* to N^6-(6-aminohexyl)-5'-AMP-Sepharose (Comer et al., 1975). Hence, with the thermophilic enzymes mentioned, on the contrary, the binding (β) increases first with increasing temperature. Destruction of the gel occurs at temperatures as low as 50°C, which is evident from the presence of the nucleotide in the eluate. Figs. 8.6 A and 8.6 B show that the pH dependence of the binding of both enzymes onto the immobilized AMP is also temperature dependent (Comer et al., 1975), while Fig. 8.6 C shows the dependence of the inflexion points of the binding on temperature.

The binding of lactate dehydrogenase onto N^6-(6-aminohexyl)-5'-AMP-Sepharose is so strong that the enzyme cannot be eluted, even with 1 M potassium chloride solution at 40°C. However, a linear gradient of NADH can be used for elution. Fig. 8.7 shows a

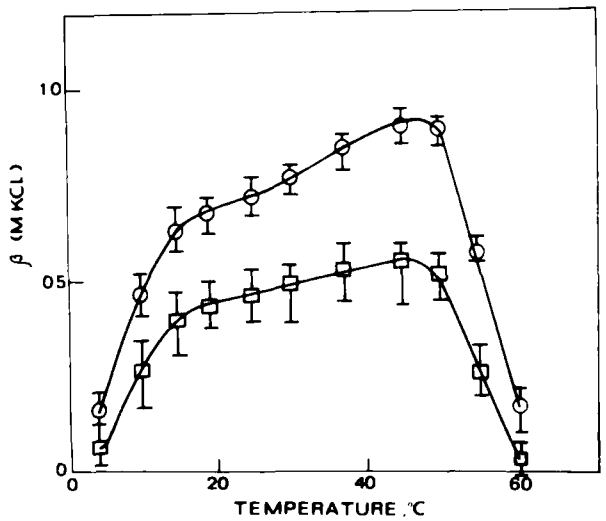


Fig. 8.5. Effect of varying temperature on binding (β) (defined in Fig. 4.6) of alcohol dehydrogenase (o) and phosphofructokinase (□) to N^6-(6-aminohexyl)-5'-AMP-Sepharose. The enzyme extract (0.5 ml, 81 U of phosphofructokinase, 20 U or 18.7 mg of alcohol dehydrogenase per millilitre) was dialysed against 10 mM potassium phosphate buffer, pH 6.8, and adsorbed onto a column of substituted AMP-Sepharose. Elution was performed with a linear salt gradient (0 to 1 M KCl, 40 ml) in 10 mM potassium phosphate buffer, pH 6.8, at 0.4 ml/min. Reproduced with permission from M. J. Comer et al., Eur. J. Biochem., 55 (1975) 201-209.

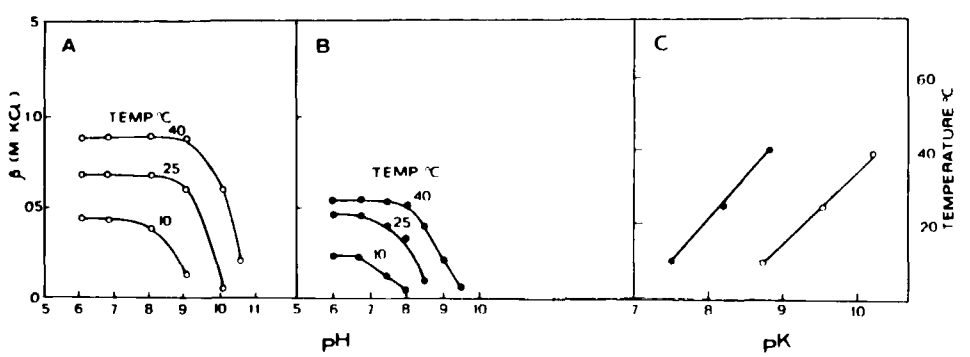


Fig. 8.6. Interdependence of binding (β) (defined in Fig. 4.6) to N^6-(6-aminohexyl)-5 '-AMP-Sepharose on both pH and temperature for (A) alcohol dehydrogenase and (B) phosphofructokinase. Conditions as in Fig 8.5. The buffer used was 10mM Tris-phosphate which was adjusted to the required pH at the relevant temperature. (C) Relationship between the point of inflection (apparent pK) and temperature for the binding of alcohol dehydrogenase (o) and phosphofructokinase (●). Reproduced with permission from M. J. Comer et al., Eur. J. Biochem., 55 (1975) 201-209.

plot of the concetration of NADH necessary for the elution of lactate dehydrogenase against temperature. With increasing temperature, the required concentration of the specific eluting agent decreases, represented by a linear Arrhenius plot. The corresponding adsorption energy is 54.6 kJ/mole (13.0 kcal/mole) (Harvey et al., 1974).

The effect of temperature on normal and diabetic levels of glycosylated hemoglobin, as determined by bioaffinity chromatography using m-aminophenyl-boronic acid on a support of cross-linked beaded agarose (GLYCO-GEL kit from Pierce, Rockford, Il, USA) was determined by Yatscoff et al. (1983). Improved separations at low temperature (4°C) of glycoproteins by concanavalin A-Sepharose bioaffinity chromatography in the presence of sodium dodecyl sulphate were described by Aono et al. (1985). Pimplicar et al. (1986) stated that, for successful bioaffinity chromatography of the anion

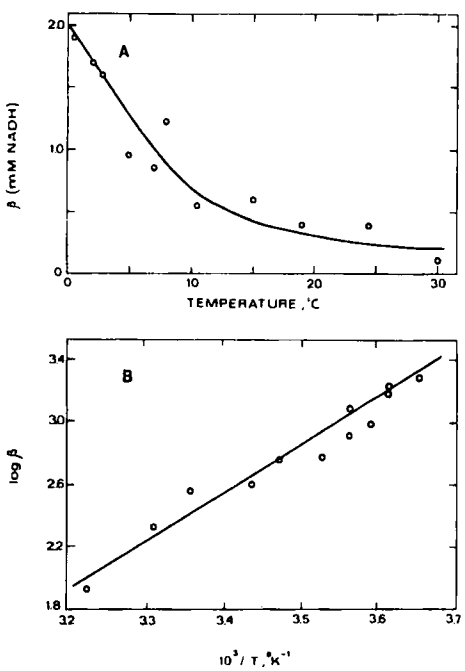


Fig. 8.7. Binding of pig heart muscle lactate dehydrogenase to N^6-(6-aminohexyl)-5'-AMP-Sepharose in response to temperature. The enzyme sample (5 U) containing 1.5 mg of bovine serum albumin (100 μl) was applied to a column (50 x 5 mm) containing 0.5 g of N^6-(6-aminohexyl)-5'-AMP-Sepharose (1.5 μmole/ml of AMP). (A) Binding was determined by the procedure reported in Fig. 3.7 using a linear gradient of NADH (0 to 5 mM, 20 ml total volume). (B) Arrhenius plot of the above data. Reproduced with permission from M. J. Harvey et al., Eur. J. Biochem., 41 (1974) 353-357.

transport protein from human erythrocyte membranes, the experiment must be carried out rapidly at 4°C. The unusually large dependence of bioaffinity purification of a glucose-containing oligosaccharide using immobilized monoclonal antibodies upon temperature has been studied by Lundblad et al. (1984) and Ohlson et al.(1988).

Fig. 8.8, published by Regnault et al. (1988), shows the effect of temperature on the adsorption capacity of fibronectin (Fn) from human plasma. The development of large-scale bioaffinity purification of Fn required the optimization of both adsorption and elution conditions. The adsorption capacity depended on the amount of gelatin coupled to the Sepharose, the residence time, the temperature and the amount of Fn loaded on the adsorbent. The maximum yield of Fn was obtained at temperatures below 10°C and yields then dropped at a uniform rate to zero at 37°C. Structual changes probably take place and the fixation sites on the immobilized gelatin are probably altered. This alteration in Fn at temperatures between 4°C and 37°C had already been reported by Brown et al. (1987).

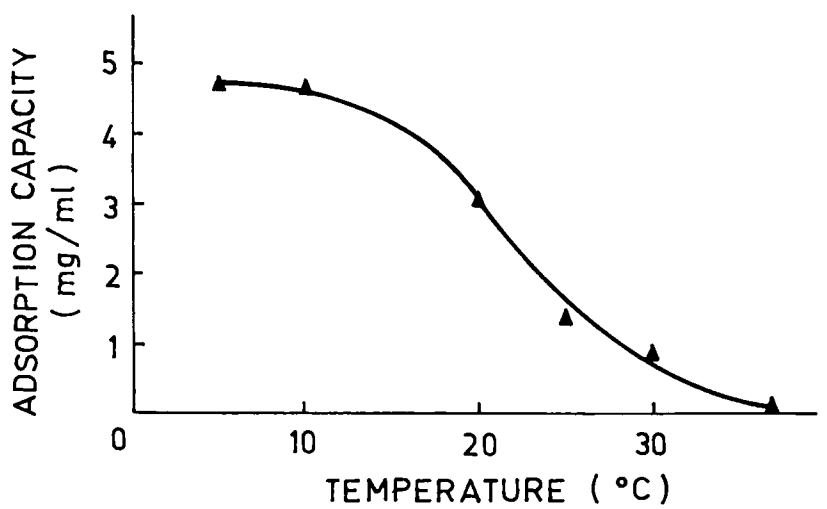


Fig. 8.8. Effect of temperature on adsorbtion capacity of fibronectin (Fn). Human plasma (100 ml) was applied to the adsorbent (2 x 1.6 cm) at a linear flow rate 50 cm/h. The column was thermostated and all solutions passed through were placed in a water bath. Each experiment was performed with an unused gelatin-Sepharose gel (3 mg of gelatin/ml of gel). Reproduced with permission from V. Regnault et al., J. Chromatogr., 432 (1988) 93-102.

The dependence of biospecific adsorption and desorption on temperature has also been studied by Rozie et al. (1991). They determined the adsorption and desorption characteristics of bacterial α-amylases on cross-linked potato starch at 4°C and 20°C. Equilibrium experiments revealed that the degree of adsorption that can be achieved at equilibrium increases with decreasing temperature. Adsorption equilibrium constants decrease with increasing temperature, showing that association is caused by van der Waals forces.

In conclusion to this section on the effect of temperature in bioaffinity chromatography, it should be stressed that the effect of temperature should always be borne in mind, because reproducible results can be obtained only if the temperature is carefully controlled, especially in analytical applications. The influence of surface character of protein molecules on their stability has been discussed in Section 4.3.

8.2.3 Effect of pH, ionic strength and nature of molecules suitable for biospecific sorption

The catalytic effect of enzymes is usually limited to a narrow range either side of the so-called optimum pH, which reflects the ionization of both the enzyme and its substrate. A shift from the optimum pH range results in a decrease in both the rate of the enzymatic reaction and the affinity of the system for the substrate. Ionic and hydrophobic interactions, operative in site where the enzyme binds with its substrate or inhibitor, are further considerably influenced by ionic strength. From this it follows that both pH and ionic strength are important factors in both sorption and desorption. The effect of pH and of ionic strength on the sorption of proteinase chymotrypsin on immobilized benzyloxycarbonylglycyl-D-phenylalanine, determined by Turková et al. (1976), is shown in Fig. 8.9. A decrease in pH and an increase in the ionic strength shift the equilibrium in favour of free chymotrypsin. Kasai and Ishii (1975) determined an increase in the dissociation constant of trypsin with immobilized glycylglycyl-L-arginine from 0.23 to 0.33 mM on addition of 0.3 M sodium chloride, and to 0.75 mM on addition of 1.0 M sodium chloride.

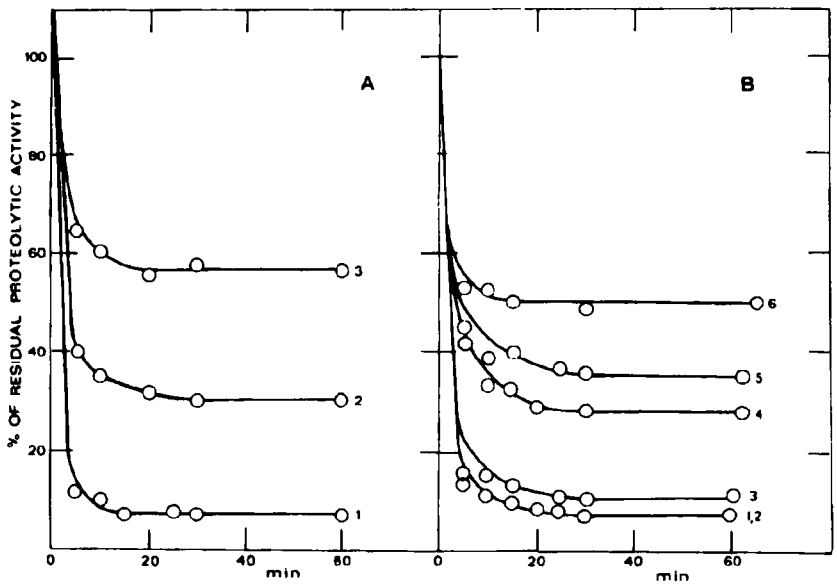


Fig. 8.9. Adsorption of chymotrypsin. (A) To Z-Gly-D-Phe-NH_2-Spheron as a function of pH: (1) 0.1 M Tris-HCl buffer, pH 8.0;(2) 0.1 M Tris-HCl buffer, pH 7.2; (3) 0.1 M Tris-maleate buffer, pH 6.0. (B) To Z-Gly-D-Phe-NH_2-Spheron as a function of ionic strength, with Tris-HCl buffers, pH 8.0, of the following concentrations: (1) 0.05 M; (2) 0.1 M; (3) 0.25 M; (4) 0.35 M; (5) 0.5 M; (6) 1M. Data from J. Turková et al., Biochim. Biophys. Acta, 427 (1976) 586-593.

The effect of pH on the binding of lactate dehydrogenase on affinity sorbents such as N^6-(6-aminohexyl)-5 '-AMP-Sepharose and 6-aminohexanoyl-NAD^+-Sepharose is shown in Fig. 8.10 (Lowe et al., 1974b). Up to pH 8 the interaction of the enzyme with the immobilized ligand is independent of pH. Depending on the nature of the immobilized affinant, above pH 8 the amount of the bound enzyme decreases with pH. With N^6-(6-aminohexyl)-5 '-AMP-Sepharose this decrease is characterized by an apparent pK value of about 9.7, and with 6-aminohexanoyl-NAD^+-Sepharose by a pK of 8.5. This difference in pK values may be caused by the effect of different preparations of the affinity adsorbents. While in the former support an already spaced nucleotide, N^6-(6-aminohexyl)-5 '-AMP, is attached to Sepharose, in the latter the attachment of NAD^+ to 6-aminohexanoyl-Sepharose may leave residual charged groups on the carrier. Winer and Schwert (1958) showed that the binding of NAD^+ to lactate dehydrogenase in free

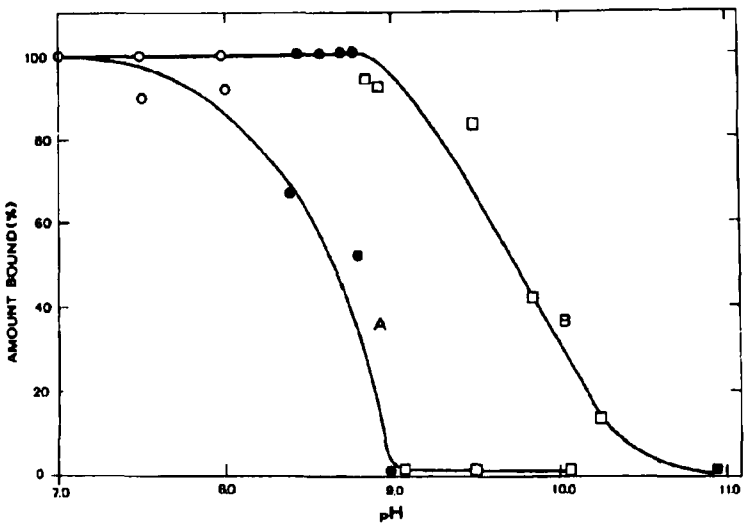


Fig. 8.10. Effect of pH on the binding of pig heart muscle lactate dehydrogenase to (A) ε-aminohexanoyl-NAD^+-Sepharose and (B) N^6-(6-aminohexyl)-5 '-AMP-Sepharose. The results are expressed as percentages based on determinations at pH 6.5: (A) β/β_{max} at pH 6.5 determined from linear KCl gradient (0 to 1 M); (B) percentage of enzyme activity eluted by NADH pulse (200 μl, 5mM). The equilibration buffers used were adjusted to a constant conductivity (3.3 $m\Omega^{-1}$) by the addition of 1 M KCl, (o) 10mM KH_2PO_4-KOH; (●) 10 mM tricine-KOH; (□) 10mM glycine-KOH and (■) 10 mM K_2HPO_4. The enzyme sample (100 μl) plus bovine serum albumin (1.5 mg) was applied to a column containing 0.5 g of N^6-(6-aminohexyl)-5'-AMP-Sepharose (1.5 μmole/ml of AMP) according to the procedure reported in Fig. 3.7. Reproduced with permission from C. R. Lowe et al., Eur. J. Biochem., 41 (1974) 347-351.

solution is affected by the group on the surface of enzymes with pK≈ 9.7, which is in good agreement with the value determined for N^6-(6-aminohexyl)-5 '-AMP-Sepharose.

In order to check this assumption, Lowe et al. (1974b) determined titration curves for 6-aminohexanoyl-NAD^+-Sepharose, unmodified Sepharose, 6-aminohexanoyl-Sepharose and, for comparison, also for corresponding derivatives of cellulose. On the basis of the curves obtained they demonstrated a distinct carrier effect (see Fig. 8.11).

If the affinity adsorbent is prepared from a charged affinant, then a decrease in its affinity for the complementary macromolecule takes place when the ionic strength increases. For sorption a low ionic strength should be used, which then has the undesirable result of sorbing an increased amount of contaminating proteins; these are also increasingly sorbed owing to the ionic bond with the ligand.

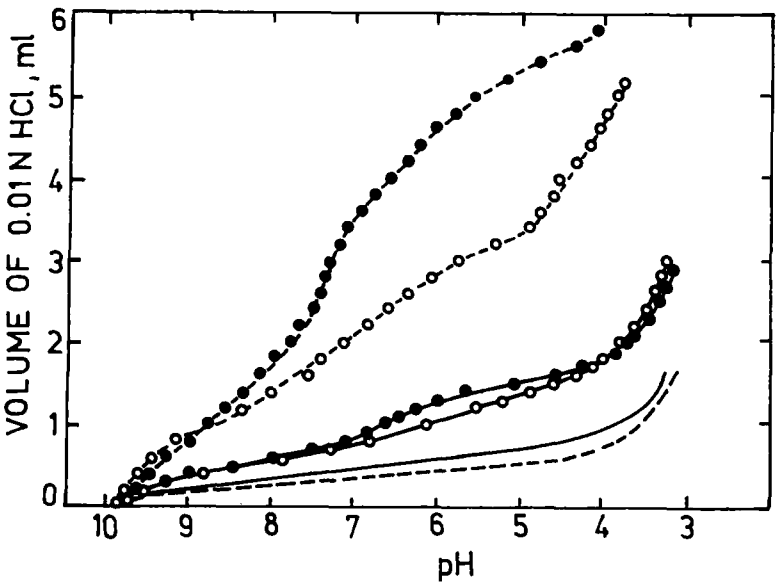


Fig 8.11. Titration curves of 6-aminohexanoyl-NAD^+ polymers. The solid lines represent Sepharose 4B alone; o, coupled to 6-aminohexanoic acid; •, as 6-aminohexanoyl-NAD^+-Sepharose. The broken lines represent cellulose alone; o and • as above. Reproduced with permission from C. R. Lowe et al., Eur. J. Biochem., 41 (1974) 347-351.

The practical utilization of pH during the sorption of neutral protease from *Bacilus subtilis* on Sepharose with linked glycyl-D-phenylalanine (through a spacer 23 atoms long, formed by combination of triethylenetetramine, succinic acid and ethylenediamine) is shown in Fig. 8.12. The maximum adsorption of neutral protease occurs at pH values corresponding to minimum values of K_m. Between pH 5 and 6.5 neutral protease is effectively adsorbed and is thus separated both from subtilisin and other proteins present in the culture filtrate. An effective separation of neutral protease from subtilisin does not take place at higher pH values. Neutral protease is set free from the complex with the immobilized affinant by increasing the pH to values at which the binding of substrate is already weak, but at which the enzyme is still not denatured. The optimum pH for the elution of neutral protease depends on its affinity towards the affinity ligand and on the pH dependence of this affinity, as well as on the concentration of the immobilized ligand (Walsh et al., 1974). Neutral protease is a metalloenzyme and is therefore inhibited by 1,10-phenanthroline and EDTA. The presence of these chela-

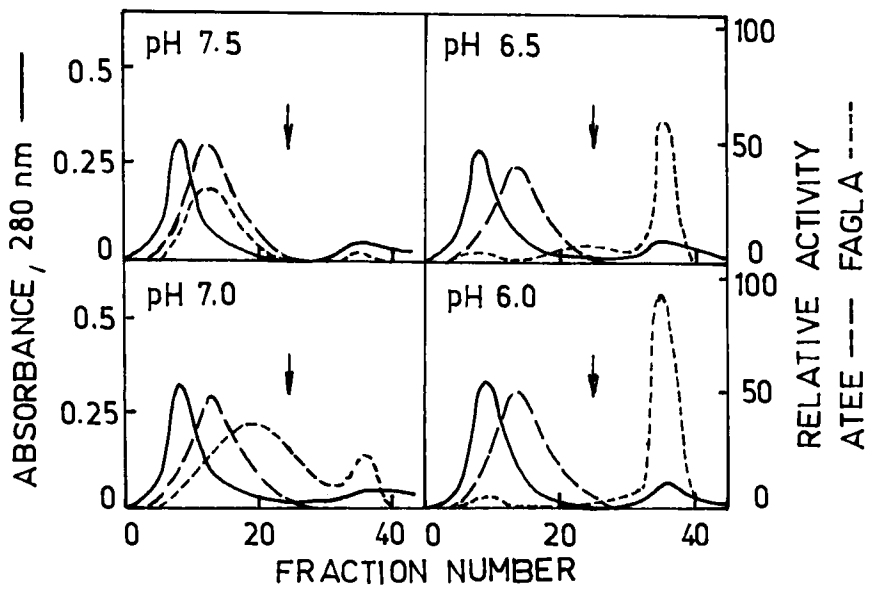


Fig. 8.12. Effect of pH on the adsorption of neutral protease on Sepharose 4B substituted with triethylenetetramine, succinic anhydride, triethylenetetramine and chloroacetyl-D-phenylalanine. Crude enzyme (10 mg) was dissolved in 0.1 ml of equilibrating buffer and applied to an affinity column (220 x 6 mm) equilibrated with 100 mM sodium chloride, 10 mM calcium chloride, containing 5 mM Tris (pH 7.5 or 7.0) or 5 mM 2-(N-morpholino)ethanesulphonic acid (pH 6.5 or 6.0). After elution for 1h at 25 ml/h, each column was washed with 100 mM sodium chloride, 10 mM calcium chloride, 50 mM Tris (pH 9.0), as indicated by the arrows. Neutral protease and subtilisin are identified by catalytic activities toward 3-(2-furylacryloyl)-glycyl-L-leucinamide (FAGLA) and acetal-N-tyrosine ethyl ester (ATEE). Reproduced with permission from K. A. Walsh et al., Methods Enzymol., 34 (1974) 435-440.

ting agents at a concentration of 1 - 5mM prevents the adsorption of the enzyme onto the specific sorbent mentioned.

It should be borne in mind that the optimum pH for the sorption of a particular enzyme need not be identical with the optimum pH for catalysis. Kasai and Ishii (1975) found pH 5.6 to be optimum for the sorption of trypsin onto Sepharose with coupled glycylglycylarginine, which is not the same pH as that required for catalysis. It is therefore useful to determine optimum conditions for the formation of each individual complex of the isolated substance with the immobilized affinant. These values can be obtained most easily by using a batchwise arrangement, described in Fig. 8.9.

Fig. 8.13 shows the retardation of L-histidinol phosphate aminotransferase on L-histidinol phosphate-coated agarose, which increases with increasing concentration

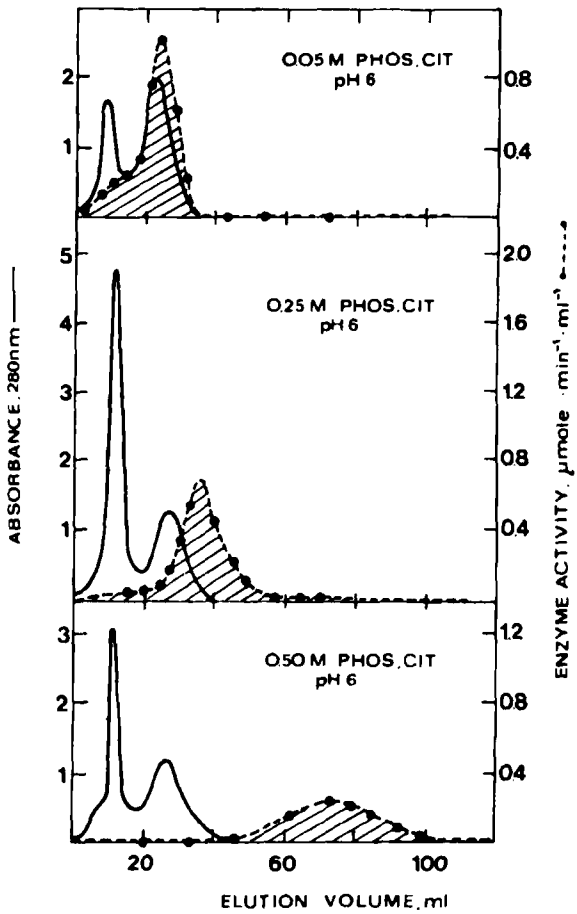


Fig. 8.13. Increased retardation of L-histidinol phosphate aminotransferase on L-histidinol phosphate-coated agarose upon increasing the concentration of a phosphate-citrate buffer. Buffers used contained Na_2HPO_4 (at the indicated concentration) and were adjusted to pH 6.0 with citric acid. Reproduced with permission from S. Shaltiel et al., Biochemistry, 13 (1974) 4330-4335.

of the phosphate-citrate buffer (Shaltiel et al., 1974). In this instance, however, this increase in retardation cannot be attributed to the increase in the ionic strength, because the position of the enzyme peak does not change if the ionic strength of the 0.05 M phosphate buffer is increased by addition of 0.9 M sodium chloride solution.

A retardation of the enzyme then takes place not only in the phosphate-citrate medium, but also in a sulphate or phosphate buffer; however, this retardation takes

place, of course, to varying degrees. In the series of carboxylic acids used for the adjustment of the pH of 0.5 M disodium hydrogen orthophosphate solution to 6.0, it was found that the retardation of the enzyme increases with increasing valency in the order acetate < succinate < citrate. The effect of individual ions on the hydrophobic interactions can be expressed by the following scheme (from Pharmacia promotional literature):

⇐ Increasing salting-out effect

Anions: PO_4^{3-}, SO_4^{2-}, $CH_3.COO^-$, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-

Cations: NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}

Increasing chaotropic effect ⇒

At constant ionic strength, the effect of ions on adsorption depends strongly on the nature of the ions, some of them causing a decrease and others an increase, which is attributed to lyotropic rather than purely electrostatic effects. The effect of anions classified in the order of decreasing adsorption, thiocyanate = iodide > chloride > acetate > citrate, is identical with Hofmeister' s series of neutral salts (Green, 1931).

If the high ionic strength of the starting buffer does not impair the formation of the biospecific complex, it is advantageous to use it because it decreases the non-specific adsorption of polyelectrolytes on any charged groups which may possibly occur on the coupled affinant. It is therefore recommended to add a ca. 0.5 M sodium chloride solution to the sorption buffer. The effect of ionic strength on the binding of β-galactosidase on Sepharose with attached p-aminophenyl-β-D-thiogalactopyranoside is shown in Table 8.3. With increasing ionic strength, the amount of the sorbed enzyme decreases, but, by contrast, its specific activity increases (Robinson et al., 1972).

The necessity for adding metal ions or other specific factors to the sorption medium is illustrated in Table 8.4. The binding of mitochondrial ATPase (dispersed with Triton X-100) on Sepharose with covalently bound inhibitor depends on the presence of magnesium ions and ATP (Swanljung and Frigeri, 1972).

Table 8.3.

Effect of ionic strength of buffer on β-galactosidase binding onto a column of agarose substituted with p-aminophenyl-β-D-thiogalactopyranoside

(Bed dimension, 114 x 17 mm; flow-rate, 2.0 ml.min^{-1}.cm^{-2}.)

Ionic strength	*Protein bound (mg)*	*Activity bound (units)*	*Specific activity (units/mg)*
0.010	75	6000	80
0.020	32	5500	190
0.035	14	3600	320
0.050	1.2	350	340

The requirement of a full supplement of manganese and calcium ions is necessary for the biospecific sorption of concanavalin A to Sephadex or for the optimum binding of carbohydrates to Con A (Karlstam, 1973). The presence of calcium is important, e.g. for bioaffinity chromatography of G-actin-binding protein on DNAse I-bound agarose (Hosoya et al., 1982), or for bioaffinity purification of human tissue factor (Guha et al., 1986). Clostridial collagenase did not bind to immobilized collagen in the absence of calcium. Evans (1985) exchanged calcium ions for lanthanide ions and he described the lanthanide-enhanced bioaffinity chromatography of this collagenase. Enzymes from halophilic bacteria can function at high salt concentrations (2 - 4 M sodium or potassium chloride) but are inactivated at low salt content (< 1M sodium or potassium chloride).

Table 8.4.

Effect of Mg^{2+} and ATP on the amount of ATPase bound to ATPase inhibitor-Sepharose column

An extract containing adenosine 5' -triphosphatase (ATPase) was applied to ATPase inhibitor-Sepharose column equilibrated with 0.2 M sucrose, 15 mM Tris-N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (Tris-TES) buffer (pH 6.6), 3mg/ml Triton X-100 and additions as indicated in the table. The amount of enzyme bound was calculated as the amount eluted with 0.2 M sucrose, 45 mM Tris-TES buffer (pH 8.75), 0.5 M KCl, 1 m M EDTA and 0.3 mg/ml Triton X-100.

Additions	*ATPase applied (nmole/min)*	*ATPase bound (nmole/min)*	*Yield (% bound of that applied)*
None	336	39	12
1 mM $MgSO_4$	192	35	18
0.5 mM $MgSO_4$ + 0.5 mM ATP	678	221	33

Thus, the presence of a high salt concentration should be necessary for the biospecific adsorption of these enzymes. Leicht (1978) described enhanced sulphate-mediated bioaffinity purification of glutamate dehydrogenase on $NADP^+$-Sepharose from a crude extract of halophilic bacteria. He reported similar results for the isolation of glucose-6-phosphate dehydrogenase from crude extracts of *Escherichia coli*. The effects were shown to be biospecific, suggesting that the strength of the interaction between the protein and immobilized coenzymes is a function of the sulphate concentration.

Bioaffinity chromatography is a particularly attractive method for isolating receptors because the method presumably mimics the receptor interactions existing in nature and can therefore, be expected to show high specificity. Pardoe and Burness (1980) used this method for the purification of human erythrocyte receptors for encephalomyocarditis and influenza viruses by the use of commercially available wheat germ agglutinin (WGA)-Sepharose. They used detergents to solubilise membranes and they transferred the solubilised material directly to the bioaffinity column without intermediate purification. Detergents apparently had no gross effect on the binding of receptor on WGA-Sepharose since identical profiles were obtained when glycophorin was chromatographed in their presence or absence. Doudeur and Jacquet (1980) studied the interactions of lectins attached to Sepharose 4B with membrane glycoproteins in the presence of ionic (sodium deoxycholate, DOC and sodium dodecyl sulphate,SDS) and non-ionic (Triton X-100) detergents at constant detergent concentrations in relation to pH and ionic strength. Fig. 8.14 shows these effects on the efficiency of galactoprotein binding to beads coated with concanavalin A (Con A) and *Ricinus* lectin (RCA). The same galactoglycoproteins and immobilized lectins were studied by the same authors at different detergent concentrations. On beads containing concanavalin A, the efficiency of binding and elution of glycoproteins was unaffected by the concentration of Triton X-100. By contrast, concentrations greater than 0.1% of SDS or 0.5% of DOC caused a marked decrease in the binding and elution efficiencies. On *Ricinus communis* beads, the efficiencies of binding and elution were unaffected by the concentrations of Triton X-100 or DOC. However, in the presence of SDS these efficiencies were dramatically decreased at concentrations greater than 0.05%. The

activities of lectins and their derivatives after immobilization on polyacrylic hydrazide-Sepharose in solutions of detergents Nonidet P40, Triton X-100, dimethyldodecylglycine, DOC and dodecyltrimethylammonium bromide, at concentrations 0.1, 1.0 and 2.5%, have been studied by Lotan et al. (1977). The effects of detergents on the specific binding of fetuin to and elution from columns of immobilized lectins were less severe when compared with lectins in solution, suggesting that the lectins are stabilized by covalent attachment to agarose beads. Nonionic detergents did not affect the binding efficiency of the immobilized lectins tested at concentrations used for membrane solubilization, while cationic and zwitterionic detergents caused significant inhibition of ConA - and soybean agglutinin (SBA)-Sepharose activities. In DOC (>1%) only RCA-Sepharose retained its activity, whereas the activities of the other lectins were

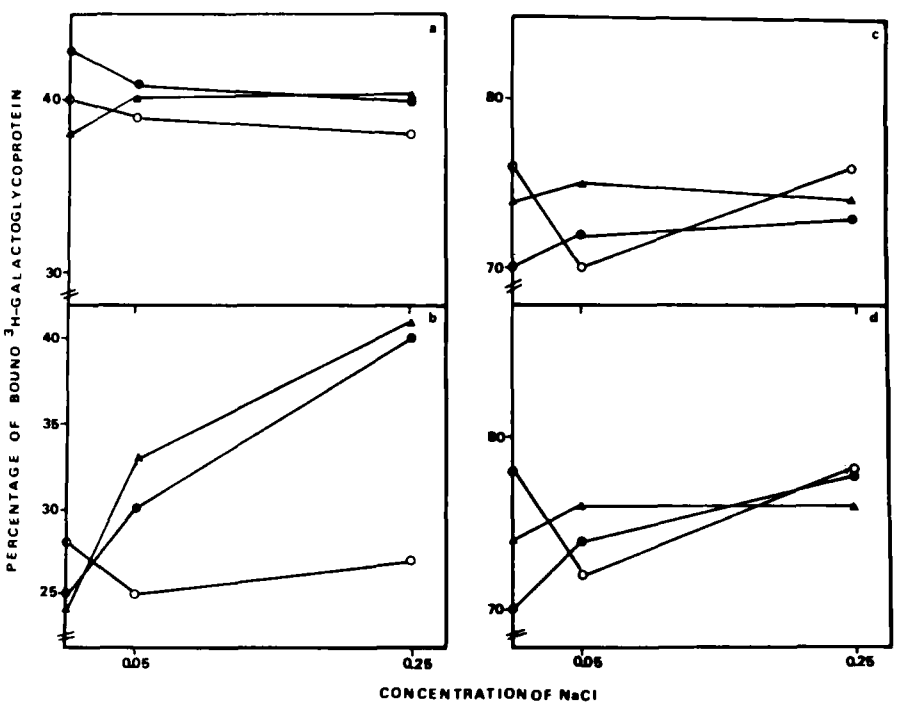


Fig. 8.14. Effects of pH and ionic strength on efficiency of [^{3}H]galactoglycoprotein binding to concanavalin A beads (left) and Ricinus lectin beads (right) in the presence of a constant concentration of detergent. The binding was performed using 0.02M Tris-HCl buffer at pH 7 (a,c) or pH 7.8 (b,d) containing different concentrations of sodium chloride and DOC (0.25%), (●), Triton X-100 (0.25%) (▲), or SDS (0.05%) (o). Each point in the figure represents the average value obtained from four separate experiments. Reproduced with permission from M. Doudeur and M. A. Jacquet, J. Chromatogr., 195 (1980) 197-203.

reduced dramatically. A low concentration of SDS (0.05%) inhibited only the activity of immobilized SBA; but at higher concentration (0.1%) and prolonged periods of incubation (16h, 23°C) most of the lectins were inactivated. The conditions for the optimum use of detergents in lectin bioaffinity chromatography were detailed in the same paper. A rapid method for removal of detergents from protein solution has been described by Horigome and Sugano (1983).

8.2.4 Practice of sorption

Immunoadsorption is a simple and effective method for purifying antigens and antibodies. Zoller and Matzku (1976) analysed a number of possibilities for eliminating the effects of non-biospecific binding. For antigen purification they recommended pre-elution with a pH 10.0 buffer of high ionic strength (1.0 M). A major problem in purifying antibodies is the non-biospecific binding of immunoglobulin G (IgG). This can be eliminated by using a non-biospecific immunoadsorbent column, where specific antibodies are not, or are only weakly bound, and IgG with irrelevant specificity but exhibiting high non-biospecific interaction is retained.

Sairam (1981) summarized the isolation of hormonal proteins and their antibodies by bioaffinity chromatography. Hormones can be effectively concentrated from very dilute solutions such as urine or other fluids, including culture media. The occasional reduction in flow rates is mostly attributable to small amounts of lipid materials that may be present in some serum samples or to extracts that occlude the top surface of the gel bed. This can be avoided by centrifugation at high speed and careful handling. Concerning dye-ligand affinity chromatography, Metcalf et al. (1981) found that compounds such as fatty acids, nucleotides and lipids must be removed from protein samples before chromatography on immobilized Cibacron Blue 3G-A in order to produce reliable and reproducible results.

The differences in the properties of the same affinant attached to agarose or cellulose is shown in Fig. 8.11. McConathy et al. (1985) studied the effect of varying cyanogen bromide concentrations and the pH used for activation of Sepharose 4B-CL on the binding capacity of immobilized antibodies against lipoprotein B (Anti-LP-B). Table

8.5 shows the percentage of coupled anti-LP-B and immunosorbent capacity for apolipoprotein B (ApoB) in mg per ml of agarose, as determined by electroimmunoassay.

Table 8.5.

Effect of varying cyanogen bromide concentration and pH on the binding capacity of immobilized antibodies against lipoprotein B (Anti-LP-B).

The same preparation of affinity-purified anti-LP-B (1 mg/ml of gel) was used in this study.

Cyanogen bromide concentration (mg/ml of gel)	*pH*	*Anti-LP-B coupled (%)*	*Immunosorbent capacity (mg ApoB per ml of gel)*
50	8.0	90	0.86
50	7.5	85	0.76
50	7.0	78	0.65
34	8.0	81	0.78
34	7.5	77	0.65
34	7.0	74	0.61
16	8.0	68	0.45
16	7.5	66	0.33
16	7.0	57	0.28

To minimize the non-biospecific adsorption of trypsin-, chymotrypsin- and subtilisin-digest fragments of fibronectin to actin-Sepharose and other protein-Sepharose derivatives, Keski-Oja and Yamada (1981) pre-washed the columns extensively with 8M urea and 1M KBr in 20 mM Tris/HCl buffer pH 8.0 and then equilibrated with column buffer (20 mM Tris/HCl buffer pH 8.0, 50 mM KCl, 5 mM $MgCl_2$). They then washed the columns first with 2 column volumes of bovine serum albumin (1 mg/ml) in column buffer, followed by extensive washing with the column buffer. Burtscher et al. (1986) prepared a biospecific adsorbent for ADP-ribosyl-transferase (EC 2.4.2.30) by coupling 3-aminobenzamide to Sepharose 4B. Using this material, ADP-ribosyl-transferase from human placenta was purified from crude extract to homogeneity within a few hours. However, both the hydroxyapatite and the phosphocellulose steps were necessary for this purification method. Applying the crude extract directly onto 3-aminobenzamide-Sepharose did not yield a homogeneous enzyme.

Phillips and Frantz (1988) isolated antigen-specific lymphocyte receptors by high-performance immunoaffinity chromatography, using immobilized monoclonal antibodies directed against the antigen. The use of the antigen to protect the molecular shape and structure of the receptor during isolation appeared to be a convenient method and provided additional sites for attachment to the immobilized antibody. In this way the receptor did not have to suffer from both the solubilization and interaction with an antibody.

8.3 CONDITIONS FOR ELUTION

While the substances that have no affinity for the attached affinant are usually eluted with the hold-up volume, the biospecifically adsorbed material usually requires displacement with a specific elution agent or by a change in solvent composition, pH, in temperature, light, or using electrophoretic or magnetic fields. Fig. 8.15 shows the elutions of alcohol dehydrogenase and phosphofructokinase from the column of the AMP-Sepharose by stepwise elution with biospecific agents (A), using a KCl gradient (B) and a pH gradient (Comer et al., 1975).

8.3.1 Elution with competitive affinity ligands

The scheme for the formation of complexes in a system containing an isolated enzyme (E), specifically sorbed on an immobilized ligand (L), and a soluble inhibitor (I) is illustrated in Fig. 8.16 (Akanuma et al., 1971). The presence of the inhibitor in the mobile phase can affect the migration of the enzyme through the column in three different ways:

(1) Competitive effect. If the ternary complex (ELI) is less stable than the binary complex (EI), the increase in the concentration of the inhibitor (I) increases the proportion of the enzyme in the mobile phase, thus reducing the retardation of the enzymes through the column.

(2) Non-competitive effect. If the stability of the ternary complex (ELI) is approximately the same as that of the binary complex (EI), the binding of the inhibitor (I) will not affect the affinity of the enzyme for the immobilized ligand. In this instance the presence of an inhibitor will have no effect on the retardation of the enzyme.

(3) Uncompetitive effect. If the stability of the ternary complex (ELI) is greater than that of the binary complex (EI), then the presence of the free inhibitor (I) will decrease

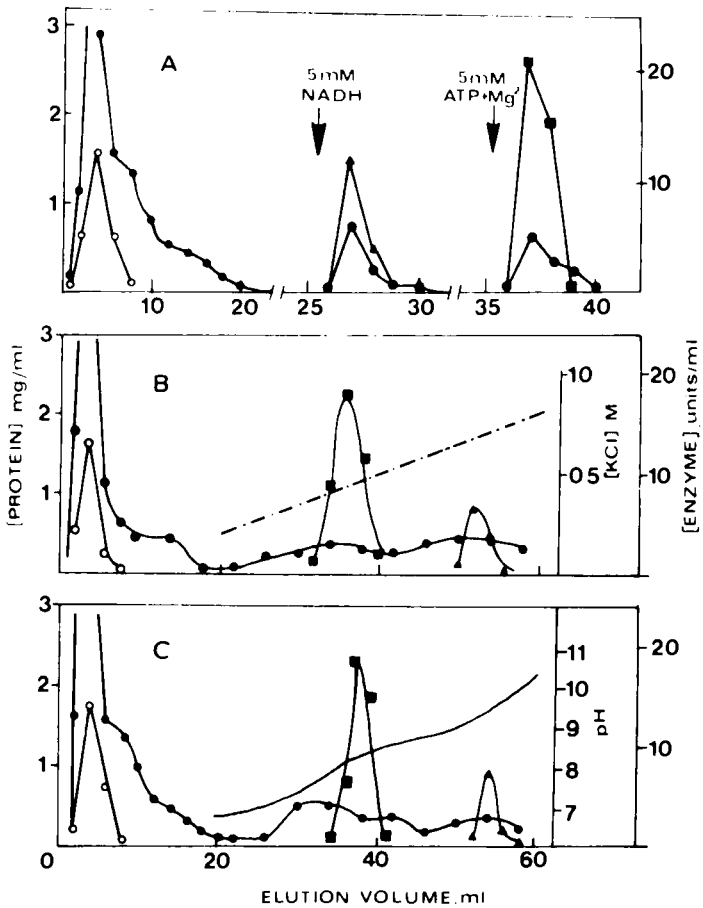


Fig. 8.15. Specific elution of alcohol dehydrogenase and phosphofructokinase from N^6-(6-aminohexyl)-5 '-AMP-Sepharose. (A) The enzyme extract (0.5 ml) (40.5 U of phosphofructokinase, 10 U or 9.9 mg of alcohol dehydrogenase per millilitre) was dialysed exhaustively against 10mM phosphate, pH 6.8, containing 0.2 M KCl (this buffer was also used for equilibration) and adsorbed on a column of the AMP-Sepharose. The matrix was then washed sequentially with (a) equilibration buffer (24 ml); (b) 5 mM NADH in the same buffer (5 ml); (c) buffer (5 ml); (d) 5 mM ATP, 5 mM Mg^{2+} in buffer (5 ml), flow rate 0.4 ml/min. (B) Using a KCl gradient with conditions essentially as in A. Following adsorption, the column was washed with 10 mM phosphate, pH 6.8, (20 ml) containing 0.2 M KCl. The linear gradient (0.2 to 0.8 M KCl) (40 ml) was applied in the same buffer. (C) Using a pH gradient with conditions essentially as in A, except that the sample was equilibrated against 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate (pH 6.8). Following adsorption, the column was washed with the same buffer (20 ml). A pH gradient (40 ml) was then applied (10 mM hydroxyethylpiperazine-ethane sulphate, 5 mM glycylglycine, pH 10.4). (●) Protein; (o) glyceraldehyde-3-phosphate dehydrogenase; (▲) alcohol dehydrogenase; (■) phosphofructokinase. Reproduced with permission from J. J. Comer et al., Eur. J. Biochem., 55 (1975) 201-209.

the proportion of the enzyme in the mobile phase and thus increase the binding of the enzyme. If the ternary complex is less stable than the binary complex, an increase in the concentration of the free affinity ligand will lead to the elution of the enzyme from the column.

Many examples of elution with competitive ligands can be found in the literature. Porath (1972) described the isolation of trypsin from pancreatic extracts, carried out on Sepharose with bonded trypsin inhibitor. Chymotrypsin was liberated using a solution of the competitive inhibitor of tryptamine, while trypsin was eluted with a benzamidine solution. For the elution of enzymes from the affinity matrix with a solution of competitive inhibitor it is important that the latter should be present in a higher concentration than that of the affinant bound in the matrix. This is true under the assumption that both the free and the bound inhibitor have approximately equal affinities towards the isolated enzyme. It is also possible to elute the enzyme with a solution of affinant that actually has a higher affinity. In many instances of the elution of specifically adsorbed enzymes with buffers that contain high concentrations of competitive inhibitor, the enzyme is obtained at a greater dilution than if the elution is produced by changes in pH or ionic

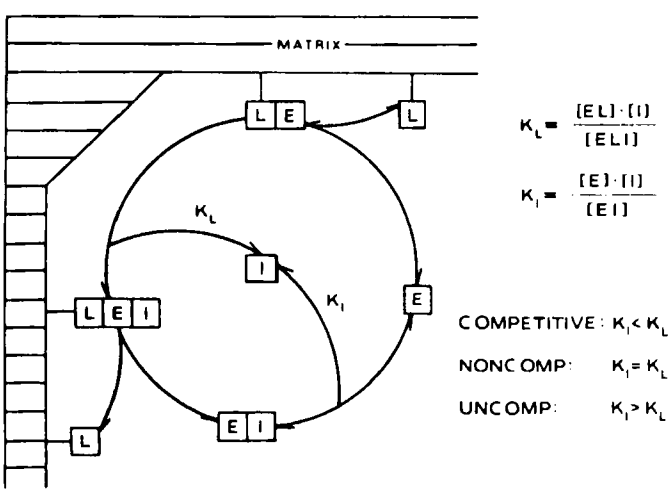


Fig. 8.16. Possible molecular species of a given enzyme within the affinity matrix. L, Covalently fixed affinity ligand to matrix; I, unfixed inhibitor; E, enzyme. Reproduced with permission from H. Akanuma et al., Biochem. Biophys. Res. Commun., 45 (1971) 27-33.

strength. This phenomenon is especially striking in interactions that involve high affinities, when elution from the affinity sorbent may represent a time-dependent process, even when high concentrations of the competitive affinant in the buffer are employed (Lowe and Dean, 1974). The rate of dissociation of the enzyme from the stationary phase is a first-order process, depending on the concentration of the complex alone, and independent of the concentration of the free competing inhibitor. The free substrate or the inhibitor reduces the tendency of the enzyme to re-associate with the immobilized ligand by preferential formation of a soluble complex, assuming that they are present in a sufficiently high concentration.

If the affinity of the complex is very high ($K_L \leq 10^{-7}$M), the time necessary for complete dissociation of the complex may be considerable. In order to decrease the amount of the enzyme bound to the immobilized affinity ligand to one-half of the original value, the so-called half-life, $t_{1/2}$, should be known, which is given by the expression

$$t_{1/2} = \frac{\ln \frac{E_o}{[E]}}{k_{-1}} = \frac{ln2}{k_{-1}} = \frac{0.693}{k_{-1}} \qquad (8.1)$$

where [E] is the concentration of the free enzyme, E_o is the initial concentration of the bound enzyme and k_{-1} is the rate constant of dissociation of the complex:

$$E + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EL$$

The relationship of the elution with time can be circumvented by temporarily interrupting the flow through the column after the soluble inhibitor has been allowed to soak into it. The necessary time is given by the nature of k_{-1}; as it is difficult to determine k_{-1}, in practice this is done more or less empirically. An alternative means of enhancing specific elution consists in inducing simultaneous changes in pH, ionic strength and temperature. As the affinity of adsorption decreases with increasing temperature, the temperature increase may substantially affect the elution with the

competitive inhibitor. The effect of various elution systems on lactate dehydrogenase bound to AMP-Sepharose (Ohlsson et al., 1972) is evident from Fig. 8.17. While a quantitative elution of lactate dehydrogenase with 0.5 mM NADH takes 1 h, only 30% of it is eluted after 20 h with 0.5 mM NAD^+ + 0.5 mM L-lactate.

An example of gradient elution with a specific eluent is shown in Fig. 8.18, which illustrates the elution of native lysozyme specifically sorbed on tri-(N-acetylglucosamine)-Sepharose by means of gradients of tri-(N-acetylglucosamine) having a variety of steepnesses (Cornelius et al., 1974). The gradient of the specific elution reagent was always applied after washing with the starting buffer. The amount of the native enzyme applied onto the column, the conditions of the columnar arrangement, and the composition of the buffer were the same in all three instances. The gradients differed only in the different rate of change of concentration of tri-(N-acetylglucosamine), as it is evident from the course of the procedure. Protein recoveries were approximately 90%. Lysozyme was eluted with all three gradients at a $5x10^{-5}$M

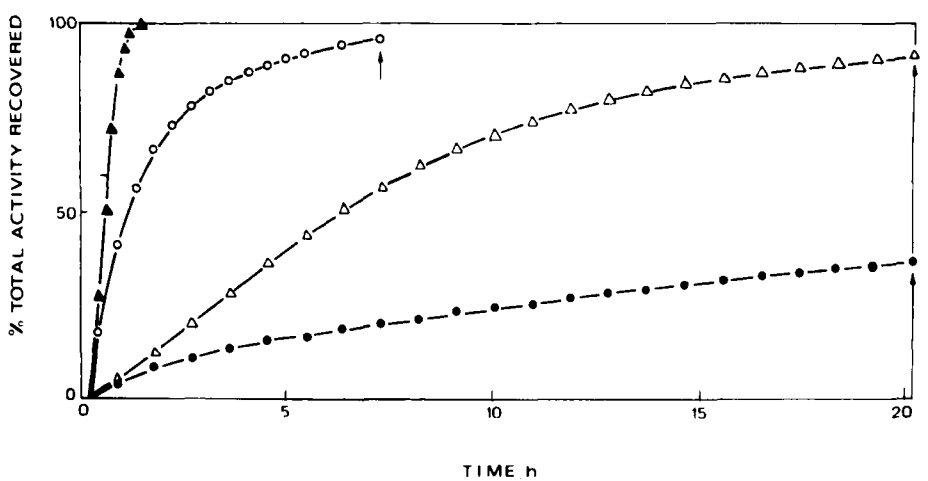


Fig. 8.17. Efficiency of different eluent systems on ox heart lactate dehydrogenase (LDH) bound to an AMP-Sepharose column (40 x 15 mm containing 1.0 g of wet gel). LDH (0.1 mg) in 0.5 ml of 0.1 M phosphate buffer, pH 7.5, was applied. The following systems in the same buffer were used: 0.5 mM NAD^+ + 0.5 mM L-lactate (●); 0.5 mM NAD^+ + 0.5 mM pyruvate (Δ); 0.5 mM oxidized NAD-pyruvate adduct (o); and 0.5 mM NADH(▲). The arrows indicate a pulse of 2.0 mM NADH to permit elution of the remaining bound enzyme. Corrections were made for the inhibition effects in enzyme assays; 5.5-ml fractions were collected at a rate of 6 ml/h. Reproduced with permission from R. Ohlsson et al., FEBS Lett., 25 (1972) 234-238.

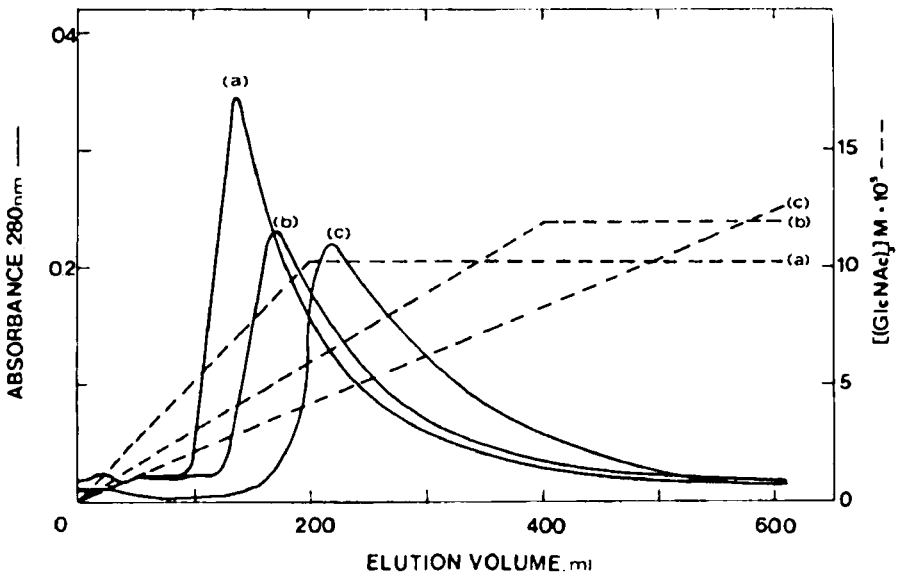


Fig. 8.18. Effect of varying the tri-(N-acetylglucosamine) [(Glc NAc)3] gradient on the elution of native lysozyme from tri-(N-acetylglucosamine)-Sepharose. Fifteen milligrams of native protein in 1 ml of starting buffer (0.1 M ammonium acetate, pH 7.0) were applied to a 260 x 17 mm column. The gradients were begun after washing the column with ca. 5 column volumes (5 x 35 ml), finishing with 0.1 mM (GlcNAc)3 in starting buffer. The gradients were formed using (a) 100 ml of starting buffer vs. 100 ml of finishing buffer, (b) 200 ml vs. 200 ml and (c) 300 ml vs. 300 ml. The flow rate was 23 ml/h. Reproduced with permission from D.A. Cornelius et al., Methods Enzymol., 34 (1974) 639-645.

concentration of tri-(N-acetylglucosamine), which agrees with the association constant for the binding of tri-N-acetylglucosamine-lysozyme ($K_{assoc} = 10^5$ l/mole).

From the theoretical point of view, however, a more advantageous elution with a competitive affinity ligand has numerous practical limitations, mainly related to availability and price. For example, lactate dehydrogenases can be eluted from immobilized N^6-(6-aminohexyl)-5'-AMP-agarose with a solution of competitive ligand of NADH of relatively high concentration (1.5 mM), or with a potassium chloride solution of medium concentration (0.13 mM). The elution of adsorbed substances by changing the pH, ionic strength, or temperature is therefore still the most commonly used procedure.

8.3.2 Elution by change of pH, ionic strength or the presence of molecules suitable for elution

The isolation of alcohol dehydrogenase and phosphofructokinase from a partly purified extract of *Bacillus stearothermophilus* on AMP-Sepharose using a pH gradient is shown in Fig. 8.15 (C). Acid pH gradient elution in high-performance immunoaffinity chromatography (HPIAC), used by Babashak and Phillips (1988), is shown in Fig. 8.19. The advantages of this HPIAC are the use of streptavidin-coated glass beads as support and of monoclonal antibody which is biotinylated using the reagent biotin hydrazine (see Sections 3.3 and 3.8). Short columns (30 x 4.6 mm I.D.) packed with 2-μm fluid-impervious silica microspheres with surface-bound Protein A were used by Várady et al. (1988) for the rapid separation and quantitation of immunoglobulins. By using morpholinoethanesulphonic acid (MES), 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) and acetic acid buffer with a linear decrease in pH from 6.0 to 4.0 and an increase in magnesium chloride concentration to 200 mM for elution, the subclasses of human IgG were separated at 40°C above pH 4.0 in 3 min. The micropellicular Protein A exhibited in this procedure high adsorption capacity (e.g. 4.5 mg human IgG per ml of wet bed volume). One-step purification of guinea pig liver transglutaminase using a monoclonal-antibody immunoadsorbent has been described by Ikura et al. (1985). Active enzyme was recovered by alkaline buffer desorption, followed by quick neutralization. Table 8.6 shows many dissociating agents used with different efficiencies for the elution of a variety of antigens from various immunoadsorbents.

Elution of alcohol dehydrogenase and phosphofructokinase from AMP-Sepharose using a KCl gradient is shown in Fig. 8. 15 (B). The isolation of papain sorbed on agarose with bonded glycylglycyl-O-benzyl-L-tyrosyl-D-arginine is an example of elution with decreased ionic strength of the elution buffer. Blumberg et al. (1970) displaced papain from the specific complex by using water.

If ionic interactions are responsible for biospecific complex formation then the elution of substances to be isolated from biospecific adsorbents would depend on the ionic strength and not on the nature of the types of salts used. If hydrophobic interactions

are prevalent, then suitable salt solutions could be used for elution. Jennissen and Heilmeyer (1975) demonstrated that the elution strength of the anions corresponded directly to the order of Hofmeister 's series, and that of the cations corresponded to the reverse order with respect to the salting-out or salting-in effect. The salts can penetrate directly into the interphase between adsorbed substance and affinant, which leads to desorption.

Fornstedt (1984) used analytical bioaffinity chromatography to investigate the dissociation of antigen-antibody complexes. Human serum albumin (HSA) coupled to Sepharose 4B and anti-HSA antibody was used as a model system. At extremely low or high pH, in the presence of highly concentrated chaotropic ions at pH 7 (2M trichloric acid-NaOH), or by elution with 100% ethylene glycol after pretreating with high-pH buffer, most of the bonds could be ruptured. A powerful resolution of the complex by a polarity-reducing agent (ethylene glycol) could take place after preceeding release of some key sites at the binding area by altering the degree of ionization of the groups

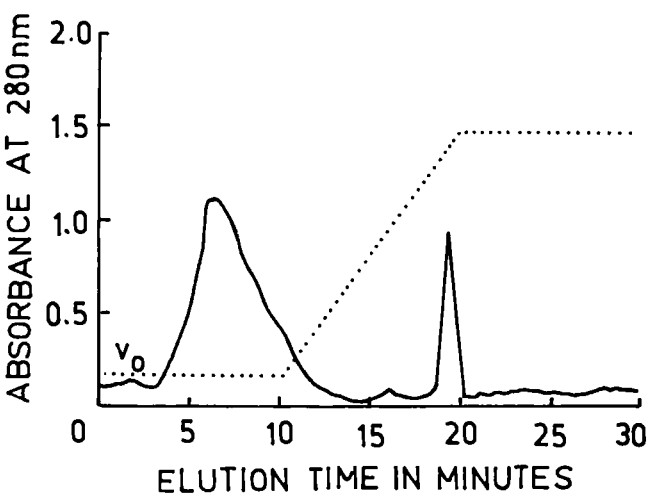


Fig. 8.19 Chromatogram of an HPIAC isolation of the B27 antigen isolated from detergent solubilized membranes. The chromatogram was developed using an acid pH gradient elution from pH 6.5 to 1.0. The trace was produced on a 10 cm x 4.6 mm I.D. HPIAC column run at 0.5 ml/min in 0.9% sodium chloride-0.1 M sodium acetate buffer (pH 6.5) and monitored at $O.D._{280\ nm}$ with the detector set at 0.005 a.u.f.s. The column was maintained at 4°C throught out the run. The dotted line indicates the pH gradient profile. V_o indicates the void volume of the column. Reproduced with permission from J. V. Babashak and T. M. Phillips, J. Chromatogr., 444 (1988) 21-28.

involved. Hill (1972) achieved elution of antibodies from immunoadsorbents by decrease of polarity using dioxan in admixture with weak organic acids.

In connection with the elution of antibodies, the findings of Murphy et al. (1976) concerning the increase in affinity of antibodies during immunization, are interesting. For example, antibodies against glucagon could initially be eluted effectively from the immunosorbent with 4.25 M ethanol in 4mM hydrochloric acid; but if they were prepared from the serum of the same rabbit one year later, 0.1 M acetic acid, adjusted to pH 2.2 with formic acid, had to be used. Table 8.6 shows a wide variety of elution buffers tested for their ability to reversibly dissociate the antigen-antibody complexes. It has already been shown, in many examples, that the specific complex of the isolated substances with the immobilized affinity ligand can also be decomposed after steric modification; for example with urea, guanidine salts or chaotropic ions, namely thiocyanate, trichloroacetate and trifluoroacetate. These reagents disrupt the hydrogen bonds or change the structure of water in the proximity of hydrophobic regions. However, when these reagents are employed, it should be borne in mind that the components of the complex might be irreversibly destroyed during liberation. It is known, however, that with immobilized enzymes the attachment of proteins to solid supports usually brings about an increase in their stability. By choosing a suitable concentration, temperature and exposure time, minimum conformational changes in the adsorption site can be achieved during desorption, which consequently also induces reversible conformational changes of whole molecules, both of the isolated substances and the immobilized affinant. It is practicable to decide the minimal concentration required for elution on the basis of a preliminary experiment in which the concentration and the biological activity are determined. For example, elution with 1.5 and 2 M guanidine hydrochloride solution was used successfully for the isolation of thermolysin and subtilisin after their specific sorption on benzyloxycarbonyl-L-phenylalanyltriethylenetetraminyl-Sepharose. Both enzymes are stable under the given elution conditions, and affinity chromatography, mentioned above, yielded preparations of high specific activity (Fujiwara et al., 1975). Guanidine hydrochloride solution was used successfully as an eluting agent by Gospodarowicz (1972), who even used a 6

Table 8.6.

Comparison of different dissociating agents desorbind the antigens from their immunoadsorbents

Antigens	*Elution*	*Recovery [%]*	*Reference*
Aminopeptidase N	2 mM Tris-HCl, pH 8.0	70	
Dipeptidylpeptidase IV	2 mM Tris-HCl, pH 8.0	56	
Sucrase-isomaltase	2 mM Tris-HCl, pH 8.0 a	40	1
Lactase-phlorizin hydrolase	1 mM K_2PO_4, pH 7.4	32	
Maltase-glucoamylase	1 mM K_2PO_4, pH 7.4	32	
Martix vesicle alkaline phosphatase	2-Amino-2-methyl-1-propanol, 0.6M	80-100	2
	$MgCl_2$, 1 M	25	
	NaCl, 3 M	1	
	Urea, 6M	0	
Glucocerebrosidase from exctracts of human tissues	Glycine-HCl 0.2 M	<5	3
	KCNS 2.0 M	<5	
	Ethylene glycol 60%	10	
	Ethylene glycol 70%	28	
	Ethylene glycol 80%	96	
Human lipoproteins containing apolipoprotein B (ApoB)	3 M NaSCN	100	4
	50 mM Glycine-HCl, pH 3.2	42	
	Water, pH 6.0	9.5 b	
	0.7 mM NaOH, pH 9.6	33	
	1.0 mM NaOH, pH 10	58	
Bacillus anthracis lethal factor	0.05 M Na_2PO_4, pH 7.0	0	5
	4.0 M NaCl in 0.05 M Na_2PO_4, pH 7.0	14.6	
	0.1 N Acetic acid, pH 3.0	86.4	
	1.0 M Glycine-HCl, pH 2.0	88	
	2.0 M NaSCN in 0.05 M Na_2PO_4, pH 7.0	83	
	4.0 M NaSCN in 0.05 M Na_2PO_4, pH 7.0	100	

a) All containing 0.1% Triton X-100

b) Values are expressed as percentage recovered ApoB in comparison with that recovered by NaSCN

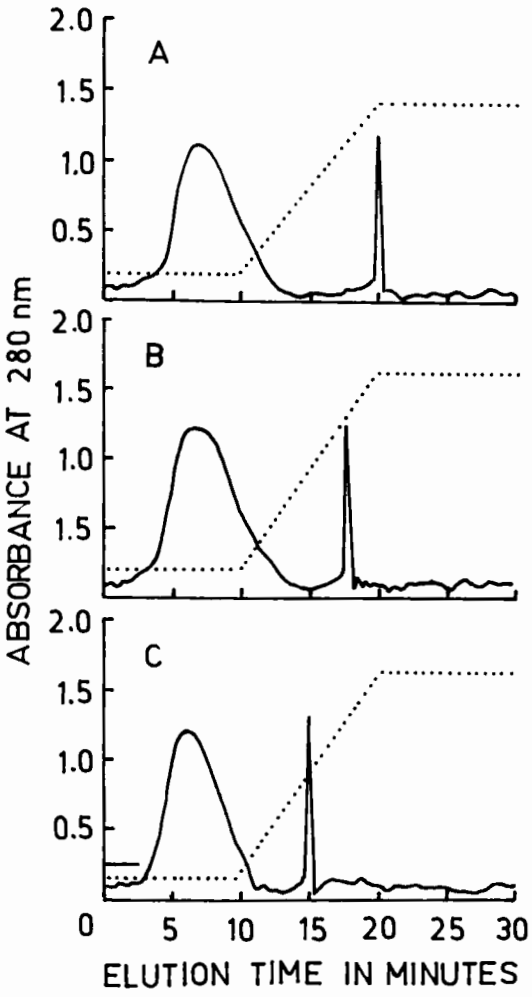
1) Danielsen et al. (1982). 2) Hsu et al. (1985). 3) Aerts et al. (1986).
4) Mc Conathy et al. (1985). 5) Machuga et al. (1986).

M solution of pH 1.5 for the elution of the luteinizing hormone from an agarose column with a bonded fraction of anti-luteinizing hormone immunoglobulin. Immediately after elution, the pH of the hormone solution was adjusted to 7.3 and dialysis was carried out against a 0.2 M ammonium hydrogen carbonate or 0.01 M phosphate buffer of pH 7.3, containing 0.9% of sodium chloride.

The HPIAC column used by Babashak and Phillips (1988) for the isolation of B27 human leukocyte antigen, wnich is shown in Fig.8.19, was used by the same authors for the isolation of the same antigen, and this is shown in Fig. 8.20. The difference is in the elution: instead of pH chaotropic gradient elution, buffer containing sodium thiocyanate from 0 to 2.5 M was used at flow rates (A) of 0.5 ml/min, (B) 1 ml/min and (C) 1.5 ml/min. The elution peak produced by the acid elution is broader than the same peak produced by chaotropic elution. The flow rates of the column did not affect the elution profile over the range tested. The shift in peak position, seen with increasing flow rates, was due to the increase in speed with which the released antigen was washed through the column. The increase in column flow rate also resulted in changes in the area under the second peak, which decreased as the antigen was eluted in a smaller volume, at a greater speed. Speeds greater than 1.5 ml/min resulted in an inefficient binding of the antigen to the immobilized antibody during the initial phase of the immunoaffinity procedure. Dot-blot analysis of the immunoaffinity isolated antigen demonstrated that superior results are obtained with chaotropic elution at flow rates of 1-1.5 ml/min.

8.3.3 Elution by change of temperature

The human blood group Ii antigens are defined by certain monoclonal or oligoclonal IgM autoantibodies which are cold agglutinins (CA). Anti-I reacts preferentially with adult red blood cells while anti-i agglutinates cord erythrocytes more strongly. The Ii antigenic determinants on the erythrocytes are oligosaccharide structures on glycosphingolipids and glycoproteins. The cold-reacting autoantibodies are clinically important since they are primarily responsible for vast majority of autoimmune CA anemias of both the transient and chronic varieties. They may also play a secondary role in the genesis of, or clinical manifestations associated with other disease states, including



8.20. Effects of flow rate on the elution profiles of an HPIAC isolation of the B27 antigen using a chaotropic gradient elution buffer. The running conditions are as described in Fig. 8.19 except that the flow rates were (A) 0.5 ml/min; (B) 1 ml/min and (C) 1.5 ml/min. The dotted line indicates the chaotropic ion gradient. Reproduced with permission from J. V. Babashak and T. M. Phillips, J. Chromatogr., 444(1988) 21-28.

sickle cell disease and various other hematological disorders and nonhematological malignancies. Their extremely selective reactions also render them useful reagents in studying the molecular basis of specific physiological disorders, i.e. chronic cold agglutinin disease (CCAD), and in studying antigenic changes associated with cell maturation, differentiation and malignant transformation. Glick and Oppenheim (1985)

described the rapid purification of anti-I and anti-i cold antibodies from the sera of patients with CCAD. The purification procedure shown in Fig. 8.21 is based on thermal bioaffinity chromatography using desialated orosomucoid-Sepharose 4B conjugated beads. Anti-I or anti-i antibodies were separated from the whole sera in all of the seven samples tested, with a recovery in most cases of 100% of the cold hemagglutinating activity.

The use of a linear temperature gradient for the differentiation of a mixture of yeast alcohol dehydrogenase, glycerokinase, hexokinase and lactate dehydrogenase in bioaffinity chromatography on N^6-(6-aminohexyl-5'-AMP-Sepharose was described by Harvey et al. (1974). It is interesting that glycerokinase and yeast alcohol dehydrogenase were eluted in the order expected on the basis of their apparent energies of adsorption, with a high recovery (70-90%), while lactate dehydrogenase required a pulse of 5 mM

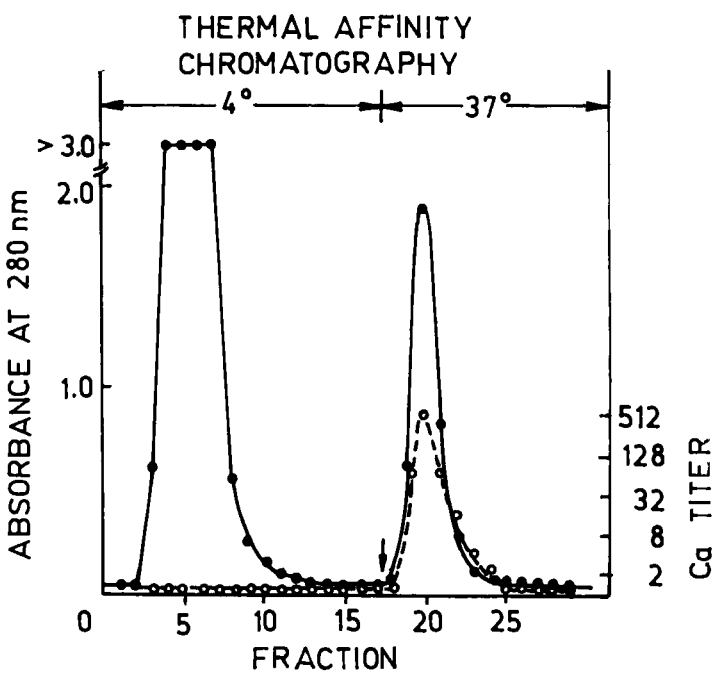


Fig. 8.21. Purification of cold agglutinins by thermal bioaffinity chromatography. High titre anti-I or anti-i sera were titrated to pH 6.8, membrane filtered, and subsequently passed through a desialated orsomucoid Sepharose 4B column (1.5 x 10 cm). The column was washed extensively with sodium phosphate buffer (PBS)pH 6.8 at 4°C and then eluted with PBS pH 7.4 at 37°C. Fractions (5 ml) were collected and monitored for absorption at 280 nm (●) and for cold hemagglutinating activity (o). Reproduced with permission from J.Glick and J.D.Oppenheim, Vox Sang., 49 (1985) 49-57.

NADH before applying the temperature gradients. The advantage of this elution is that the eluted enzymes are not contaminated with elution reagents (salts, nucleotide pulse), and they can be utilized directly for kinetic studies. For firmly bound enzymes, the method is suitable for use in combination with other techniques, such as a lower concentration of ligands, NADH pulses or a gradient of ionic strength.

Babashak and Phillips (1988) also studied the elution of B27 antigen from an HPIAC column (streptavidin-coated beads) in relation to temperature. Elutions and regenerations at room temperature were determined over 1 - 2 week intervals (5 - 10 elutions), at 4°C for 4 - 6 months (10 - 40 elutions) and at 37°C for 1 week (2 - 10 elutions and regeneration in all cases, depending on the elution agent). It was possible to store HPIAC columns at 4°C for up to 6 months or longer, and for 1 - 2 weeks at room temperature. The operating temperature of bioaffinity chromatography often affects the functional life of biospecific adsorbents, which should be borne in mind. The effects of organic solvents and temperature, including sub-zero temperatures, on desorption from immunoadsorbents have been studied by Anderson et al. (1978, 1979).

8.3.4. Photocontrol of bioaffinity chromatography

Those compounds that show reversible colour formation upon irradiation with light are called photochromic compounds. Spiropyran compounds, which are in the class of photochromic compounds, show a drastic polarity change under light irradiation. Karube et al. (1978) modified Sepharose 4B with N-(ω-aminohexyl)-L-aspartic acid (AHA) and spiropyran. The AHA-spiropyran gel showed reverse photochromism. It was coloured red in the dark (polar form) and was bleached upon irradiation with visible light (apolar form). Asparaginase was bound to the AHA-spiropyran gel under visible light and was released in the dark. The optimum conditions for photocontrolled binding and release of asparaginase were 0.05M phosphate buffer and pH 7.0. Since no interaction was observed between the agarose gel modified with spiropyran compound and asparaginase, it can be concluded that asparaginase binds to the immobilized AHA moiety. Fig. 8.22 shows that the spiropyran derivatives on the AHA-agarose gel were

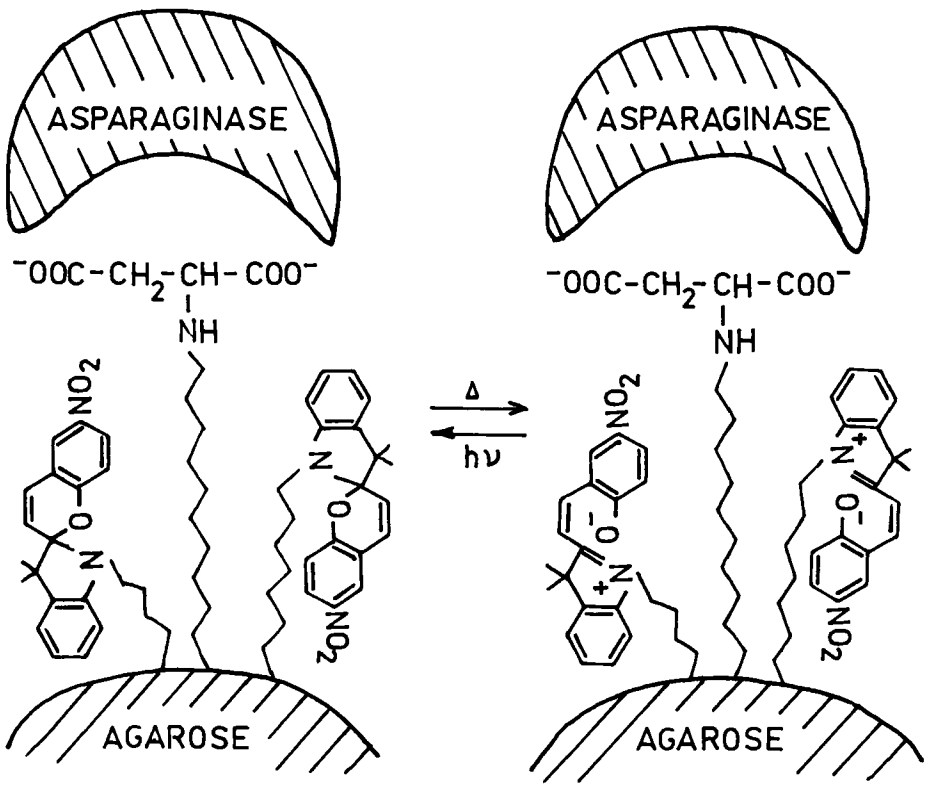


Fig. 8.22. Schematic diagram of the N-(ω-aminohexyl)-L-aspartic acid-spirogen-Sepharose under visible light (hν) and in the dark (Δ). Reproduced with permission from I. Karube et al., Biotechnol. Bioeng., 20 (1978) 1775-1783.

ionized in the dark. The ionic strength around the immobilized AHA increased in the dark. Therefore, asparaginase might be forced out by ionic repulsion in the dark.

8.3.5 Elution in an electric field

In some cases the elution of adsorbed materials from bioaffinity matrices can be the most critical step in bioaffinity chromatography. In some cases it is not possible to achieve the elution of highly biologically active compounds by conventional elution procedure. As a consenquence of this impossibility the powerful tool of bioaffinity chromatography could sometimes not be used. Morgan et al. (1978) developed the technique of electrophoretic desorption. This makes it possible to avoid the severe, e.g. chaotropic, conditions often used for the dissociation of biospecific complexes exhi-

biting strong interaction; e.g. in the case of haptens with immunoadsorbents. The method combines the principles of electrophoresis and biospecific interactions described by Nakamura et al. (1960). Morgan et al. (1978) placed bioaffinity matrices on the top of the polyacrylamide gels, and slightly modified gel electrophoresis equipment was employed. Electrodes were situated in buffer compartments on opposite sides of bioaffinity matrix. The technique involves applying an electric field across a loaded bioaffinity matrix after the usual adsorption and wash steps of bioaffinity chromatography have been performed.

The patterns of desorption reported in Morgan et al.'s paper for human serum albumin (HSA) from Cibacron Blue F3G-A-Sepharose, for ferritin from anti-ferritin antibody-Sepharose, and for oestriol-16α-glucuronide from anti-oestriol-16α-glucuronide antibody-Sepharose are the same, and appear to be typical of all electrophoretic desorptions. These patterns take the form of an initial peak of desorbed material, which is followed by a 'trail' that decreases until no more material remains on the bioaffinity matrix. The amount of material represented by the height of the initial peak is related to the time between the washing of the bioaffinity matrices and the commencement of the desorption. The relative mobilities (as measured by the position of the initial peak) of compounds that have been electrophoretically desorbed from bioaffinity matrices are those obtained for the compounds in the absence of any bioaffinity matrix. Thus the desorption process begins immediately the voltage is applied. The proposed mechanism of desorption is that the observed initial peak represents material which is unbound at the start of the desorption. This material has dissociated from the ligand after the washing procedure has removed the excess, free material. The amount of material observed in this peak depends on both time and association constant K_a. The 'trail' that follows the unbound material into the gel represents material which dissociates and migrates continuously in the ensuing period. This dissociation persists until no material remains on the support. The desorption is thus passive procedure. Ions will move in the field towards the gel only when dissociated; if they re-associate with immobilized ligand then their migration rate may be proportionally retarded. Morgan et al. (1978) therefore

predict that the rate at which a material can be desorbed will be directly related to the rate of dissociation for that material and the immobilized ligand.

In an experiment in which the desorption of HSA on two gels of different diameters is compared under identical conditions, it is seen that the greater the surface area between the gels and bioaffinity suport the faster is the rate of desorption. This is because the increased surface area increases the probability of migration into the gel without the occurrence of re-association, since the distance across which the ions have to migrate is less than with a smaller surface area in contact with the gel. Thus the rate of desorption with narrow gels is slower than for wide gels, for the same amount of affinity matrix.

In accordance with the postulated mechanism, factors affecting the binding of the material to the affinity support, such as changes in pH, or changes in temperature will also influence the observed rates of desorption. Any compound adsorbed non-covalently by a bioaffinity (or other) interaction to an immobilized support should be capable of being desorbed by this electrophoretic method, provided that:

(i) the interaction is reversible;

(ii) the compound to be desorbed is charged.

Morgan et al. (1980) also used electrophoretic desorption of HSA from Cibacron Blue F3GA-Sepharose 4B for the investigation of important controlling factors, such as the electric current, the matrix thickness, the buffer molarity, and temperature. The results demonstrate some of the advantages of this technique, including the mild conditions, high yield and small desorbate volume.

A review of the principles, methodology and applications of electrophoretic elution from biospecific adsorbents has been published by Yarmush and Olson (1988). A comprehensive list of applications shows that the materials recovered by electrophoretic elution have included steroids, enzymes, antibodies, virus particles and viruses. The application of this technique to the recovery of live cells from bioaffinity supports is made more attractive by the fact that many cells have high electrophoretic mobilities and are known to be stable in a buffer containing nonelectrolyte media (e.g. sucrose or mannitol). In addition, electrophoretic elution would also be ideal for recovering

multi-subunit proteins which may have difficulty in reassembling after dissociation in chemical eluents. In view of these considerations, the comparative safety of electrophoretic elution may be especially attractive for systems in which conventional elution methods have proved to be inadequate or disadvantageous. De Bortoli and Roggero (1985) showed in their review that many electrophoretically eluted materials completely retain their affinity and capacity, including identical antigenicity and infectivity of isolated viruses. The same review also gives the scale and time-frame of the applications mentioned.

Fig. 8.23 illustrates the fundamentals of an electrophoretic elution device with product recovery over a semipermeable membrane. The electric field causes dissociated ligate to migrate into an elution chamber. The elution chamber is bounded by a semipermeable membrane that retains the larger product molecules but allows small ions to pass and complete the electric circuit.

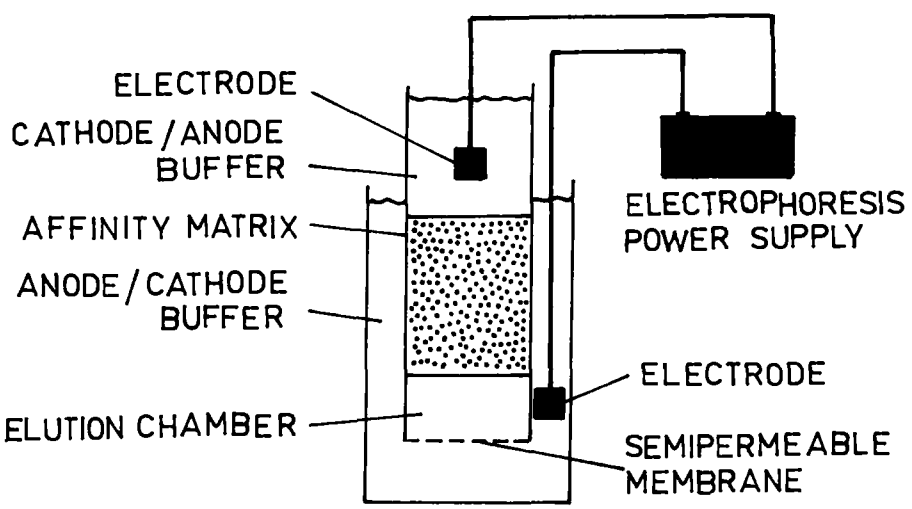


Figure 8.23. Electrophoretic elution device with product recovery over a semipermeable membrane. The membrane retains the product molecule but allows small ions to pass and complete the electric circuit. Reproduced with permission from M. L. Yarmush and W. C. Olson, Electrophoresis, 9 (1988) 111-120.

Flat-bed electrophoresis equipment for desorbing substances tightly bound to bioaffinity matrix using isoelectric focusing has been described by Haff et al. (1979). For preparative work, this method typically involves the following steps.

(1) Prepare a flat bed of inert gel supporting a pH gradient. (2) Perform the adsorption and wash steps of bioaffinity chromatography in a separate vessel. (3) Replace a small zone of the inert gel with the saturated bioaffinity matrix. (4) Apply an electric field to elute the material from the bioaffinity matrix into the inert gel for isoelectric focusing. (5) Remove the zone of inert gel containing the focused material. (6) Separate the focused material from the inert gel. Since additional separation is achieved in the isoelectric focusing gel, this method offers extremely high product purities.

Kašička and Prusík (1983) developed the third electrophoretic recovery method, isotachophoretic focusing, which involves elution into a discontinuous system of electrolytes chosen so that the mobility of the desorbed substance is intermediate between those of the leading and terminating electrolytes. Because of its lower mobility, ligate that elutes into the high-mobility leading electrolyte acquires a velocity less than that of the electrolyte boundary. Because (1) the current is constant throughout the capillary and (2) the electrical conductivity of the terminating electrolyte solution is less than that of the leading electrolyte solution, the electric field is constrained to be greater in the terminating solution. For this reason, ligate that elutes into the terminating solution travels faster than the boundary and eventually accumulates or focuses as a new zone. The location of this zone can be determined using tracking dyes or other types of sensors. Fig. 8.24 shows a plan of the apparatus constructed by Prusík and Kašička (1985) for micropreparative isotachophoretic desorption. Electrodesorption methods for releasing polypeptides and proteins from high-bioaffinity sorbents have been surveyed by Prusík and Kašička (1987). New applications of desorption isotachophoresis are summarized and demonstrated there. The desired properties of a bioaffinity adsorbent especially suited for desorption isotachophoresis were formulated on the basis of experimental data.

Takeo and Nakamura (1972) found that the electrophoretic mobility of glucan phosphorylases was diminished due to the specific interaction if glycogen was

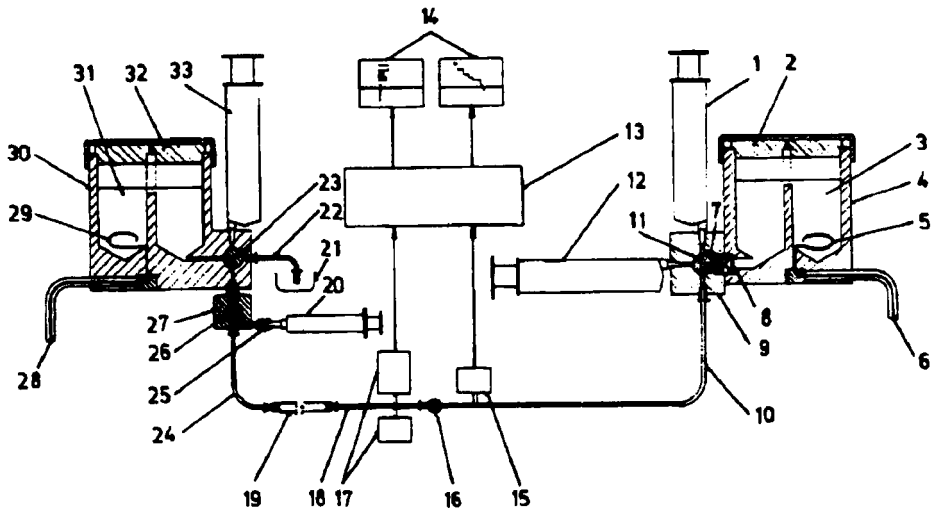


Fig. 8.24. Apparatus for micropreparative isotachophoretic desorption. 1 = Polypropylene 20-ml syringe for the leading electrolyte; 2 = cover of the electrode compartment for the leading electrolyte; 3 = leading electrolyte; 4 = electrode vessel for the leading electrolyte; 5 = Pt electrode; 6 = high-voltage cable; 7 = deaeration channel; 8 = semipermeable membrane (Kuprophan); 9 = body of the valve of the leading electrolyte; 10 = polytetrafluoroethylene (teflon, PTEF) tubing, I. D. 1 mm; 11 = PTFE core of the valve of the leading electrolyte; 12 = polypropylene 20-ml syringe for filling the capillary and rinsing the sample; 13 = electronics for the UV detector and the potential gradient (PG) detector; 14 = recorders of the UV and PG signals; 15 = PG detector; 16 = PTEF coupling; 17 = source and sensor of the UV detector; 18 = PTFE tubing, I. D. 1 mm; 19 = polyethylene tubing, I. D. 3 mm; 20 = polypropylene syringe for the sample and deaeration; 21 = waste reservoir; 22 = PTFE tubing, I. D.1 mm; 23 = PTFE core of the valve for the terminating electrolyte; 24 = PTFE tubing, I. D. 1 mm; 25 = needle valve joint; 26 = segment with the affinity sorbent; 27 = body of the sorption element; 28 = high-voltage cable; 29 = Pt electrode; 30 = electrode vessel for the terminating electrolyte; 31 = terminating electrolyte; 32 = cover of the vessel for terminating electrolyte; 33 = polypropylene 20-ml syringe for the terminating electrolyte. Reproduced with permission from Z. Prusík and V. Kašička, J. Chromatogr., 320 (1985) 81-88.

embedded in the suppport gel. Bøg-Hansen (1973) and Hořejší with Kocourek (1974) proposed the term "affinity electrophoresis" (AE) for this procedure, since it combines the bioaffinity technique and electrophoresis. A section on AE was printed in Chapter 7 of the first edition of this book (Turková, 1978). Reviews on AE have been published by Hořejší (1981, 1984) and Takeo (1984); on AE of glycoproteins by Bøg-Hansen (1983); and on qualitative and quantitative applications of AE to the study of protein-

ligand interactions by Hořejší and Tichá (1986). AE is advantageous for analytical purposes because it requires a small amount of the sample and permits the analysis of many samples simultaneously in a short time. However, it can not be applied to neutral substances, nor to substances having a net charge of zero. It is also only limited to analytical applications.

Shimura and Kasai (1982) described separation technique for biomolecules under the name "affinophoresis". This technique requires a carrier macromolecule, an "affinophore", which contains both an affinity ligand for a certain biologically active molecule and many charges, either positive or negative, in order to migrate rapidly in an electric field. When a mixture of substances is subjected to electrophoresis in the presence of the affinophore, the biomolecule having an affinity with the affinity ligand forms a complex with the affinophore. This results in a change in the apparent electrophoretic mobility. If the biomolecule accelerates sufficiently, it can be separated from other materials. Shimura and Kasai separated e.g. *Streptomyces griseus* trypsin using m-aminobenzamidine attached to soluble cationic dextran containing DEAE - groups. An anionic affinophore based on poly-L-lysine as matrix was used for the affinophoresis of anhydrochymotrypsin by Shimura and Kasai (1986). A review on progress in affinophoresis has been published by Shimura (1990).

8.3.6 Elution in a magnetic field

The advantages of magnetized supports, which are attracted by a magnetic field, prepared under the commercial name Magnogel AcA, and their applications, have already been discussed in Section 5.4. Molday and Mackenzie (1982) prepared immunospecific ferromagnetic iron-dextran reagents for the magnetic separation of cells. The magnetic particles used were in the size range of 30 - 40 nm and had an electron dense core of about 15 nm. They were stable against aggregation in physiological buffer, showed little non-specific binding to cells and had a magnetic moment. After their periodate oxidation and coupling of Protein A, human red blood cells labelled with immunospecific ferromagnetic particles were quantitatively retained by a simple permanent magnet and could be separated from unlabelled cells. Reported studies pointed

to the potential wide application of immunospecific ferromagnetic iron-dextran particles for the separation of specific antigen bearing cells, cell membranes and receptors.

Ugelstad et al. (1988) summarized their long-term work on the preparation of monosized magnetic particles (MMP) and their use in selective cell separation. Several types of magnetic particles have been prepared. A preferred method involves, as a first step, the introduction of oxidizing groups, chemically bound to the surface of the pores of macroreticular particles. Under appropriate conditions Fe^{2+} is continuously transported from an outer phase into the pores, where it is oxidized and precipitates as very fine grains of magnetic iron hydroxid-oxide compounds which form magnetic iron oxides on heating. The magnetic particles which up to now have been most widely used in cell separation are called M-450 and they are produced by Dynal A. S., Oslo, Norway. Magnetic beads have been used in clinical applications for the depletion of tumour cells in the equipment shown in Fig. 8.25.

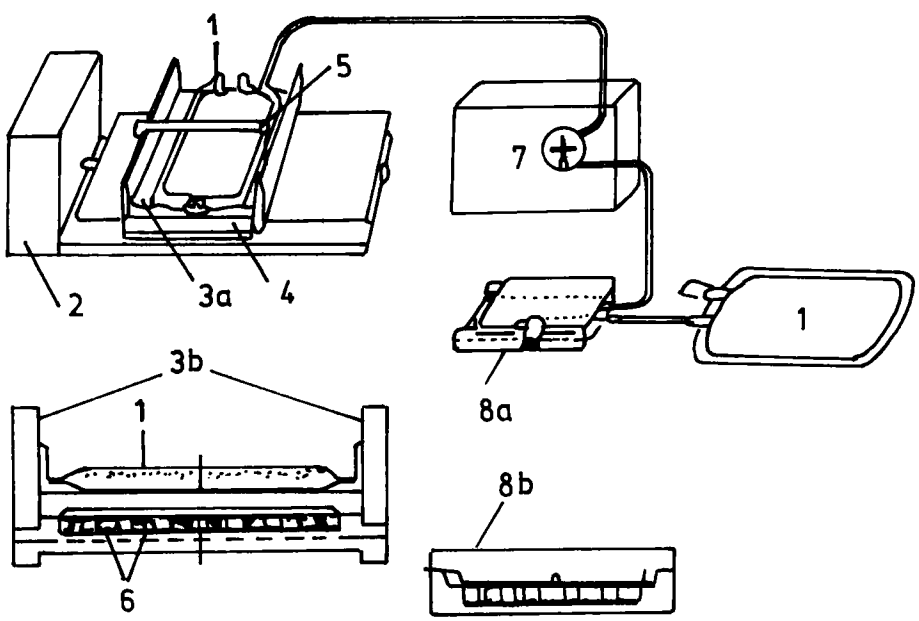


Fig. 8.25. Cell separation unit for clinical use. 1, 600 ml Fenwall blood bags; 2, rocking device; 3a, platform supporting blood bag above bank of magnets; 3b, enlarged cross-section of 3a; 4, removable iron plate; 5, handle for stepwise regulation of distance between platform and magnets; 6, cobalt samarium magnets; 7, peristaltic pump; 8a, magnetic trap; 8b, enlarged cross-section of 8a. Reproduced with permission from J. Ugelstad et al., Makromol. Chem., Macromol. Symp., 17 (1988) 177-211.

Mononuclear cells and MMP are introduced into a standard 600 ml Fenwall blood bag. The bag is fixed in a cassette which is placed on the platform of a rocking device. During the incubation with MMP the bag is shielded from the magnetic field by a removable iron plate. After incubation, the bag is lowered stepwise towards the magnets in order not to detach beads from the cells during transport of the MMP-rosetted cells to the bottom of the bag. The supernatant cell suspension is then sucked out with the help of a peristaltic pump. To remove beads that might escape the bag, the cell suspension is transported through a magnetic trap before it is collected in a blood bag. A second cycle of purging is carried out by adding a new batch of polyclonal antibody coated MMP and repeating the procedure.

Special types of MMP have been developed for the isolation of organelles and viruses. Special modifications of the M-450 beads, where the beads have a non-magnetic polymer shell outside the magnetic core, have proved to be very effective for the selective separation of organelles and viruses. The special magnetic device used in this case involves separation in a "magnetic flow system". After incubation, the suspension contained in a 15 ml isolation chamber is placed within the magnetic field generated by an electromagnet. The shape of the magnetic field is designed so that the beads will exist as single particles and are contained within the chamber, while the washing buffer flows through the chamber and removes unbound and nonspecifically bound components. The gentle washing conditions prevent losses due to vesicularization of the specifically bound vesicles, which has been a severe problem with previous methods.

A method for rapid purification to near homogenity of sequence-specific DNA binding proteins based on magnetic separation has been descibed by Gabrielsen et al. (1989). They described the magnetic DNA bioaffinity purification of yeast transcription factor by using monodisperse superparamagnetic particles (Dyna beads M-280) with attached Streptavidin mixed with biotinylated tRNA gene fragment. The high biotin binding capacity of these magnetic DNA bioaffinity beads resulted in near 100% binding with the amounts of DNA.

Fine magnetic particles (ferrofluid) were prepared by a co-precipitation method using the oxidation of Fe^{2+} with nitrite by Shinkai et al. (1991). The particles were activated

with (3-aminopropyl)triethoxysilane in toluene and the activated particles were combined with some enzymes by using glutaraldehyde. Enzyme-immobilized magnetic particles had sizes between 4 - 70 nm and the size could be changed by the ratio of the amount of Fe^{2+} to that of nitrite. Proteins could be immobilized at the level between about 70 and 200 mg/g support. To decrease intraparticle diffusion limitation of substrate transport within the carriers, fine particles, the size of which is as small as possible, are preferably used as a support. However, in repeated use the separation becomes more difficult. Magnetic particles can easily be recovered by the magnetic field. Using the published method, the magnetic particle size could be controlled according to the application required.

8.4 ESTABLISHMENT OF OPTIMUM CONDITIONS AND SATURATION EFFECT

In order to discover the optimum conditions, Porath and Kristiansen (1975) recommended preliminary experiments for the determination of the bed capacity. This determination can best be carried out by frontal chromatography on a column bed with a total volume of a few millilitres. The UV absorption of the effluent is measured at a suitable wavelength and small fractions are collected. The activity of the fractions is determined, and the capacity is calculated from the retention of activity. In addition, frontal chromatography will also provide information about the possible presence of several components with identical affinities but different retention volumes. Sometimes two activities may be determined simultaneously; for example that of trypsin and chymotrypsin, during elution from a column of immobilized soybean trypsin inhibitor. Information obtained in the preliminary experiment is then valuable for planning the main experiment, which should achieve maximum purification in a bed of a given volume of the sorbent used.

In some instances it was observed that, when freshly prepared sorbents were used, some of the activity of the isolated substance was lost, evidently due to irreversible sorption. Thus, for example, Gorecki et al. (1974) described the variability of penicillin-Sepharose conjugate. p-Aminobenzylpenicillin coupled to Sepharose was a very stable

conjugate, which preserved its ability to adsorb D-alanine carboxypeptidase specifically, even after storage in water in the presence of 0.02% sodium azide at 4°C for several months. During the use of this sorbent, the authors found, however, that only about 60% of all preparations of substituted gels are suitable for immediate use in the bioaffinity chromatography of D-alanine carboxypeptidase. That is, some of the conjugates sorbed the enzyme irreversibly and no enzymatic activity could be eluted, even when high concentrations of salts were applied. Exhaustive saturation of this gel with a definite amount of crude enzyme (about a 10-fold amount of enzyme with respect to that commonly used in normal purification procedures) converted this penicillin-Sepharose into a normal reversible adsorbent, suitable for the bioaffinity chromatography of D-alanine carboxypeptidase. The saturation effect was highly specific, and no other inert protein, for example bovine serum albumin, could replace D-alanine carboxypeptidase in the irreversible sorption. It was further observed that, during storage at 4°C, some of these gels characterized by irreversible sorption were converted into gels suitable for normal bioaffinity chromatography. This change can be ascribed to the decrease in the number of highly active "adsorbing sites" on the gel surface, so that the enzyme can then be bound reversibly to these modified specific sorbents.

The equilibrium constant of the biospecific complex influenced the amount lost by the dissociation of the specifically adsorbed material during exposure to the washing buffer and the heterogeneity of the substance isolated. Using two or three column volumes of washing buffer, Eveleigh and Levy (1977) determined that the heterogeneity of the antibody preparation is less than 2% in the majority of applications. These authors also studied the effect of cycling the immunosorbent, which is shown in Table 8.7.

The poor recovery of bound antigen from the column in the first cycle can be partly explained by an insufficient volume of eluent to completely dissociate the complex, but a comparison with the recovery in the subsequent cycles indicates that the lower yield represents an apparently irreversible reaction of the antigen. This "first-cycle effect" has been observed with many supports and with a variety of immunological systems, and must be considered when defining the immunosorbent capacities (Eveleigh and Levy, 1977).

Table 8.7.

Effect of cycling a 5-ml column with excess antigen and eluting with 15 ml of 2.5 M NH_4SCN

Cycle	*Amount (mg)*			*Eluted/ bound (%)*	*Amount retained (mg)*
	Applied	*Bound*	*Eluted*		
1	5.87	4.192	3.204	76.4	0.988
2	5.97	3.316	3.019	91.0	0.297
3	5.89	3.239	2.988	92.2	0.251
4	5.88	3.179	2.964	93.2	0.215
5	5.86	3.135	2.995	95.5	0.140
6	5.91	3.089	2.996	96.0	0.124

The major interfering effects in bioaffinity chromatography are due to non-biospecific binding. Zoller and Matzku (1976) studied the elimination of non-biospecifically bound proteins in immunoadsorption. In their paper various possibilities for eliminating these effects were compared and analysed. They stated that, for the purification of their antigen (α-foetoprotein), pre-elution with a pH 10.0 buffer of high ionic strength (1.0 M) was effective. A major problem in purifying anti-pepsinogen II group antibodies was eliminated by using a non-biospecific immunoadsorbent column where specific antibodies are not bound, or are only weakly bound. Wright et al. (1978) described the purification of hexokinases by bioaffinity chromatography on Sepharose-N-aminoacrylglucosamine derivatives. The capacity of the bioaffinity columns for these enzymes also depended on the purity of the applied sample: the purer the sample, the higher the capacity. This phenomenon can be expected in low-affinity systems where the non-specific adsorption of protein can readily interfere with specific interaction. Thus the more impure the fraction to be chromatographed the larger the column that is required. A more detailed discussion on non-specific sorption is given in Section 4.6.

Bioaffinity chromatographic procedures are difficult to scale up from the analytical to the preparative level when the ligand used for purification is a limiting factor. A versatile, computer-controlled bioaffinity chromatographic system has been described by Jepsen et al. (1986). It permits automatic repetition of the purification process, and

sophisticated control functions are available, based on the ultraviolet absorbance of fluid passing through the bioaffinity column. The system was used for automation and scaling up of the purification of *Plasmodium falciparum* exoantigens. An automatic time-based instrument for the preparative application of immunoaffinity system has been described by Eveleigh (1982). Many critical factors involved in the large scale preparation of biologically active molecules will be discussed in greater detail in Section 9.4.

8.5 REGENERATION AND STORAGE OF BIOAFFINITY COLUMNS

The commonest method of column regeneration for repeated use is to wash them alternately with alkaline and acidic buffers, in a similar manner to that used during their preparation. For example, Walsh et al. (1974) washed Sepharose with bonded glycyl-D-phenylalanine after the isolation of neutral protease from *Bacillus subtilis* with two column volumes of a buffer of pH 9 and then a buffer of pH 5 or 7, and finally they again equilibrated the column with the buffer used for affinity chromatography. The columns regenerated in this manner did not change their chromatographic behaviour, even after 50 runs. Benson et al. (1974) used washing with 6 M guanidine hydrochloride solution and re-equilibration with the starting buffer for the regeneration of 19-nortestosterone-17-O-succinyldiaminodi-propylaminoagarose. Turková et al. (1975, 1976) also used 6 M guanidine hydrochloride solution for the regeneration of specific sorbents.

After experiments involving elution with detergents, the Pharmacia company recommended the following washing procedure for the regeneration of sorbents:

(1) wash with 1 bed volume of distilled water, followed by 1 bed volume of ethanol;

(2) wash with 2 bed volumes of n-butanol;

(3) wash with 1 bed volume of ethanol, followed by 1 bed volume of distilled water;

(4) re-equilibrate the gel with starting buffer, ready for the next experiment.

The flow rate during washing may be similar to or higher than that used during classical chromatography, and 25-50 cm/h has been found to be suitable. The expe-

rimental conditions for high-performance bioaffinity chromatography will be discussed in Section 9.3.

The insoluble affinant, especially if it has a protein character, is often more stable when bound to a solid support than when free. In many instances, for the preservation of activity, it is best to store the suction-dried specific sorbent at low temperature in the presence of a suitable bacteriostatic agent (for example, 0.02% of sodium azide). The choice of the storage buffer depends on the properties of the bound affinant.

REFERENCES

Aerts, J.M.F.G., Donker-Koopman, W.E., Murray, G.J., Barranger, J.A., Tager, J.M. and Schram, A.W., Anal. Biochem., 154 (1986) 655-663.

Akanuma, Y., Kasuga, A., Akanuma, T. and Yamasaki, M., Biochem. Biophys. Res. Commun., 45 (1971) 27-33.

Andersson, K., Benyamin, Y., Douzou, P. and Balny, C., J. Immunol. Methods, 23 (1978) 17-21.

Andersson, K., Benyamin, Y., Douzou, P. and Balny, C., J. Immunol. Methods, 25 (1979) 375-381.

Aono, S., Sato, H., Semba, R. and Kashiwamata, S., Experientia, 41 (1985) 1084-1087.

Babashak, V. and Phillips, T.M., J. Chromatogr., 444 (1988) 21-28.

Benson, A.M., Suruda, A.J., Shaw, R. and Talalay, P., Biochim. Biophys. Acta, 348 (1974b) 317-320.

Blumberg, S., Schechter, I. and Berger, A., Eur. J. Biochem., 15 (1970) 97-102.

Bøg-Hansen, T.C., Anal. Biochem., 56 (1973) 480-488.

Bøg-Hansen, T.C., in W.H. Scouten (Editor), Solid Phase Biochemistry, Wiley, New York, 1983, pp. 223-251.

Brown, E.J., Bohnsack, J.F., O'Shea, J.J. and Mc Garr, J., Mol. Immunol., 24 (1987) 221-230.

Burtscher, H.J., Auer, B., Klocker, H., Schweiger, M. and Hirsch-Kauffmann, M., Anal. Biochem., 152 (1986) 285-290.

Comer, M.J., Craven, D.B., Harvey, M.J., Atkinson, A. and Dean, P.D.G., Eur. J. Biochem., 55 (1975) 201-209.

Cornelius, D.A., Brown, W.H., Shrake, A.F. and Rupley, J.A., Methods Enzymol., 34 (1974) 639-645.

Danielsen, E.M., Sjöström, H. and Norén, O., J. Immunol. Methods, 52 (1982) 223-232.

De Bortoli, M. and Roggero, P., Microbiologica, 8 (1985) 113-121.

Dodeur, M. and Jacquet, M.A., J. Chromatogr., 195 (1980) 197-203.

Ernst-Cabrera, K. and Wilchek, M., Trends Anal. Chem., 7 (1988) 58-63.

Evans, C.H., Biochem. J., 225 (1985) 553-556.

Eveleigh, J.W. and Levy, D.E., J. Solid-Phase Biochem., 2 (1977) 45-78.

Ferre, J., Yim, J.J. and Jacobson, K.B., J. Chromatogr., 357 (1986) 283-292.

Fornstedt, N., FEBS Lett., 177 (1984) 195-199.

Fujiwara, K., Osue, K. and Tsuru, D., J. Biochem., Tokyo, 77 (1975) 739-743.

Gabrielsen, O.S., Hornes, E., Korsnes, L., Ruet, A. and Öyen, T.B., Nucl.Acid. Res., 17 (1989) 6253-6267.

Glick, J. and Oppenheim, J.D., Vox Sang., 49 (1985) 49-57.

Gorecki, M., Bar-Eli, A. and Patchornik, A., Methods Enzymol., 34 (1974) 398-401.

Gospodarowicz, D., J. Biol. Chem., 247 (1972) 6491-6498.

Green, A.A., J. Biol. Chem., 93 (1931) 495-516.

Guha, A., Bach, R., Konigsberg, W. and Nemerson, Y., Proc. Nat. Acad. Sci. U.S., 83 (1986) 299-302.

Haff, L.A., Lasky, M. and Manrique, A., J. Biochem. Biophys. Meth., 1 (1979) 275-290.

Harvey, M.J., Lowe, C.R. and Dean, P.D.G., Eur. J. Biochem., 41 (1974) 353-357.

Hill, R.J., J. Immunol. Methods, 1 (1972) 231-245.

Hořejší, V., Anal. Biochem., 112 (1981) 1-8.

Hořejší, V., Methods Enzymol., 104 (1984) 275-281.

Hořejší, V. and Tichá, M., J. Chromatogr., 376 (1986) 49-67.

Horigome, T. and Sugano, H., Anal. Biochem., 130 (1983) 393-396.

Hosoya, H., Mabuchi, I. and Sakai, H., J. Biochem., Tokyo, 92 (1982) 1853-1862.

Hsu, H.H.T., Munoz, P.A., Barr, J., Oppliger, I., Morris, D.C., Vaananen, H.K., Tarkenton, N. and Anderson, H.C., J. Biol. Chem., 260 (1985) 1826-1831.

Ikura, K., Sakurai, H., Okumura, K., Sasaki, R. and Chiba, H., Agr. Biol. Chem., Tokyo, 49 (1985) 3527-3531.

Jennissen, H.P. and Heilmeyer, L.M.G., Biochemistry 14 (1975) 754-760.

Jepsen, S., Hindersson, P. and Axelsen, N.H., Trans. Roy. Soc. Trop. Med. Hyg., 80 (1986) 534-536.

Karlstam, B., Biochim. Biophys. Acta, 329 (1973) 295-304.

Karube, I., Ishimori, Y., Suzuki, S. and Sato, T., Biotechnol. Bioeng., 20 (1978) 1775-1783.

Kasai, K. and Ishii, S., J. Biochem., Tokyo, 77 (1975) 261-264.

Kašička, V. and Prusík, Z., J.Chromatogr., 273 (1983) 117-128.

Katoh, S., Kambayashi, T., Deguchi, R. and Yoshida, F., Biotechnol. Bioeng., 20 (1978) 267-280.

Keski-Oja, J. and Yamada, K.M., Biochem. J., 193 (1981) 615-620.

Leicht, W., Eur. J. Biochem., 84 (1978) 133-139.

Lotan, R., Beattie, G., Hubbell, W. and Nicolson, G.L., Biochemistry, 16 (1977) 1787-1794.

Lowe, C.R. and Dean, P.D.G., Affinity Chromatography, Wiley, New York, London, 1974, pp. 1-272.

Lowe, C.R., Harvey, M.J. and Dean, P.D.G., Eur. J. Biochem., 41 (1974a) 341-345.

Lowe, C.R., Harvey, M.J. and Dean, P.D.G., Eur. J. Biochem., 41 (1974b) 347-351.

Lundblad, A., Schroer, K. and Zopf, D., J. Immunol. Methods, 68 (1984) 227-234.

Machuga, E.J., Calton, G.J. and Burnett, J.W., Toxicon, 24 (1986) 187-195.

Male, K.B., Nguyen, A.L. and Luong, J.H.T., Biotechnol. Bioeng., 35 (1990) 87-93.

Manen, C.A. and Russell, D.H., Life Sci., 14 (1974) 1907-1915.

Mc Conathy, W.J., Koren, E., Wieland, H., Campos, E.M., Lee, D.M., Kloer, H.U. and Alaupovic, P., J. Chromatogr., 342 (1985) 47-66.

Metcalf, E.C., Crow, B. and Dean, P.D.G., Biochem. J., 199 (1981) 465-472.

Molday, R.S. and Mackenzie, D., J. Immunol. Methods, 52 (1982) 353-367.

Morgan, M.R.A., Brown, P.J., Leyland, M.J. and Dean, P.D.G., FEBS Lett., 87 (1978) 239-243.

Morgan, M.R.A., George, E. and Dean, P.D.G., Anal. Biochem., 105 (1980) 1-5.

Muller, D., Yu, X.J., Fischer, A.M., Bros, A. and Jozefonvicz, J., J. Chromatogr., 359 (1986) 351-357.

Murphy, R.F., Iman, A., Hughes, A.E., Mc Gucken, M.J., Buchanan, K.D., Conlon, J.M. and Elmore, D.T., Biochim. Biophys. Acta, 420 (1976) 87-96.

Nadin-Davis, S. and Mezl, V.A., J. Biochem.. Biophys. Meth., 11 (1985) 185-189.

Nakamura, S., Tanaka, K. and Murkawa, S., Nature, 188 (1960) 144-145.

Ohlson, S., Hansson, L., Glad, M., Mosbach, K. and Larsson, P., Trends Biotechnol., 7 (1989) 179-186.

Ohlson, S., Lundblad, A. and Zopf, D., Anal. Biochem., 169 (1988) 204-208.

Ohlsson, R., Brodelius, P. and Mosbach, K., FEBS Lett., 25 (1972) 234-238.

Pardoe, I.U. and Burness, A.T.H., J. Virol. Methods, 1 (1980) 285-298.

Pensky, J. and Marshall, J.S., Methods Enzymol., 34 (1974) 385-389.

Phillips, T.M. and Frantz, S.C., J. Chromatogr., 444 (1988) 13-20.

Pimplikar, S.W. and Reithmeier, R.A.F., J. Biol. Chem., 261 (1986) 9770-9778.

Porath, J., Biotechnol. Bioeng. Symp. No 3, (1972) 145-166.

Porath, J. and Kristiansen, T., in H. Neurath and R.L. Hill (Editors), The Proteins, 3rd. ed., Academic Press, New York, 1975, pp. 95-178.

Prusík, Z. and Kašička, V., J. Chromatogr., 320 (1985) 81-88.

Prusík, Z. and Kašička, V., J. Chromatogr., 390 (1987) 39-50.

Regnault, V., Rivat, C. and Stoltz, J.F., J. Chromatogr., 432 (1988) 93-102.

Robinson, P.J., Dunnill, P. and Lilly, M.D., Biochim. Biophys. Acta, 285 (1972) 28-35.

Rozie, H., Somers, W., Bonte, A., van Riet, K., Visser, J. and Rombouts, F.M., Biotechnol. Appl. Biochem., 13 (1991) 181-195.

Sada, E., Katoh, S. and Shiozawa, M., Biotechnol. Bioeng., 24 (1982) 2279-2282.

Sairam, M.R., J. Chromatogr., 215 (1981) 143-152.

Shaltiel, S., Henderson, G.B. and Snell, E.E., Biochemistry, 13 (1974) 4330-4335.

Shimura, K., J. Chromatogr., 510 (1990) 251-270.

Shimura, K. and Kasai, K., J. Biochem., Tokyo, 92 (1982) 1615-1622.

Shimura, K. and Kasai, K., J. Chromatogr., 376 (1986) 323-329.

Shinkai, M., Honda, H. and Kobayashi, T., Biocatalysis, 5 (1991) 61-69.

Štrop, P. and Čechová, D., J. Chromatogr., 207 (1981) 55-62.

Swanljung, P. and Frigeri, L., Biochim. Biophys. Acta, 283 (1972) 391-394.

Takeo, K., Electrophoresis, 5 (1984) 187-195.

Takeo, K. and Nakamura, S., Arch. Biochem. Biophys., 153 (1972) 1-7.

Taylor, R.F. and Marenchic, I.G., J. Chromatogr., 317 (1984) 193-200.

Turková, J., Affinity Chromatography, Elsevier, Amsterdam, 1978, pp. 144-147.

Turková, J., Bláha, K., Horáček, J., Vajčner, J., Frydrychová, A. and Čoupek, J., J. Chromatogr., 215 (1981) 165-179.

Turková, J., Bláha, K., Valentová, O., Čoupek, J. and Seifertová, A., Biochim. Biophys. Acta, 427 (1976) 586-593.

Turková, J., Vavreinová, S., Křiváková, M. and Čoupek, J., Biochim. Biophys. Acta, 386 (1975) 503-508.

Ugelstad, J., Berge, A., Ellingsen, T., Aune, O., Kilaas, L., Nilsen, T.N., Schmid, R., Stenstad, P., Funderud, S., Kvalheim, G., Nustad, K., Lea, T., Vartdal, F. and Danielsen, H., Makromol. Chem., Macromol. Symp., 17 (1988) 177-211.

Várady, L., Kalghatgi, K. and Horváth, C., J. Chromatogr., 458 (1988) 207-215.

Walsh, K.A., Burstein, Y. and Pangburn, M.K., Methods Enzymol., 34 (1974) 435-440.

Wikström, P. and Larsson, P.O., J. Chromatogr., 388 (1987) 123-134.

Winer, A.D. and Schwert, G.W., J. Biol. Chem., 231 (1958) 1065-1083.

Wright, C.L., Warsy, A.S., Holroyde, M.J. and Trayer, I.P., Biochem. J., 175 (1978) 125-135.

Yarmush, M.L. and Olson, W.C., Electrophoresis, 9 (1988) 111-120.

Yatscoff, R.W., Tevaarwerk, G.J.M., Clarson, C.L. and Warnock, L.M., Clin. Biochem., 16 (1983) 291-295.

Zoller, M. and Matzku, S., J. Immunol. Methods, 11 (1976) 287-295.

Chapter 9

Bioaffinity chromatography in the isolation, determination or removal of biologically active substances

Bioaffinity chromatography has increasingly become the method of choice for the purification or the determination of biologically active substances. The use of biospecific adsorbents is described in an ever greater number of publications. Many examples of classical, high-performance or large-scale bioaffinity chromatography of a wide range of compounds are presented in Tables 9.1, 9.2 and 9.3. The expansion of this method, which has been particularly vigorous in industrial approaches, has been mainly due to development in the field of biotechnology.

9.1 CLASSICAL BIOAFFINITY CHROMATOGRAPHY

The types of bioaffinity chromatography most frequently described in publications are performed in research laboratories. The widespread use of soft gels - e.g. agarose - or other solid supports in chromatographic procedures under similar conditions is shown in Table 9.1. The preparation of specific sorbents utilizing the exceptional properties of biologically active substances to form specific and reversible complexes has enormously facilitated the isolation of a number of antibodies, antigens and haptens, cells and cell organelles, cofactors and vitamins, many enzymes, enzyme subunits and their modified derivatives, glycoproteins and saccharides, hormones, inhibitors, lectins, lipids, nucleic acids and nucleotides, binding and transfer receptors, tissue factors, SH-containing proteins and peptides, other proteins, specific peptides and amino acids, viruses and other compounds, as described in alphabetical order. In addition to isolated substances, Table 9.1 also shows the affinity ligands used, the solid supports and the spacers, with an indication of whether it was the affinant or the solid matrix that was modified. However, in several instances published in the table, it is not certain whether biospecificity is the principle of chromatographic behaviour.

The different conditions applied during bioaffinity chromatography depend on the nature of the substances to be isolated. Even if, in some instances, a homogeneous

Table 9.1.

Low-presure bioaffinity chromatography (LPBAC)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
ANTIBODIES			
Acylphosphatase antibodies	Acylphosphatase	CNBr-activated Sepharose 4B	BERTI et al. (1982).
Aggregated human γ-globulin (IgG) or immune complexes (at 4 °C)	First component of complement (Clq)	CNBr-activated Sepharose 4B	GIBBONS et al. (1981).
Aglycosylated immunoglobulin G from mouse	Protein A	Protein A-Sepharose CL-4B	LEATHERBARROW and DWEK (1983).
Alloantibodies to allotypic determinants	a1- and b4-Positive rabbit immunoglobulin	Cellulose trans-2;3-carbonate	KENNEDY et al. (1982).
Anti- rabbit immunoglobulin G from swine conjugated with glycoprotein peroxidase	Concanavalin A	Con A-Sepharose	ARENDS (1981).
Anti-(human prolactin) immunoglobulin	Prolactin	CNBr- activated Sepharose CL-4B	HODGKINSON and LOWRY (1982).
Anti-2;4-dinitrophenyl (DNP) antibody from rabbits	1-Fluoro-2;4-dinitrobenzene or DNP-glycine	Sepharose 4B cross- linked with epichlorohydrin; reduced with $NaBH_4$ and after activation by CNBr with ethylene-diamine	HOFFMANN and KUO (1977).
Anti-acetylcholine receptor antibodies from rabbits	Acetyl- choline receptor (noncovalent binding)	CNBr-activated Sepharose 4B with α-bungarotoxin	SCHWARTZ et al. (1979).
Anti-B-50 protein antibody from rabbits	Phosphoprotein B-50 (substrate of protein kinase) from rat brain membranes	CNBr-activated Sepharose 4B	OESTREICHER et al. (1983).
Anti-bovine serum albumin (BSA) antibodies from rabbit	BSA	Trisacryl-GF-2000 with p-nitrophenylchloroformate or N-hydroxysuccinimidechloroformate	MIRON and WILCHEK (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Anti-carbohydrate antibodies from rabbits immunized with vaccine of nonviable cells of Streptococcus bovis	p-Aminophenyl-β-D-glucoronide	CNBr-activated Sepharose 4B and AH- Sepharose	PAZUR et al. (1982).
Anti-cholecystokinin antibody	Cholecystokinin	CH-Sepharose 4B	PENKE et al. (1986).
Anti-DNA antibodies from sera of lupus patients	DNA or Cibacron blue F3GA	DNA cellulose or Affi-Gel Blue	SUENAGA et al. (1986).
Anti-DNA antibodies from serum of patients with systematic lupus erythematosus	Highly polymerized calf thymus DNA	Agarose-poly-L-lysine	NICOTRA et al. (1982).
Anti-DNA antibodies from systemic lupus erythematosus serum	Cibacron blue F3GA and Procion red HE3B	Matrex Gel Blue A and Matrex Gel Red A	POLLARD and WEBB (1982).
Anti-DNP antibodies from goat	DNP-Rabbit serum albumin	Sepharose 4B after periodate oxidation with polyacrylhydrazide	MIRON and WILCHEK (1981).
Anti-ε-dinitrophenyl (DNP) antibodies from goat	ε-DNP-lysine	Trisacryl-GF-2000 with N-hydroxysuccinimidechloroformate	MIRON and WILCHEK (1985).
Anti-ferritin IgG	Ferritin	CNBr-activated Sepharose 4B	IMAGAWA et al. (1982).
Anti-glycolipid antibodies from rabbits	2-Hydroxy-3-N-palmitoylamido-4-O-β-galactosyl butyric acid with N';N'-carbonyldiimidazole	Hexamethylene amino-controlled pore glass	UCHIDA and NAGAI (1980).
Anti-gum arabic antibodies from rabbits	Gum arabic from acacia shrub	AH-Sepharose 4B	PAZUR et al. (1986).
Anti-human chorionic gonadotropin (hCG) Fab'	hCG	CNBr- activated Sepharose 4B	HASHIDA et al. (1985).
Anti-human Fab antibodies from rabbits	Human IgG	CNBr- activated Sepharose 4B	WESTON et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Anti-human serum albumin antibodies from goat	Human serum albumin	Porous silica gel (pore diameter; 500 Å) with γ-aminopropyltrimethoxysilane and glutaraldehyde	ROY and KUNDU (1979).
Anti-*I* and anti-*i* cold antibodies from the sera of patients with chronic cold agglutinin disease	Desialated orosomucoid	CNBr- activated Sepharose 4B	GLICK and OPPENHEIM (1985).
Anti-N^6-(Δ^2-isopentenyl)adenosine antibody	N^6-(Δ^2-isopentenyl)adenosine	Sepharose 4B with 1;4- butanediol diglycidyl ether	MC LENNAN and RANEY (1985).
Anti-rhamnose antibodies from rabbits immunized with vaccines of Streptococcus mutans	p-Aminophenyl α-L-rhamnopyranoside	CNBr-activated Sepharose 4B	PAZUR et al. (1983).
Anti-Streptococcus group A antibodies from rabbits	p-Aminophenyl-β-D-N-acetyl-glucosaminide after diazotisation	Sepharose with glycyl-tyrosine	POULSEN and JOHANSEN (1977).
Antibodies (IgG) against purified erythrocyte membrane Ca^{2+} pumping ATPase	Ca^{2+} -ATPase	CNBr-activated Sepharose 4B	VERMA et al. (1984).
Antibodies against "Facteur thymique serique" (FTS = Pyr-Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn) - from rabbits	Synthetic FTS	Ultrogel AcA22 with glutaraldehyde	JAMBON et al. (1981).
- - (8-lysine)-vasopressin from rabbit antiserum	(8-Lysine)-vasopressin	Sepharose 4B or Spheron P-300	VANĚČKOVÁ et al. (1975).
- - 2'-deoxycytidine 5'-monophosphate (pdC) in conjugate with thyroglobulin from rabbits	pdC and oligo-pdC (via phosphate group)	AH-Sepharose and cellulose (carbodiimide technique)	REDDY and JACOB (1983b).
- - 5'-O-phosphoryl-2'-deoxyadenylyl-(3'⇒5')-2'-deoxythymidine (pdApdT) in conjugate with thyroglobulin from rabbits	pdApdT and 2'-deoxycytidine 5'-monophosphate (via phosphate group)	AH- Sepharose (carbodiimide technique)	REDDY and JACOB (1983a).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
- - acetylcholine receptor from plasma of patients with myasthenia gravis	Acetylcholine receptor	CNBr-activated Sepharose 4B with α-bungarotoxin	LANG et al. (1982).
- - acetylcholine receptor (AChR) from rabbits	Reduced and carboxymethylated AChR	Sepharose 2B	BARTFELD and FUCHS (1979).
- - actin from human smooth muscle antibodies	Actin	Activated CH-Sepharose 4B	RIISOM et al. (1982).
- - aminopeptidase N (EC 3.4.11.-) - from rabbits	Aminopeptidase N from Escherichia coli	Indubiose AcA-34 activated with glutaraldehyde	MURGIER et al. (1977).
- - angiotensin II	(Ile5)-angiotensin II	Affi- Gel 102	HERMANN et al. (1986).
- - Bacillus subtilis levansucrase (EC 2.4.1.10) - from rabbits	Levansucrase	Act Ultrogel AcA22 (glutaraldehyde activated)	PETIT-GLATRON et al. (1987).
- - basic pancreatic trypsin inhibitor (BPTI) - from rabbits	BPTI and trypsin	Sepharose 4B after CNBr- activation	KUROWSKA and SZEWCZUK (1988).
- - benzylpenicilloylbovine-γ-globulin (BPO- BGG) conjugates from rabbits	Sodium-penicilin-G	AH-Sepharose 4B	SCHNEINER et al. (1978).
- - biotin (biotinyl bovine serum albumin) from rabbits	Lipoic acid	Sepharose 4B after CNBr-activation with ethylenediamine (carbodiimide technique)	HARMON (1980).
- - bovine prothrombin fragment 1 from rabbits	Bovine prothrombin fragment 1	CNBr-activated Sepharose	MADAR et al. (1981).
- - calmodulin-dependent cyclic nucleotide posphodiesterase - from rabbits	Protein A and phosphodiesterase	Protein A-Sepharose and CNBr-activated Sepharose 4B	KINCAID (1988).
- - carcinoembryonic antigen (CEA) from perchloric extract of colonic tumor liver metastasis	CEA after periodate oxidation	CNBr-activated Sepharose 4B with human serum albumin (after coupling of CEA reduction with sodium borohydride)	BURTIN and GENDRON (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
- - cloned antigens of Plasmodium falciparum from human plasma	Lysates of bacteria expressing P.falciparum proteins	CNBr-activated Sepharose 4B	CREWTHER et al. (1986).
- - CN1 fragment of histone H3 from anti-H3 globulins from rabbits	Fragment of histone H3 (peptide 1-90:CN1)	Activated CH-Sepharose 4B	ABSOLOM and VAN REGENMORTEL (1977).
- - collagens - from rabbits	Bovine types I and II collagens	Glutaraldehyde-activated Indubiose AcA 2	RICARD- BLUM et al. (1982).
- - cortisol 21-acetate-3-(O-carboxymethyl)oxime - bovine serum albumin from rabbits	Cortisol-3-(O-carboxymethyl)oxime	AH-Sepharose 4B (carbodiimide technique)	LEWIS and ELDER (1985).
- - cortisol-21-acetate-3(O-carboxymethyloxime) coupled to serum albumin - from rabbit	Cortisol-3(O-carboxymethyloxime) or 11-deoxycortisol-3(O-carboxymethyloxime)	AH-Sepharose 4B (carbodiimide technique)	LEWIS and ELDER (1988).
- - dextran	Insolubilized dextran	Sephadex	ROBBINS and SCHNEERSON (1974).
- - digoxin from rabbits	Ouabain-human serum albumin conjugate	Affi-Gel 10	FREYTAG et al. (1984).
- - dinitrophenylated (DNP) bovine immunoglobulin from pigs	DNP-serum albumin	Sepharose 4B after CNBr-ac- tivation	FRANEK et al. (1979).
- - double-stranded DNA from serum of patients with systemic lupus erythematosus	Double-stranded T5 DNA	Sepharose 4B with poly-L-lysine	GILLIAM et al. (1980).
- - erythrocyte	Erythrocyte	Spheron 300	TLASKALOVA et al. (1975).
- - fluorescein isothiocyanate (attached to keyhole limpet hemocyanin) from mice	Florescein	Sepharose 4B	BALLARD et al. (1983).
- - fluoresceinated rabbit serum albumin from goat	Fluoresceinated ovalbumin	CNBr-activated Sepharose 4B	FONG et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
- - glycolipid galactocerebroside from rabbits	Glycolipid hapten of liposomes	Liposomes	ALVING and RICHARDS (1977).
- - glycolipid gangliotetrasylceramide from rabbits	Gangliotetraosylceramide	Polystyrene latex beads G 2401 (coated aggregates formed during incubation at 37 °C for 60 min were precipitated by centrifugation)	TAKI et al. (1982).
- - human growth hormone (HGH) from rabbits	HGH	Affi-Gel 10	O'SULLIVAN et al. (1979).
- - human polymorphonuclear collagenase from rabbits	Collagenase	Sepharose 4B	CHRISTNER et al. (1982).
- - human serum albumin from goats	Human serum albumin	CM-Bio-Gel A (carbodiimide technique)	HARPER et al. (1982).
- - human Tamm-Horsfall glycoprotein from rabbits	Tamm-Horsfall glycoprotein	CNBr-activated Sepharose 4B	HUNT et al. (1985).
- - influenza virus from rabbits	Deoxycholate- split virus	CNBr-activated Sepharose 4B	WATANABE et al. (1979).
- - interleukin 3 from rabbits	Protein A	Protein A-Sepharose CL-4B	MAY and IHLE (1986).
- - L-alkaline phosphatase from rabbits	B- Alkaline phosphatase	CNBr-activated Sepharose 4B	LEHMANN (1980).
- - L-cell colony-stimulating factor (CFS)	CFS (purified by ConA-Sepharose)	CNBr-activated Sepharose 4B	SHADDUCK et al. (1979).
- - major glycoprotein (128 kDa) involved in thrombogenicity of elastin-associated microfibrils - from rabbits	Protein A	Protein A-Sepharose	FAUVEL-LAFEVE et al. (1988).
- - myoglobin from rabbits	Myoglobin	Sepharose 6B with divinylsulphone	SARKAR and MANDAL (1985).
- - myotoxin from rabbit	Myotoxin a from Crotalus viridis viridis venom	Affi-Gel 10	BOBER and OWNBY (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
- - N^6-(Δ^2-isopentenyl) adenosine (iA) from rabbits	iA after periodate oxidation	Sepharose 4B with ethylenediamine	HUMAYUN and JACOB (1974).
- - Naja naja siamensis toxin 3 from horse refined globulin	N.n.siamensis toxin 3	CNBr-activated Sepharose 4B with bovine serum albumin (carbodiimide technique)	KUKONGVIRI-YAPAN et al. (1982).
- - native ovine follitropin from crude antisera from rabbits	Lutropin derivative with covalently-linked subunits	CNBr-activated Sepharose	POIRIER et al. (1984).
- - Nesseria gonorrhoeae and Nesseria meningitidis lipopolysaccharides	Lipopolysaccharides	CNBr-activated Sepharose 4B	RODAHL and MAELAND (1984).
- - nicotinic acetylcholine receptor (AcChR)	AcChR from Torpedo californica (cross-linking with dimethyl suberimidate better than with dimethyl 3;3'-dithiobispropionimidate)	CNBr-activated Sepharose 4B with curarimimetric neurotoxin	HINMAN et al. (1985).
- - oestradiol-3-(carboxymethyl)-ether/bovine serum albumin conjugate from rats	Oestrone-3-(carboxymethyl)- ether	AH-Sepharose 4B (carbodiimide technique)	AL-AZAWIE et al. (1985).
- - oestriol-16α-glucuronide	Oestriol-16α-glucuronide-bovine serum albumin	CNBr- activated Sepharose 4B	MORGAN et al. (1978).
- - ovalbumin from rabbits	Ovalbumin	CNBr-activated Sepharose	NYGREN and STENBERG (1985).
- - peptide corresponding to six C-terminal amino acids of polyoma virus middle-size tumor antigen (Lys-Arg-Ser-Arg-His-Phe) coupled to bovine serum albumin	Lys-Arg-Ser-Arg-His-Phe	CH-Sepharose 4B (carbodiimide technique)	WALTER et al. (1981).
- - periodate oxidized N^6-(Δ^2-isopentenyl)adenosine (i6A)-bovine serum albumin conjugate from rabbits	i6A phosphorylated by cyanoethyl phosphate (coupled through 5'phosphate)	AH-Sepharose 4B	SENAPATHY et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
- - periodate-oxidized 5'-nucleotide conjugates with bovine serum albumin from rabbits	5'-Nucleotide after periodate oxidation (reduced with $NaBH_4$)	Sepharose 4B after CNBr-activation with hexamethylenediamine	EICHLER and GLITZ (1974).
- - rabbit and human immunoglobulin (IgG)	Antigen	Bio-Gel P-300 with glutaraldehyde	TERNYNCK and AVRAMEAS (1972).
- - rabbit Fc fragment of antibodies from donkey	Rabbit IgG	Sepharose with 1;4-bis(2;3-epoxypropoxy)butane	O'SULLIVAN et al. (1979).
- - rabbit immunoglobulin G (IgG) from swine labeled with ferritin (measured using alkaline phosphatase- labeled antibodies against ferritin)	Rabbit IgG	Normal glass tubes (75x12mm) with triethoxypropylamine activated with glutaraldehyde	RAUTERBERG et al. (1984).
- - rat liver cathepsin D (EC 3.4.23.5) from rabbit	Cathepsin D	CNBr-activated Sepharose 4B	YONEZAWA et al. (1987).
- - regulatory subunits (R) of type I (RI) and type II (RII) of c AMP-dependent protein kinases	Bovine skeletal muscle RI and bovine heart muscle RII	CNBr-activated Sepharose 4B after coupling cross-linked with 25mM glutaraldehyde (final concentration 5mM)	KAPOOR and CHO-CHUNG (1983).
- - Schistosoma mansoni soluble worm antigen preparation (SWAP) from pooled sera from Schistosoma mansoni patients	SWAP	CNBr-activated Sepharose 4B	BOCTOR and SHAHEEN (1986).
- - sDNA from human sera of patients with systemic lupus erythematosus	Soft roe DNA	Bromoacetyl cellulose with ovalbumin (carbodiimide technique)	MANAK and VOS (1978).
- - sheep; rabbit and human immunoglobulin (IgG) and bovine serum albumin (BSA) from rabbit and sheep	Human; sheep and rabbit IgG and BSA	Magnetically responsive polyacrylamide (4%) agarose (4%) beads Magnogel activated with glutaraldehyde	GUESDON et al. (1978).
- - soybean lipoxygenase-1 and -2 (EC 1.13.11.12) from rabbits	Lipoxygenase-1 or -2	CNBr-activated Sepharose 4B	VERNOOY-GERRITSEN et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
- - Streptococcus mutans strain SS1083 from rabbits	Formalin-killed cells of Streptococcus mutans SS1083	Triethylaminoethyl cellulose Cellex T	MC KINNEY et al. (1978).
- - target cells KMT-50 (culture cells of methylcholanthrene-induced rat fibrosarcoma) from rabbit serum	Protein A	Protein A-Sepharose CL-4B	SATO et al. (1979).
- - testosterone-3-(O-carboxymethyl)oxime-bovine serum albumin from rabbits	5α-Dihydrotestosterone-3-(O-carboxymethyl)oxime-bovine serum albumin	Sepharose 4B after CNBr-activation	ENGLEBIENNE and DOYEN (1983).
- - tetanus and diphtheria toxins from crude equine tetanus antitoxin	Tetanus and diphtheria toxins	Spherosil XOB-015 with glutaraldehyde	RELYVELD (1981).
- - tobramycin from rabbits	Tobramycin	Sepharose CL-4B with 2-fluoro-1-methylpyridinium toluene-4- sulfonate	NGO (1986).
- - tripeptidyl peptidase II (TPP II) of human erythrocytes from rabbits	TPP II	AH-Sepharose (carbodiimide technique)	BALOW and ERIKSSON (1987).
- - vimentin - from guinea pig	Vimentin	Glutaraldehyde-activated Ultrogel AcA22	BALL and SINGER (1981).
Antibodies specific for protein serotype antigens of meningococci	Outer membrane of meningococci	CNBr-activated Sepharose 4B	BRAKSTAD and MAELAND (1985).
Antibodies specific to complement components C3; C4 and C5b-9 from rabbit antisera	Protein A	Protein A-Sepharose	BHAKDI and KAYSER (1981).
Antibodies to a format determinant of the synthetic myelin basic protein didecapeptide S82	Didecapeptide S82	AH-Sepharose 4B or CH-Sepharose 4B	DAY and HASHIM (1984).
Antibodies to antigenic sites of myoglobin	Peptides of five myoglobin antigenic sites	Sepharose CL-4B	TWINING and ATASSI (1979).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Antibodies to β-lipotropin and β-endorphin	Citraconyl derivative of β-endorphinylthioglycine	ω-Aminohexyl-Sepharose 4B	BLAKE et al. (1982).
Antibodies to human serum albumin (HSA)	HSA	CNBr-activated Sepharose 4B	SHIMIZU et al. (1978).
Antibodies to outer membrane proteins from rabbits	Outer membrane from Escherichia coli	Epoxy-activated Sepharose 6B (coupled in presence of dioxane)	HENRIKSEN and MAELAND (1986).
Antibody against glycosphingolipid (galactosyl (β1 ⇒ 3) N-acetylgalactosaminyl (β 1 ⇒ 4) galactosyl (β 1 ⇒ 4) glucosylceramide) from rabbits	Oligosaccharide (reductamination method with $NaBCNH_3$)	AH-Sepharose 4B	TAKI et al. (1981).
Antibody against human growth hormone (HGH) from goat	HGH	CNBr-activated Sepharose 4B	FORNSTEDT (1984).
Antigen-specific IgM and IgG antibodies (review)	Protein A	Sepharose	LANGONE (1982).
Antispermine antibodies	Spermine	Activated CH-Sepharose 4B	BARTOS et al. (1979).
Antisulfoglycolipid antibodies from immune serum	Glycolipids	Aminopropyl or aminoaryl controlled pore glass with hydroxysuccinimidylazidobenzoate or methyl-4-azidobenzidimate (coupling by irradiation)	LINGWOOD (1984).
Antithyroglobulin autoantibodies from human and rabbit sera	Human thyrogloblin	Sepharose CL-6B activated with 1;1'-carbonyldiimidazole	HEARN et al. (1981).
Bovine antibodies to Brucella abortus lipopolysaccharide	Alkali-treated lipopolysaccharide from Brucella abortus	Sepharose 4B	STILLER and NIELSEN (1983).
Carbohydrate-specific immunoglobulins	Glycoprotein fetuin	Sepharose 4B	SELA et al. (1975).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cell line (71A7) secreting monoclonal mouse immunoglobulin G1 antibody specific for concanavalin A	Concanavalin A	Con A- Sepharose 4B	SPURLL and OWEN (1980).
Circulating immune complexes (CIC) - removal by extracorporeal affinity column from plasma of patients with histologically confirmed malignant disease	Highly purified Protein A from Staphylococcus aureus	Prosorba columns (Protein A bound to silica matrix)	MESSERSCHMIDT et al. (1988).
Complement-binding immune complexes in sera of mink infected with Aleutian disease virus	Equine complement Clq	Sepharose 4B with covalently attached equine IgG	BURGER et al. (1983).
Digoxin-specific antibodies from rabbits	Quabain (analog of digoxin) after periodate oxidation conjugated with human serum albumin (reduced with cyanoborohydride)	Affi-Gel 10	FREYTAG et al. (1984).
DNA Antibodies	Cibacron blue F3GA	Affi-gel blue	EMLEN and BURDICK (1983).
DNA-Binding immunoglobulins	DNA (adsorbed)	Ag(lys)n (beaded agarose with covalently bound poly-L-lysine)	DE BARI et al. (1984).
F (ab')$_2$ fragments of goat anti-mouse immunoglobulin G (IgG) against Fc fragment of murine IgG	Murine IgG	Sepharose 4B	TARKOWSKI et al. (1984).
F(ab')$_2$ from anti-human chorionic gonadotropin IgG digested with pepsin	Human chorionic gonadotropin	CNBr-activated Sepharose 4B	INOUE et al. (1985a).
Fab'-fragment from digoxin-specific antibody conjugated with β-galactosidase	Quabain (analog of digoxin) after periodate oxidation conjugated with human serum albumin (reduced with cyanoborohydride)	Affi-Gel 10	FREYTAG et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Fluorescein-labeled specific anti-hemoglobin antibody from goat	Mouse hemoglobin	CNBr-activated Sepharose 4B and after coupling cross-linked with glutaraldehyde	ANSARI et al. (1981).
Guinea pig (anti-insulin) Fab'-peroxidase conjugate	Insulin after mercaptosuccinylation	Activated Thiol-Sepharose 4B	RUAN et al. (1985).
Guinea pig IgG isotypes	Protein A	Protein A-Sepharose CL-4B	RICARDO et al. (1981).
Horseradish peroxidase conjugated to antibody (rabbit IgG) with glutaraldehyde	Concanavalin A	Con A-Sepharose	ARENDS (1979).
Human IgG antibody	Human IgG	Eupergit C; Separon HEMA 1000; ACT Ultrogel; Affi-Gel 10; CNBr-Sepharose; Epoxy-Sepharose; Reacti-Gel 6X; CDI-Glycophase; NHS-Glycophase	TAYLOR (1985).
IgG fraction of EDP 208 pilus-specific antisera from rabbits	Protein A	Protein A-Sepharose	WOROBEC et al. (1985).
IgG subclasses (IgG1 and IgG2) from guinea pig serum	Staphylococcal protein A	Protein A-Sepharose CL-4B	MARTIN (1982).
IgG subclasses from rat	Protein A	Protein A-Sepharose CL-4B	NILSSON et al. (1983).
IgG subclasses from rat	Protein A	Protein A-Sepharose CL-4B	NILSSON et al. (1982).
Immune complexes from sera of patients with large variety of autoimmune and malignant diseases	Rheumatoid factor	Polystyrene tubes coated with human IgG (by use of glutaraldehyde)	GILEAD et al. (1981).
Immunoglobulin	Protein A	Sepharose CL-6B; Fractogel HW-65; Trisacryl GF-2000; LiChrospher Si 500 diol I and II	HEARN (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Immunoglobulin A - from human serum and colostrum	Jacalin (lectin from jackfruit Artocarpus integrifolia)	Separose 4B after CNBr-activation	ROQUE-BARREIRA and CAMPOS-NETO (1985).
Immunoglobulin from human placenta	Histidine	Sepharose 4B with 1;4-butanediol diglycidyl ether	KANOUN et al. (1986).
Immunoglobulin G against adenosine kinase from rabbits	Adenosine kinase (purified by AMP-Sepharose 4B)	CNBr-activated Sepharose 4B	JURANKA and CHAN (1985).
Immunoglobulin G against NADP-specific glutamate dehydrogenase (NADP-GDH) from rabbit antiserum	Purified NADP-GDH with subunits linked together with dimethylsuberimidate	CNBr- activated Sepharose 4B	YEUNG et al. (1981).
Immunoglobulin G Fc fragment-binding glycoprotein from human blood platelets	Fc Fragment from human IgG	CNBr-activated Sepharose 4B	CHENG and HAWIGER (1979).
Immunoglobulin G from human serum	F(ab')$_2$ antibody fragments prepared by pepsin digestion	CNBr-activated Sepharose 4B	KENNEDY and BARNES (1981).
Immunoglobulin G from rabbit anti-DT diaphorase (EC 1.6.99.2) serum	Protein A	Protein A-Sepharose CL-4B	RAFTELL and BLOMBERG (1980).
Immunoglobulin G subclasses (IgG2 and IgG1)	Protein A	Protein A-Sepharose	ESCRIBANO et al. (1982).
Immunoglobulin G to human ferritin from rabbits	Ferritin	CNBr-activated Sepharose 4B	IMAGAWA et al. (1982).
Immunoglobulin M and complement component C4b-binding protein from human serum	Native calf thymus DNA	Cellulose CF 11	ABDULLAH et al. (1985).
Immunoglobulins G against human chorionic gonadotropin; carcinoembryonic antigen; α-foetoprotein or casein from rabbits	Protein A	Protein A-Sepharose CL-4B	KARDANA et al. (1979).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Immunoglobulins IgG1 and IgG2 from serum of goat immunized against rabbit tubular basement membrane	Staphylococcal protein A	Protein A-Sepharose CL-4B	DUHAMEL et al. (1980).
Measles-specific IgM antibodies from nonimmunized and immunized measles patients (detection)	Goat antibody against human IgM and Protein A of Staphylococcus aureus	Polystyrene microtitration plates and Protein A-Sepharose CL-4B	TISCHER and GERIKE (1986).
Monoclonal anti-dinitrophenol (DNP) antibodies	p-Nitrophenyl-casein	CNBr-activated Sepharose 4B	STANLEY et al. (1983).
Monoclonal antibodies against carcinoembryonic antigen	Protein A	Protein A-Sepharose CL-4B	WAGENER et al. (1983b).
Monoclonal antibodies against inactivated preparation of tickborne encephalitis virus	Protein A	Protein A-Sepharose	STEPHENSON et al. (1984).
Monoclonal antibodies against low-density lipoprotein receptor - from mouse ascites fluid and tissue culture medium	Protein A	Protein A-Sepharose	SCHMITZ et al. (1988).
Monoclonal antibodies from ascitic fluids	Cibachrom Blue F3GA	DEAE Affi-Gel Blue	BRUCK et al. (1982).
Monoclonal antibodies to carboxypeptidase A from mice	Carboxypeptidase A	Sepharose after periodate oxidation with polyacrylhydrazide or Sepharose after CNBr-activation	SOLOMON et al. (1984).
Monoclonal antibody against human tumor antigen (250 kDa glycoprotein/proteoglycan from melanoma cells)	Protein A	Protein A-Sepharose CL-4B	LEE et al. (1986).
Monoclonal antibody to human platelet intracellular membranes from ascites fluid	Protein A	Protein A-Sepharose	HACK et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Monoclonal IgG against elongation factor EF-G	Fab fragment of monovalent immunoglobulin (coupling by use of SH group)	AH- Sepharose 4B with N-maleonyl-β-alanine	PRISYAZHNOY et al. (1988).
Monoclonal immunoglobin G1 against Protein A	Protein A	Protein A Avi Gel F	BLOOM et al. (1989).
Monoclonal immunoglobulin M (IgM) from serum of patient with Waldenstrom's macroglobulinemia	Concanavalin A	Con A- Sepharose	CHUA et al. (1984).
Monospecific anti-Pseudomonas immunoglobulins from serum and lung lavage fluid from patients with cystic fibrosis	Pseudomonas aeruginosa lipopolysaccharide	CNBr-activated Sepharose 4B or Epoxy-activated Sepharose 6B	FICK Jr. et al. (1980).
Monospecific anti-Salmonella liposaccharide antibodies	Liposaccharide activated by benzoquinone	AH-Sepharose 4B	GIRARD and GOICHOT (1981).
Monospecific antibodies to hepatis B surface antigen (HBsAg) from human plasma containing HBsAg	HBsAg (control immunoadsorbent were prepared in same fashion as CPG-HBsAg from HBsAg-negative; normal human plasma)	Controlled pore glass (CPG) Bioglass 1500 -adsorbtion	DODD and KOBITA (1978).
Plasmin-degradeted immunoglobulin G (plasmin-treated IgG digest)	Fragment from elastase-digested plasminogen constituting three N-terminal triple-loop structures (high affinity lysine-binding sites of plasminogen)	CNBr-activated Sepharose 4B	HARPEL et al. (1989).
Polysomes enriched in mRNA for low density lipoprotein (LDL) receptor after incubation with anti-LDL receptor IgG from rabbit (polysome/antibody slurry)	Protein A	Protein A-Sepharose	RUSSELL et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Rabbit (anti- human chorionic gonadotropin) Fab'-peroxidase conjugate	Human chorionic gonadotropin	CNBr-activated Sepharose 4B	RUAN et al. (1985).
Rabbit (anti-human IgG) Fab'	Human IgG	CNBr-activated Sepharose 4B	INOUE et al. (1985b).
Rabbit anti-DNP IgG	Dinitrobenzene (DNP)	Glass coverslips with poly-L-lysine	MICHL et al. (1983).
Rabbit anti-human chorionic gonadotropin Fab'-β-D-galactosidase conjugate	Goat (anti-rabbit IgG) IgG	CNBr-activated Sepharose 4B	INOUE et al. (1985a).
Receptor monoclonal antibody (blocks attachment of major group of human rhinoviruses)	Protein A	Affi-Gel protein A	COLONNO et al. (1986).
Sex hormone binding globulin from human serum of pregnant females	5α-Dihydrotestosterone	Epoxy-activated Sepharose 6B	STALLA et al. (1982).
Soluble immune complexes from human serum	Protein A	Protein A-Sepharose	VIRELLA et al. (1981).
Sperm antibodies mixed with preparations of donor sperm in serum of infertility patients (detection)	Human immunoglobulins (IgG; IgA or IgM)	Rabbit antihuman immunobeads	SHULMAN et al. (1985).
Thyroxine (T4) and triiodothyronine (T3) after incubation with anti-T4antibody or anti-T3antibody and antigen-β-D-galactosidase conjugate (determination)	IgG fractions of goat antiserum to rabbit IgG reduced with 2-mercaptoethylamine	Activated Thiol-Sepharose 4B	YAMAMOTO et al. (1981).
Xenogeneic antibodies with reactivity for surface determinants of guinea pig line-10 hepatocarcinoma	Formalin-treated line-10 tumor cells	DEAE-cellulose = DE 52 fibers	METHEWS et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Fucose-binding lectin from Ulex europeus	p-Aminophenyl-α-L-fucoside	Copolymer N-[tris(hydroxymethyl)methyl] acrylamide/acrylamide-6-hexanoic acid (A-6-NH-Trisacryl H) and N-[tris(hydroxymethyl)methyl] acrylamide/methacryloyl-glycylglycine (MGG-Trisacryl H) (carbodiimide technique)	RACOIS and BOSCHETTI (1978).
Phosphate carrier from pig heart mitochondria	Mersalyl (sodium o-((3-(hydroxymercuri)-2-methoxypropyl)-carbonyl)- -phenoxyacetate) coupled using N-ethoxycarbonyl-2-ethoxy-1;2-dihydro- quinoline	AM Ultrogel A-4-R with epichlorohydrin and ammonia	TOURAILLE et al. (1981).
ANTIGENS AND HAPTENS			
2-Adenovirus structural proteins and herpes simplex virus glycoprotein	Monoclonal antibodies to these proteins	Monolayers of Staphylococcus aureus Cowan strain A on plastic tissue culture plates after drying under vacuum without freezing	RANDALL (1983).
2-Amino-3-methylimidazo(4;5-f) quinoline (IQ) from foodstuffs and metabolites from body fluids (determination)	Monoclonal antibodies against IQ	CNBr-activated Sepharose 4B	SKIPPER et al. (1987).
Acylated and radiolabelled synthetic derivative of residues 75-83 of bovine myelin basic protein (S79)	S79-reactive antibodies	CNBr-activated Sepharose 4B	DAY et al. (1981).
Adipocyte plasma membrane antigens from rat intrinsic membrane proteins	IgG from antiserum from rabbits against intrinsic membrane proteins	CNBr-activated Sepharose 4B	PILLION et al. (1980).
Aflatoxin derivatives from urine of people	IgM monoclonal antibody against aflatoxins	Sepharose 4B	GROOPMAN et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Albuterol in human plasma (determination)	Immunoglobulin G (IgG) from rabbit antisera against O-(3-carboxypropionyl) derivative of albuterol covalently linked to bovine serum albumin	CNBr-activated Sepharose 4B	ONG et al. (1989).
Allergen from Kentucky Blue Grass pollen (KBG-R)	Monoclonal antibodies against KBG-R	Activated Sepharose 4B	EKRAMODDOULLAH et al. (1986).
α-Crystallin (one of major proteins of lens) and its subunits -from foetal calf lenses	Monoclonal antibodies	CNBr-activated Sepharose 4B	STEVENS and AUGUSTEYN (1988).
α-Fetoprotein (AFP) from ascites of patient with hepatoma	Monoclonal anti-AFP antibodies from ascites of mice	Sepharose 4B	NISHI and YAMAZAKI (1983).
α-Hemolysin (AH) from Escherichia coli	Anti-AH monoclonal antibodies	Affi-Gel 15	BOHACH and SNYDER (1986).
Antigen epitope	Monoclonal antibodies	CNBr-activated Sepharose 4B	GAMBLE (1985).
Antigen from colorectal carcinoma cell line SW948 and from malanoma WM9 cells	Monoclonal antibody 16B-13 (from immunization of BALB/c mice with lung tumor line)	Sepharose 4B after CNBr-activation	PAK et al. (1983).
Antigen from nematodes	Antibodies against Dirofilaria immitis; Toxocara canis; Angiostrongylus cantonensis or Ascaris lumbricoides crude antigens from rabbits	CNBr-activated Sepharose 4B	WELCH and DOBSON (1981).
Antigen-binding molecules from mice that were hyperimmunized with sheep erythrocytes	Anti-mouse immunoglobulins from rabbits or goats	Sepharose 4B	IVERSON et al. (1983).
Antigenic component (antigen I) of cell surface of Streptococcus mutants	IgG of rabbit antiserum against antigen I/II or antigen II	CNBr-activated Sepharose 4B	RUSSELL et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Antigenic fractions from collagenase-solubilized human glomerular basement membrane (GBM) (implicated in pathogenesis of anti-GBM renal disease)	Anti-GBM antibody	CNBr-activated Sepharose 4B	HUNT et al. (1982).
Antigenic substances from livers of mice infected with Tyzzer's disease	Antibody against crude antigen from mice	CNBr- activated Sepharose 4B	ORIKASA et al. (1984).
Antigenically active Bacillus anthracis lethal factor	Monoclonal antibodies against lethal factor component of Bacillus anthracis toxin	CNBr-activated Sepharose 4B	MACHUGA et al. (1986).
Antigens from culture medium of in vitro cultivated Plasmodium falciparum	Antibodies	CNBr-activated Sepharose 4B	JEPSEN and ANDERSEN (1981).
Antigens of Fasciola hepatica	Concanavalin A	Con A-Sepharose	HILLYER and DE ATECA (1979).
Antihemorrhagic factors (AHR) from opossum serum	Monoclonal antibody against AHR	CNBr-activated Sepharose 4B	TARNG et al. (1986).
Apolipoprotein F (ApoF) from human plasma	Anti-ApoF antibody from goat	CNBr-activated Sepharose CL-4B	KOREN et al. (1982).
β-Lipotropin from pig pituitary and precursor peptides	Monoclonal antibody against porcine β-lipotropin	Sepharose 4B	THORPE et al. (1983).
Biomphalaria glabrata/ Schistosoma mansoni shared antigens	IgG from serum of a human infected with S.mansoni	CNBr-activated Sepharose 4B	RIVERA-MARRERO and HILLYER (1985).
Biosynthetically radiolabeled sialyl oligosaccharide	Monoclonal antibody (19-9) against cancer-associated sialyl-Lea antigen	Protein A-Sepharose	HANSSON and ZOPF (1985).
Blood group A-active oligosaccharides	Anti-blood group A monoclonal antibody (IgMk)	Con A-Sepharose or CNBr-activated Sepharose	DAKOUR et al. (1986).
Carcinoembryonic antigen (CEA) of A; B; 0 blood group specificity (detection)	Immunoglobulins from anti-A;B;H (CEA) antisera	Sepharose 4B	MAGOUS et al. (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Carcinoembryonic antigen (CEA) (heat stable glycoprotein) from cancer tissue	Antibody against α1-acid glycoprotein	CNBr- activated Sepharose 4B	OCHI et al. (1982a).
Carcinoembryonic antigen (CEA) and related antigens from colorectal carcinoma and spleen tissues; bile and meconium	Monoclonal anti-CEA antibodies (purified by Protein A-Sepharose 4B) from ascitic fluids	CNBr-activated Sepharose 4B	NEUMAIER et al. (1985).
Carcinoembryonic antigen (CEA) from membrane fractions of human carcinomas	Anti-CEA immunoglobulin IgG	CNBr-activated Sepharose 4B	WAGENER et al. (1982).
Carcinoembryonic antigens	Monoclonal antibodies against carcinoembryonic antigen	CNBr-activated Sepharose 4B	WAGENER et al. (1983a).
Cardiotoxin (polypeptide containing 60 amino acids) synthetized by solid-phase methods	Antibodies against cardiotoxin from rabbits	Sepharose 4B after CNBr-activation	WONG et al. (1978).
Cholinergic nerve terminals (contained choline acetyltransferase- EC 2.3.1.6; lactate dehydrogenase- EC 1.1.1.27 and glutamate decarboxylase- EC 4.1.1.15) from rat brain	Sheep anti-cholinergic antigen 1 serum	Cellulose with nitrobenzyloxymethyl pyridinium chloride; reduced by sodium dithionite and diazotized with nitrous acid	RICHARDSON (1986).
Cholinergic nerve terminals from mammalian brain after incubation with sheep anti-cholinergic antigen 1-serum	Monoclonal anti- (sheep IgG)-antibody	Cellulose with nitrobenzyloxymethyl pyridinium chloride; reduced by sodium dithionite and diazotized with nitrous acid	RICHARDSON et al. (1984).
Chromatin derived from single human chromosome in mouse-human hybrid cell line	Immunoglobulin G against mouse and human chromatin	CNBr-activated Sepharose 4B	ALEVY and FLEISCHMAN (1980).
Chromogranin (endocrine granule protein) from human adrenal glands	Monoclonal antibody against human chromogranin	Sepharose CL 4B	WILSON et al. (1986).
Clostridium perfringens type C (CwC)	Antibody against CwC	Glutardialdehyde-activated silicate beads	LAWRENCE et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cofactor of lipase (colipase II)	Anti-colipase antibodies	Ultrogel AcA 22	LEGER et al. (1982).
Complememt attack complexes from insulin activated normal human serum	Monospecific caprine anti-human C5 immunoglobulin G	CNBr-activated Sepharose 4B	WARE et al. (1981).
Complement component C9 from human plasma	Monoclonal antibody C9-8	CNBr-activated Sepharose 4B	MORGAN et al. (1983).
Complement component Clq from human plasma	Antibodies against purified Clq from rabbits	Agarose-polyaldehyde microsphere beads (produced by encapsulating polyacrolein microspheres with agarose)	GAZITT et al. (1985).
Complement-fixing immune complexes (IC) from whole serum	Monoclonal anti-C3g antibodies	Sepharose 4B	SAMUEL et al. (1986).
Core complex of I-Ak antigens from detergent lysate of spleen cells	Hybridoma antibody 10-2.16 from ascites fluid	Sepharose CL-4B after CNBr-activation	ZECHER and RESKE (1982).
Crosslinked complexes of high mobility group proteins (HMG) 14 and 17 (from chicken erythrocyte nuclei) with DNA	Antibodies against HMG 14/17 from rabbit	Protein A-Sepharose	SHICK et al. (1985).
Cytokinins from plant extracts	Antibodies against cytokinin- bovine serum albumin conjugates (prepared by cytokininpolylysine- agarose columns)	Microcrystalline cellulose	MAC DONALD and MORRIS (1985).
Cytokinins from soy bean trifoliate leaves	Immunoglobulin G against zeatin riboside- or dihydrozeation riboside-bovine serum albumin conjugates from rabbits	CNBr-activated Sepharose 4B or Affi-Gel 10	DAVIS et al. (1986).
Cytotoxins (proteins) from human peripheral blood mononuclear cells	Monoclonal immunoglobulins against human cytotoxin	Trisacryl GF 2000 with hexamethylenediamine and N-hydroxysuccinimide	HAHN et al. (1985).
Detergent solubilized membrane proteins DR and DR-related antigens from homozygous B lymphoblastoid cells	Monoclonal antibody LKT111	Affi-Gel 10	ANDREWS et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Detergent-solubilized HLA antigens from human B lymphoblastoid cells	Monoclonal antibodies against HLA products	Sepharose CL-4B after CNBr-activation	PARHAM (1983).
Detergent-solubilized membrane antigens (I-A and H-2) from spleen cells after binding of biotinylated monoclonal antibodies	Streptavidin	Affi-Gel 15	UPDYKE and NICOLSON (1984).
Digoxin (determination in serum)	Polyclonal antibody against digoxin	Glutaraldeyde activated silica Sperosil	REH (1988).
DNA Primase-DNA polymerase complex from crude soluble extracts of yeast cells	Monoclonal antibodies against yeast DNA polymerase I	Protein A-Sepharose (coupling with dimethyl suberimidate)	PLEVANI et al. (1985).
Ductal carcinoma antigen from mice	Monoclonal antibody F36/22 (purified by Protein A-Sepharose) and wheat germ lectin	CNBr- activated Sepharose 4B and wheat germ lectin-Sepharose	PAPSIDERO et al. (1984).
Epidermal growth factor (EGF) from clonal A431 human epidermoid carcinoma cells	Monoclonal antibody against EGF	Sepharose 4B	WEBER et al. (1984).
Epidermal growth factor urogastrone from human urine	Monoclonal antibodies against urogastrone	Reacti-Gel (activated with 1;1'-carbonyldiimidazole)	HISSEY et al. (1985).
Escherichia coli heat-labile enterotoxin	Anti-cholera toxin immunoglobulin	CNBr-activated Sepharose 4B	WOLK et al. (1980).
Fibrinogen-fibrin related antigen from human plasma of normal subjects and patients with defibrination	Antibody to fibrinogen	Sepharose CL-4B	MERSKEY et al. (1980).
Gibberellins from plant extracts	Anti-gibberellin antibodies from rabbits	Affi-Gel 10	DURLEY et al. (1989).
Heat-stable antigen from Treponema Reiter	Antibodies from syphilitic patients	CNBr-activated Sepharose 4B	PEDERSEN et al. (1981).
Hepatis B viral surface antigen (HBsAg) from human serum (quantitative removal)	Anti-HBsAg IgM	Regenerated cellulose acetate filters	MARCINIAK et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Hepatitis B surface antigen (HBsAg) from blood and plasma	Human antiserum to HBsAg	Sepharose 2B after CNBr-activation	ARESON et al. (1980).
High mobility group protein 17 (HMG-17) from chromatin	Anti- HMG-17 IgG	CNBr-activated Sepharose 4B	MALIK et al. (1984).
Human choriogonadotropin (hCG) from urine of patients with hydatidiform mole	Immunoglobulin G against hCG from rabbits	Sepharose 6B	CHOY et al. (1979).
Human cytotoxin	Monoclonal antibody against cytotoxin	Trisacryl-GF-2000 with N-hydroxysuccinimidechloroformate and ε-aminocaproic acid	MIRON and WILCHEK (1985).
Human fibroblast interferon (IFN-β1)	Monoclonal anti-IFN-β1 antibodies	Agarose-polyacryl hydrazide	NOVICK et al. (1983b).
Human interferon-γ (IFN-γ)	Monoclonal anti-IFN-γ antibodies	Agarose-polyacryl hydrazide	NOVICK et al. (1983a).
Human myelin basic protein (MBP)	Monoclonal antibodies against bovine MBP	CNBr-activated Sepharose 4B	TIGYI et al. (1984).
Human pregnancy zone protein (PZP)	Anti-human PZP-immunoglobulin	CNBr-activated Sepharose 4B	FOLKERSEN et al. (1978a).
Human seminal plasma No.7 antigen	Monoclonal antibody	CNBr- activated Sepharose 4B	ISOJIMA et al. (1982).
Hybrid proteins with both IgG binding and β-galactosidase activities (gene fusion vectors based on gene for staphylococcal Protein A and β-galactosidase in Escherichia coli strains containing such plasmids)	Immunoglobulin G	IgG-Sepharose	UHLEN et al. (1983).
IgG + and IgG- lymphocytes (selection) of rat mesenteric lymph node	Goat anti-rat immunoglobulin G antibody	Glass beads of 48-60 mesh coated with poly(2-hydroxyethyl methacrylate)polyamine	KATAOKA et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Immunoadsorbed migration inhibitory factor (MIF) (induced macrophage disappearance)	Anti-MIF antibody from rabbit	CNBr- activated Sepharose 4B	OCHIYA et al. (1982).
Immunoglobulin (Ig) A or M from human serum	Antibodies against human IgA or IgM from rabbits	Protein A-Sepharose (non- covalently bound)	RUSSELL et al. (1984).
Immunologically active cyanogen bromide peptide from bovine brain filament preparation	Anti-glial fibrillary acidic protein antibody	Sepharose 4B	DAHL and BIGNAMI (1979).
Indole-3-acetic acid (IAA) from Pinus Sylvestris	IgG fraction of rabbit antibodies against IAA-bovine serum albumin conjugate	Glutardialdehyde-activated silicate support	SUNDBERG et al. (1986).
Insulin-like growth factors (IGF)	Monoclonal antibodies against IGF I	CNBr-activated Sepharose	LAUBLI et al. (1982).
Intrinsic factor (IF)-cobalamin (B12) complex from human gastric juice	Type II antibody to IF-B12 complex (binding antibody) from serum of patient with pernicious anaemia	Protein A- Sepharose CL-4B (adsorbed antibody covalently attached using carbodiimide technique)	SHEPHERD et al. (1984).
Kininogens from human plasma	Antibodies against immunologically pure human albumin from rabbits (removing protein impurities) or anti-kininogen antibodies	Sepharose 4B after CNBr-activation	KARKKAINEN (1985).
Labeled thymus-leukemia antigens	Cowan 1 strain of Staphylococcus aureus (SaC1)	Glutaraldehyde fixed SaC1	PISCHEL and LITTLE (1980).
Lactoferrin (LF) from human milk and polymorphonuclear neutrophils	Monoclonal antibody against human LF	Reacti-Gel 6X	BROXMEYER et al. (1986).
Leishmania membrane glycoprotein	Monoclonal antibody	Affi-Gel 10	CHANG and CHANG (1986).
Lethal factor from venom of Vespa orientalis	Monoclonal antibody against Physalia physalis venom from mice	CNBr- activated Sepharose 4B	RUSSO et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Lewis blood group antigens from human red blood cells carrying Lewis antigen	Goat anti-Lewis antibody	Glycophase controlled- pore glass beads with reactive N-hydroxysuccinimide ester side- chains	FRANTZ et al. (1988).
Membrane proteins from cell lysates	Monoclonal antibodies	Protein A-Sepharose CL-4B (crosslinking with dimethyl pimelimidate)	SCHNEIDER et al. (1982).
Myelin basic protein (BP) from delipidated bovine brain tissue	Anti-BP antibodies from rabbits (purified by BP coupled to CNBr- activated Sepharose 4B)	CNBr-activated Sepharose 4B	REIDL et al. (1981).
Patient-specific IgE reactive allergens	Anti-human IgE antibodies from rabbits	Sepharose CL-4B	SONDERGAARD and WEEKE (1984).
Phospholipase-free cardiotoxins from venom of Elapidae snake Naja mossambica mossambica	Antibodies against phospholipase Ph3 from rabbits	CNBr-activated Sepharose 4B	DELORI and TESSIER (1980).
Plant hormone abscisic acid (ABA)	Antibodies against ABA	CNBr-activated Sepharose 4B	MERTENS et al. (1982).
Plasma membrane fraction from post-nuclear supernatant of cultured rat hepatocyte (Fao cell)	Affinity-purified sheep anti-rabbit IgG	Diazonium derivative of amino cellulose	DEVANEY and HOWELL (1985).
Plasminogen activator (PA) from Bowes melanoma tissue culture media	Monoclonal antibody against PA	CNBr-activated Sepharose 4B	REAGAN et al. (1985).
Plasminogen activator (PA) from pig heart tissue	Antibodies against human melanoma tissue PA from goats	Sepharose 4B	POHL et al. (1986).
Platelet plasma membrane proteins labelled with DNP	Anti-DNP antibodies	Sepharose 4B	ROTMAN and LINDER (1981).
Polysaccharide egg antigens	Concanavalin A and Wheat germ agglutinin	Con A-Sepharose and WGA-Sepharose	BOCTOR et al. (1982).
Pregnancy-specific β1-glycoprotein from maternal serum (PSβ1G)	IgG fraction from rabbit anti-PSβ1G-I antiserum	CNBr-activated Sepharose 4B	GRIFFITHS and GODARD (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Protein A from cell extracts of Staphylococcus aureus	Immunoglobulin G	IgG-Sepharose 4B	LOFDAHL et al. (1983).
Protein B1 from Escherichia coli ribonucleotide reductase	Monoclonal antibodies against B1 protein	Sepharose 4B	ANDERSON et al. (1986).
Protein P1 from Mycoplasma pneumoniae	Monoclonal antibodies against protein P1	CNBr-activated Sepharose 4B	LEITH and BASEMAN (1984).
Proteinase from cell-surface of human leukocytes	Antibodies specific for purified proteinase	Sepharose CL-4B activated with 1;1'-carbonyl diimidazole	HARPER et al. (1984).
Simian virus 40 (SV 40) large tumor antigen	Viral DNA extracted from infected cells (SV 40 or F161 variant) or calf thymus DNA	Cellulose or Calf thymus DNA cellulose	OREN et al. (1980).
Soluble specific antigens of systemic candidiasis	Immunoglobulin G against Candida albicans from rabbits	CNBr- activated Sepharose 4B	BENBOUZID et al. (1984).
Staphylococcal toxic shock syndrome toxin 1 (TSST-1) from patient with toxic shock syndrome	Monoclonal antibody against TSST-1 from mice	Affi-Gel 10	REEVES et al. (1986).
T Cell supressor factor (TsF) from human tonsil cells	Monoclonal antibody against TsF	CNBr-activated Sepharose 4B	STEELE et al. (1985).
Three exotoxic factors: protective antigen (PA); lethal factor (LF) and edema factor (EF) - from crude culture supernatant of Bacillus anthracis	Monoclonal antibodies against PA; LF and EF	Sepharose CL-4B after CNBr-activation	LARSON et al. (1988).
Thyroglobulin from human thyroids	Antibodies against whole human serum	Sepharose CL-6B activated with 1;1'-carbonyldiimidazole	HEARN et al. (1979).
Tissue factor apoprotein - from human brain	Polyclonal antibodies against human brain tissue factor from goat	Affi- Gel 15	RAO (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Tissue factor utilizing factor VII from human brain	Immunoglobulin G against human factor VII from goat	Affi-Gel 15	RAO and RAPAPORT (1987).
Toxoplasma antigen (protected mice against lenthal infection with Toxoplasma gondii) from Toxoplasma sonicate	Monoclonal antibody F3G3	CNBr-activated Sepharose CL-4B	SHARMA et al. (1984).
Transferin	Biotinylated antibodies	Avidin-Sepharose	BAYER and WILCHEK (1990).
Transforming growth factor-β (TGF) from bovine bone	Monoclonal antibodies against native bovine TGF	CNBr-activated Sepharose	DASCH et al. (1989).
Tumor antigen p53 from mouse neuroblastoma cells	Monoclonal PAb122 antibodies	Sepharose 4B	LEVITSKY et al. (1983).
Vitamin K-dependent proteins from human plasma	Monoclonal antibodies against human vitamin K-dependent proteins from mice	CNBr-activated Sepharose 4B	JENNY et al. (1986).
CELLS AND CELL ORGANELLES			
Animal cells (human skin fibroblasts;HeLa and erythroleukemic cells; human kidney and rat colon carcinoma cells)	Gelatin or chitosan	Gelatin or chitosan cross- linked with glutaraldehyde; gelatin magnetic beads obtained in addition of Fe_3O_4	NILSSON and MOSBACH (1980).
Anti-2;4-dinitrophenyl plague-forming cells from mouse spleen	Dinitrophenylated ovalbumin	Polymetaacrylic plastic beads	SLANKARD-CHAHINIAN and SISKIND (1979).
Antibody-bearing cells: IgG-carrying mouse spleen B lymphocytes and thymuses thymocytes (after treatment with antibodies)	Protein A of Staphylococcus aureus (SpA)	CNBr-activated Sepharose 6MB or sheep red blood cells coated with SpA	GHETIE et al. (1978).
Antibody-coated lymphocytes	Albuminated CIBA blue dextran- Protein A - conjugate	CNBr-activated Sepharose 6MB	DUFFEY et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
B cells of human lymphocyte population	Anti-human F(ab')$_2$ antibody from rabbits	Sephadex G-200 after CNBr-activation	ROMAIN and SCHLOSSMAN (1984).
Basophil leukocytes from human peripheral blood	Anti-IgE	CNBr-activated Sepharose 6MB	TOLL et al. (1981).
Brain synaptosomes (chick)	Concanavalin A	Con A-Sepharose	SHARMA et al. (1986).
Brush-border membrane vesicles induced by essential fatty acid- deficient diet - from male piglets	Phlorizin; linolenic acid and wheat germ agglutinin (WGA)	Phlorizin copolymerized at 120 °C by use of formaldehyde and urea; Linolenic acid-agarose and WGA-Ultrogel AcA22	ALESSANDRI et al. (1988).
Cell subpopulations with a fluorescein isothiocyanate (FITC) labeled ligand	Goat anti-FITC-antibody	Polyacrylamide beads (Affi-Gel Cell Sorting System; Anti-FITC)	BARAN et al. (1982).
Cells and cell membranes (review)	Lectins; antigens; antibodies etc.	Sepharose; glass beads; nylon etc.	SHARMA and MAHENDROO (1980).
Cells having IgG on their surface or cells coated with IgG antibody specific for particular membrane antigen	Protein A from Staphylococcus aureus	CNBr-activated Sepharose 6MB or Protein A-Sepharose 6MB	GHETIE and SJOQUIST (1984).
Cells with erythroid differentiation (B-G) antigens (determination)	Monoclonal antibodies	Fluorescent latex spheres	KIERAN and LONGENECKER (1984).
Cloned murine T helper (Th; type II) cells	Anti-T cell receptor monoclonal antibodies	Polystyrene latex beads (3-μm average diameter)	DE BELL et al. (1990).
Coated vesicle proteins (subcellular organelles) from pig brains	Calmodulin	CNBr-activated Sepharose 4B	LINDEN et al. (1981).
Cortical thymocytes	Lobster agglutinin 1	CNBr-activated Sepharose 6MB	HERRON et al. (1983).
Cytotoxic effector cells from mice immunized with tumor cells	Target cells	Monomolecular film of phytohaemagglutinin onto polystyrene tissue culture plate with poly-L-lysine	SILVA et al. (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Daudi lymphoblasts	Biotinylated antibodies	Avidin conjugation to CNBr-activated Sepharose 6MB	BERENSON et al. (1986b).
Dictyostelium discoideum plasma membrane fragments	Fluorescein-labeled F-actin	Sephacryl S-1000 with rabbit antifluorescein antibody	LUNA et al. (1984).
Epidermal Langerhans cells	Staphylococcus aureus cells	Falcon petri dishes	SCHULER et al. (1983).
Erythrocyte and HeLa cell plasma membranes	Polylysine	Bio-Gel P2	COHEN et al. (1980).
Erythrocyte plasma membrane	Wheat germ agglutinin (WGA)	WGA- Sepharose 6MB	KAPLAN et al. (1984).
Escherichia coli cells (genetic selection technique - directed evolution of λ receptor)	Soluble starch	Sepharose 6B with 1;4-butanediol diglycidyl ether	FERENCI and LEE (1982).
Eukaryotic messenger ribonucleoprotein particles from chick embryonic muscles	Oligo (dT)	Oligo-dT-cellulose	JAIN et al. (1979).
Externally labeled (^{125}I) normal mesenteric node lymphocytes	Anti-immunoglobulin (purified by Sepharose CL-4B with coupled immunoglobulins)	Polyvinylchloride microtiter plate	TAMURA et al. (1984).
Fluorescein labeled normal human T cells or T cell line (eluted with fluorescein-L-lysine)	Antibodies against fluoresceinated rabbit serum albumin from goat	Plastic culture dishes	FONG et al. (1981).
Hepatocytes from rat	Five galactoside aglycon groups	Copolymerization procedure of ligands with acrylamide and bisacrylamide	WEIGEL et al. (1979).
Human and rabbit peripheral blood cells	Polyethylene glycol	Epoxy-activated Sepharose 6B	MATSUMOTO et al. (1984).
Human and rabbit peripheral blood cells	Glycidyl ethers of poly(propylene glycol)	Several kinds of agarose bead	MATSUMOTO and SHIBUSAWA (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Human erythrocytes with blood group specifity for adsorbed lectin	Hog gastric mucin blood group A + H substance and after coupling adsorption of lectins from Ulex europaeus; L.tetragenol-obus; D.biflorus; Phaseolus lunatus or H. comatia	Sephadex G-25 or Sepharose 4B after CNBr-activation	PEREIRA and KABAT (1979).
Human lymphocytes (fractionation of T and B cells)	Helix pomatia A hemagglutinin and wheat germ agglutinin	CNBr- activated Sepharose 6MB	HELLSTROM et al. (1984).
Human lymphoid cells	Monoclonal antibodies	Separon	TLÁSKALOVÁ-HOGENOVÁ et al. (1986).
Human natural killer cells	Monolayers of sensitive target cell lines	60-mm polystyrene tissue culture plates coated with poly- L-lysine	JENSEN et al. (1979).
Human umbilical cord blood mononuclear cells	Peanut agglutinin	CNBr-activated Sepharose 6MB	ROSENBERG et al. (1983).
Immunoselective cell populations after labelling with biotinylated antibodies	Biotinylated antibody	Avidin-coupled sheep erythrocytes (rosette formation)	WORMMEESTER et al. (1984).
Lymphocyte subpopulation of mature T lymphocytes from human peripheral blood	Soybean agglutinin	CNBr-activated Sepharose 6MB	HERTZ et al. (1985).
Lymphoid cells from mouse and rat	Anti-mouse Ig or anti-rat Ig antibodies from rabbits	Magnetic polyacrylamide(4%)-agarose(4%)-iron oxide(7%) beads Magnogel 44	ANTOINE et al. (1978).
Lymphoid cells from peripheral blood	Immunoglobulin fraction of rabbit anti-mouse IgM serum	CNBr-activated Sepharose 6MB or glass beads or plastic beads (noncovalently adsorbed)	HUBBARD et al. (1984).
Mast cells from human lung tissue	Goat anti-human immunoglobulin E	CNBr-activated Sepharose 4B	VAN OVERVELD et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Microvillus membrane vesicles	Antibodies to aminopeptidase; sucraseisomaltase and lactase	CNBr-activated Sepharose 4B	CARLSEN et al. (1982).
Monocytes from human peripheral blood	Gelatin	Gelatin cross- linked with glutaraldehyde	SJOGREN et al. (1983).
Mouse leukaemia L1210 cells	Serum protein transcobalamin II	Submicrometre latex particles (amide-modified polystyrene latex particles 0.35 μm with glutaraldehyde)	TAKAHASHI et al. (1980).
Murine cytotoxic T lymphocytes	Lectins	CNBr-activated Sepharose 4B	KIMURA et al. (1979).
Neurones from chick embryo sympathetic ganglia	α-Bungarotoxin	Sepharose 6MB after CNBr-activation with hexamethylenediamine; p-nitrobenzoyl azide reduced by sodium dithionite and diazotized with sodium nitrite in presence of HCl	DVORAK et al. (1978).
Nucleated erythroid cells	Human transferrin	Bio-Gel P6	JUCKETT and HULTQUIST (1983).
Outer segment plasma membranes	Concanavalin A	Polystyrene beads with 4% divinylbenzene	KAMPS et al. (1982).
Phosphorylcholine binding lymphocytes	Phosphorylcholine	Gelatin coated plates via the cleavable crosslinking reagent N- succinimidyl 3-(2-pyridyldiothio)propionate	CAMBIER and NEALE (1982).
Photosystem II reaction center complex from photosystem II particles from spinach chloroplasts	Peripheral 33-kDa polypeptide from spinach chloroplasts	Affi-Gel 15	ISOGAI et al. (1987).
Plasma membrane vesicles of human plateles	Wheat germ agglutinin (WGA)	WGA agarose beads	KAMBAYASHI et al. (1981).
Polysomes from human B lymphoblastoid cells	Monoclonal antibody against separated heavy chain from papain-treated detergent-solubilized HLA-DR antigen	Protein A-Sepharose CL-4B	KORMAN et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Polysomes from Xenopus ovaries	5;6-Iodotyrosine	Activated CH- Sepharose 4B	KIDSON and FABIAN (1985).
Red blood cells from human blood	Protein A; goat antirabbit Ig antibody or wheat germ agglutinin	Ferromagnetic iron dextran microspheres with glutaraldehyde	MOLDAY and MOLDAY (1984).
Reticulocytes	Human transferrin	Sepharose CL-2B	JUCKETT and HULTQUIST (1983).
Ribosomes from rabbit reticulocyte lysates	Heparin	CNBr-activated Sepharose 4B	HRADEC and KŘÍŽ (1978).
Right-side-out and inside-out submitochondrial particles from pig heart	Cytochrome c	CNBr-activated Sepharose	GODINOT and GAUTHERON (1979).
Sodium-dependent D-glucose transport system	Phlorizin	Phlorizin polymer	LIN et al. (1981a).
Specifically-labeled cells	Protein A from Staphylococcus aureus	Ferromagnetic iron-dextran particles	MOLDAY and MAC KENZIE (1982).
Subcellular particles and organelles or antibody-organelle complexes (review)	Anti-organelle antibodies or antibody-binding reagents	Nonporous solid supports with low levels of nonspecific binding	RICHARDSON and LUZIO (1986).
Surface Ig positive cells (mostly B-cells) from rat peripheral blood mononuclear cells	Monoclonal antibodies against rat Ig κ light chain	Dextrin Reppal PZ 9 (enzymatically hydrolysed starch mixed with ferrofluid)	SCHRODER et al. (1986).
T cells (CD8 positive) - from human peripheral blood	Tetramolecular monoclonal antibody complex: anti-CD8 mouse IgG1;twoo rat anti-mouse IGg1 and anti-FITC (fluorescein isotiocyanate) mouse IgG1	Glass beads coated with FITC-bovine serum albumin	THOMAS and LANSDORP (1988).
Translating ribosomes from Escherichia coli MRE-600	Periodate-oxidized poly(U)	Cellulose hydrazide after treatment of KNO_2 in presence of HCl with dihydrazide of dithiodiglycolic acid	BARANOV (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Tumor cells from bone marrow	Polyclonal antibody	Dynabeads M-450	UGELSTAD et al.. (1988).
Tumour cells	Monoclonal antibodies (anti-tumour osteogenic sarcoma)	Polyhexylcyanoacrylate nanoparticles	ILLUM et al. (1983).
Viable cell populations (lymphoid subpopulations)	Biotinylated goat anti-mouse immunoglobulin	Avidin conjugation to CNBr-activated Sepharose 6MB or to Bio-Gel P-30 after activation by carbodiimide or glutaraldehyde	BERENSON et al. (1986a).
COFACTORS AND VITAMINS			
Biotin	Avidin	Sepharose 4B	BODANSZKY and BODANSZKY (1970).
Cobalamin (Cbl; vitamin B12) and Cbl analogs from bacterial lysates	Cbl-binding protein (hog gastric nonintrinsic factor)	CNBr-activated Sepharose 4B	KOLHOUSE and ALLEN (1978).
Coenzyme A	p-Acetoxymercurianiline	Sepharose 6B	MATUO et al. (1975).
Coenzyme A	CoA-Binding protein	Sepharose 6B	CHIBATA et al. (1974a).
Enzyme cofactor NAD from yeast extract	Alcohol dehydrogenase (EC 1.1.1.1)	CNBr-activated Sepharose 4B	DAS et al. (1975).
Flavin-adenine dinucleotide	p-Acetoxymercurianiline	Sepharose 6B	MATUO et al. (1975).
Flavins	Riboflavin-binding apoprotein from hen egg white	Azide derivative of Bio-Gel P-150	KOZIK and ZAK (1982).
Molybdenum cofactor from xanthine oxidase	Glutathione-2-pyridyl disulphide	Activated Thiol-Sepharose 4B	MENDEL and ALIKULOV (1983).
Riboflavin 5'-phosphate and its analogues	Apoflavodoxin	Sepharose 4B	MAYHEW and STRATING (1975).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
ENZYMES			
Acetylcholinesterase (EC 3.1.1.7) from chicken, rat, calf and human brain and from electric organ of Torpedo marmorata	10-Methyl 9-[3-(6-aminocaproyl)amino]-propylaminocridinium bromide hydrobromide	Sepharose 4B	VALLETTE et al. (1983).
Acetylcholinesterase (EC 3.1.1.7) from rabbit brain	Monoclonal antibody against human red blood cell cholinesterase and phenyltrimethylamonium iodide	CNBr-activated Sepharose 4B	MINTZ and BRIMIJOIN (1985).
11-S Acetylcholinesterase (EC 3.1.1.7) from Electrophorus electricus	9-(β-Aminopropylamino)acridine	Sepharose 4B after CNBr-activation with 6-aminocaproic acid	TAYLOR et al. (1983).
Acetylcholinesterase (EC 3.1.1.7) from bovine erythrocytes and electric eel	N-Methylacridine	Sepharose 2B	SEKAR et al. (1980).
Acetylcholinesterase (EC 3.1.1.7) from adult rat brain	Concanavalin A and m-carboxyphenyldimethylethylammonium iodide	Con A-Sepharose and Sepharose 4B with hexamethylenediamine	RAKONCZAY et al. (1981).
Acetylcholinesterase (EC 3.1.1.7) from cobra (Naja naja oxiana) venom	N,N,N-Trimethyl-(m-aminophenyl)-ammonium chloride	AH-Sepharose with succinic anhydride	RABA et al. (1979).
Acetylcholinesterase (EC 3.1.1.7.) from pig brain (solubilized with 1% Triton X-100)	1-Methyl-9-[Nβ-(ε-aminohexanoyl)-β-aminopropylamino] acridium bromide hydrobromide	Sepharose 4B after CNBr-activation	REAVIL et al. (1978).
Acetylcholinesterase (EC 3.1.1.7) from mouse brain	N-Methyl-3-aminopyridinium iodide	Affi-Gel 202	ADAMSON (1977).
Acetylcholinesterase (EC 3.1.1.7) from Hemicentrotus pulcherrimus embryos	Trimethyl (p-aminophenyl) ammonium chloride hydrochloride	Sepharose 2B with 3,3'-diaminodipropylamine and succinic anhydride; repeated twice	AKASAKA et al. (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Acetyl Coenzyme A carboxylase from chick liver	Avidin	Sepharose CL-4B after CNBr activation	BEATY and LANE (1982).
^{32}P-Labelled acetyl-CoA carboxylase (EC 6.4.1.2) from 32P-labelled rat epididymal fatpads	Avidin (monomeric form)	Sepharose CL-4B after CNBr-activation	HOLLAND et al. (1985).
N-Acetyl-β-glucosaminidase (2 acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase; EC 3.2.1.30) from human urine	p-Aminophenyl-N-acetyl-β-D-thioglucosamidide	N-Hydroxysuccinimide ester of succinylated aminoalkyl agarose	BAMBERG et al. (1975).
N-Acetyl-β-glucosaminidase	Antibody to human N-acetyl-β-glucosaminidase	CNBr-activated Sepharose 4B	ERICKSON and SANDMAN (1977).
β-Acetylhexosaminidase (EC 3.2.1.52) from porcine kidney	2-Acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosyl- amine	Sepharose 4B	KYOSAKA et al. (1980).
β-N-Acetylhexosaminidase (EC 3.2.1.30) from rabbit sperm cytoplasmic droplets	Desialylated Cowper's gland mucin or desialylated fetuin	Sepharose 4B after CNBr-activation	FAROOQUI and SRIVASTAVA (1979).
N-Acetylhexosaminidase from human tissues	Concanavalin A	Con A-Sepharose	BRATTAIN et al. (1977).
Acylphosphatase (EC 3.6.1.7) from guinea-pig muscle	Anti-(horse muscle acylphosphatase)antibodies	CNBr-activated Sepharose 4B	LIGURI et al. (1984).
Adenosine monophosphate aminohydrolase (EC 3.5.4.6) from rat liver	ATP after periodate oxidation	Sepharose 4B after CNBr-activation with adipic acid dihydrazide	SMITH et al. (1977b).
Adenosine 5'-phosphosulphate sulphohydrolase (EC 3.6.2.1) from mitochondrial-lysosomal suspension of bovine liver	N^6(6-Aminohexyl)-adenosine 5'-monophosphate	5'AMP-Sepharose 4B	ROGERS et al. (1978).
Adenosine triphosphatases (ATPases):			

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
$(Ca^{2+} + Mg^{2+})$-Dependent adenosine triphosphatase from human erythrocyte membranes	Calmodulin from bovine brain	CNBr-activated Sepharose 4B	GIETZEN et al. (1980).
$(Ca^{2+} - Mg^{2+})$-ATPase (EC 3.6.1.3) from bovine aortic smooth muscle	Calmodulin	CNBr-activated Sepharose 4B	FURUKAWA and NAKAMURA (1984).
ATPase (EC 3.6.1.3) from rat liver mitochondria	N^6-(6-Aminohexyl)-5'-AMP	Sepharose 4B	LE DEAUT et al. (1978).
Ca ATPase of sarcoplasmic reticulum vesicles	Reactive red-120	Reactive red-120 agarose	COLL and MURPHY (1984).
Calcium-stimulated Mg-dependent adenosine triphosphatase (ATPase) (EC 3.6.1.3) - from rat liver plasma membranes	Concanavalin A	Concanavalin A-Ultrogel	LOTERSZTAJN et al. (1981).
Calmodulin-stimulated Ca^{2+}-transport ATPase ($(Ca^{2+} + Mg^{2+})$-ATPase) from pig antrum smooth muscle	Calmodulin	CNBr-activated Sepharose 4B	DE SCHUTTER et al. (1984).
DNA-Dependent ATPase II from Escherichia coli	Denaturated deoxyribonucleic acid	DNA-cellulose	RICHET and KOHIYAMA (1978).
H^+ -Translocating adenosine triphosphatase from Rhodospirillum rubrum	8-(6-Aminohexyl)amino-ADP	Sepharose 4B after CNBr-activation	WEBSTER and JACKSON (1978).
Na; K-ATPase (EC 3.6.3.1) from rectal glands of spiny dogfish	Concanavalin A	Con A-Sepharose	ESMANN (1980).
Vacuolar proton-translocating ATPase (H^+ATPase) from bovine kidney	Monoclonal antibody against H^+ATPase	Protein A-Sepharose coupling with dimethylpimelimidate	GLUCK and CALDWELL (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Adenylate cyclase from Bordetella pertussis	Genetically engineered VU-8 calmodulin in which 3 glutamic acid residues (82-84) substituted with 3 lysine residues	CNBr-activated Sepharose 4B	HAIECH et al. (1988).
Adenylate cyclase (EC 4.6.1.1) from bovine brain	Cibacron Blue F3GA and calcium-dependent regulatory protein	Affi-Gel Blue and CNBr-activated Sepharose 4B	WESTCOTT et al. (1979).
Adenylate cyclase (EC 4.6.1.1) from cardiac muscle of rabbits	7-O-Hemisuccinyl-7-deacetylforskolin	Sepharose CL-4B with ethylenediamine	PFEUFFER and METZGER (1982).
Agarase from Littorina mandshurica	Agarose	Bio-Gel A-5m	USOV and MIROSHNIKOVA (1975).
L-Alanine: 4,5-dioxovalerate transaminase from rat liver mitochondria	L-Alanine	AH-Sepharose 4B (carbodiimide technique)	SHANKER and DATTA (1986).
Aldolase from rabbit liver and muscle	Cibacron Blue F3GA	Affi-Gel Blue or Blue Sepharose	SYGUSCH et al. (1984).
Alkylglycerol monooxygenase from liver microsomes	p-Aminobenzylidene derivative of 1-O-hexadecylglycerol	CH-Sepharose 4B	ISHIBASHI and IMAI (1985).
Aminolaevulinate dehydratase (EC 4.2.1.24) from bovine liver	Aminolaevulinate	Sepharose 4B after CNBr-activation or Sepharose 4B with ethylenediamine and succinic anhydride	STELLA and DEL BATLLE (1977).
δ-Aminolaevulinate dehydratase (EC 4.2.1.24) from Neurospora crassa	p-Aminophenylmercuric acetate	Sepharose 4B after CNBr-activation	CHANDRIKA and PADMANABAN (1980).
L-Aminolevulinate dehydratase (ALAD - EC 4.2.1.24) from spinach	Monoclonal antibodies against ALAD	Sepharose after CNBr-activation	LIEDGENS et al. (1980).
Amylases:			
α-Amylase (EC 3.2.1.1) from rat pancreatic acinar carcinoma	α-Glucohydrolase inhibitor	AH-Sepharose 4B (2x sodium borohydrid was added)	REDDY et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
α-Amylase from germinating Vigna mungo seeds	Cycloheptaamylose (or = β-cyclodextrin)	Epoxy-activated Sepharose 6B	KOSHIBA and MINAMIKAWA (1981).
α-Amylase from crude triticale and wheat extracts	Cycloheptaamylose	Epoxy-activated Sepharose 6B	WESELAKE and HILL (1982).
α-Amylase (EC 3.2.1.1) from pig pancreas	α-Amylase/trypsin double-headed plant inhibitor from ragi (adsorption)	Trypsin coupled to CNBr-activated Sepharose 4B	SHIVARAJ and PATTABIRAMAN (1981).
α-Amylase from Helix pomatia	Glycogen	Shellfish glycogen cross-linked with CNBr and hexamethylenediamine	MARSHALL and WOLOSZCZUK (1978).
Amylase (EC 3.2.1.1) from rat pancreas and salivary glands	α-Glucohydrolase inhibitor	AH-Sepharose 4B (2x sodium borohydride was added)	BURRILL et al. (1981).
Amylase (EC 3.2.1.1) from hog pancreas	Yam amylase inhibitor	DEAE-cellulose (tightly adsorbed)	SHARMA and PATTABIRAMAN (1982).
Amylase isoenzymes from human parotid gland and pancreas	Concanavalin A	Con A-Sepharose 4B	TAKEUCHI (1979).
β-Amylase from barley	Anti-barley β-amylase IgG	Activated Ultrogel AcA 22	BUREAU and DAUSSANT (1983).
β-Amylase from mustard (Sinapis alba L.) cotyledon	Immunoglobulin G against β-amylase from rabbits	CNBr-activated Sepharose	SUBBARAMAIAH and SHARMA (1987).
Amyloglucosidase from commercial preparations	p-Chloromercuribenzoate	Cellulose with epichlorohydrin and hexamethylenediamine	KUČERA (1979).
Amyloglucosidase from Aspergillus after periodate oxidation condensed with galactosides containing amino group	Ricinus communis agglutinin	CNBr-activated Sepharose 4B	MIKHAILOV et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
α-L-Arabinofuranosidase (EC 3.2.1.55) from Aspergillus niger	Arabin	Epoxy-activated Sepharose 6B or arabin cross-linked by epichlorohydrin	WAIBEL et al. (1980).
Arachidonate 5-lipoxygenase from porcine leukocytes	Monoclonal anti-5-lipoxygenase antibody (purified by Protein A - Sepharose)	Affi-Gel 10	UEDA et al. (1986).
Arginase (EC 3.5.3.1) from mouse liver	Procion Red HE-3B	Matrex Gel Red A	SPOLARICS and BOND (1988).
Arginase (EC 3.5.3.1) from livers of man; bovine; pig; dog; guinea pig; rat and mouse	Anti-human arginase immunoglobulin	CNBr-activated Sepharose 4B	BRUSDEILINS et al. (1985).
Arylsulphatase (variant form) from human urine (EC 3.1.6.1)	Concanavalin A	Con A-Sepharose	ISHIBASHI et al. (1980).
Arylsulphatase (EC 3.1.6.1) from rabbit liver	Arylsulphatase A monomer subunit	CNBr-activated Sepharose 4B	VAN ETTEN and WAHEED (1980).
Arylsulphatase A and B (EC 3.1.6.1) from rat brain	Cibacron Blue F3GA	Sepharose 6B treated with epichlorohydrin and sodium borohydride	AHMAD et al. (1977).
Arylsulphatase (EC 3.1.6.1) from chicken caecal	Concanavalin A and cyclic AMP	Con A-Sepharose and cyclic AMP-agarose	FAROOQUI and HANSON (1987).
Arylsulphatase (EC 3.1.6.1) from Klebsiella aerogenes	L-Tyrosine or anti-arylsulfatase-immunoglobulin G from rabbits	Beads of curdlan type polysaccharide 13140; β-1,3-glucan with hexamethylenediamine	MUROOKA et al. (1977).
Arylsulphatase A (EC 3.1.6.1) from human urine	Concanavalin A or arylsulfatase subunit	Con A-Sepharose Affi-Gel 10	LAIDLER et al. (1985).
Asparaginase (EC 3.5.1.1) from Escherichia coli	N-(ω-aminohexyl)-L-aspartic acid	Sepharose 4B (modified by spiropyran)	KARUBE et al. (1978).
L-asparaginase from Proteus vulgaris	N-(ω-Aminohexyl)-L-aspartic acid	Sepharose 6B	CHIBATA et al. (1974b).
Aspartase (EC 4.3.1.1)	N-(ω-Aminohexyl)-L-asparatic acid	Sepharose 6B	TOSA et al. (1974).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
L-Aspartase from Escherichia coli	Procion Red HE3B	Matrex Gel Red A	KARSTEN et al. (1985).
Aspartate transcarbamoylase (EC 2.1.3.2) from Escherichia coli	Procion red HE3B or Cibacron blue F3GA	Matrex dye gels	WEST et al. (1985).
Aspartate transcarbamylase (EC 2.1.3.2) from wheat germ	N-(3-Carboxypropionyl)aminodecane	Sepharose 4B	YON and SIMMONDS (1975).
Aspartate transcarbamylase (EC 2.1.3.2) from germinated seedlings of Phaseolus aureus	L-Aspartate	Sepharose 4B	RAO et al. (1979).
Bacteriophage f2 replicase	Single-stranded f2 RNA	Cellulose	FEDOROFF and ZINDER (1971).
Bifunctional enzyme imidazoleglycerolphosphate dehydratase (EC 4.2.1.19) and histidinol phosphate (EC 3.1.3.15) from Salmonella typhimurium	Antibodies against homogeneous bifunctional enzyme (M_r = 46 000) from rabbits	Sepharose CL-4B	STAPLES and HOUSTON (1980).
Branched-chain amino acid transaminase (EC 2.6.1.42) from pig-heart	Cycloserine	CNBr-activated Sepharose 4B	KORPELA and SAARINEN (1985).
Carbonic anhydrase (EC 4.2.1.1)-enriched and -depleted membranes from chick brain	p-Sulfamylbenzoic acid	CNBr-activated Sepharose 6MB with hexamethylenediamine	SHARMA and BABITCH (1984).
Carbonic anhydrase B and C from hemolysate of human erythrocytes	p-Aminobenzenesulfonamide after diazotization or p-carboxybenzenesulfonamide	Sepharose 4B with Gly-Tyr or with ethylenediamine	JOHANSEN (1976).
Catalase (EC 1.11.1.6) - from bovine liver	Procion HE3B dye	Red-A Matrex Gel	JOUVE et al. (1986).
Catalase from rat liver phenylalnine hydroxylase (EC 1.14.16.1)	Antibodies against bovine catalase from rabbits	CNBr-activated Sepharose 6MB	WEBBER and WHITELEY (1980).
Cellobiase (EC 3.2.1.21) from Trichoderma viride	Concanavalin A	Con A-Sepharose	KMÍNKOVÁ and KUČERA (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cellobiases from Trichoderma koningi; lignorum and viride; Aspergillus foetidus and niger; and Geothrihum candidum;	Cellulose	Avicel and ball-milled cotton linters	RABINOVITCH et al. (1982).
Cellulase from Thermomonospora fusca - endoglucanase E2 (after cloning of gene) - from Streptomyces lividans	Microcrystalline cellulose	Avicel type PH-102 microcrystalline cellulose	GHANGAS and WILSON (1988).
Cellulases from Trichoderma reesei	Amorphous cellulose	Whatman CF 11 cellulose powder	NUMMI et al. (1981).
Extracellular cellulase endo-β-glucanase from Chaetomium celluloticum	Concanavalin A	Con A-Sepharose 4B	FAHNRICH and IRRGANG (1984).
Chalcone flavanone isomerase (EC 5.5.1.6) from Petunia hybrida	Blue Dextran	Blue Dextran Sepharose	VAN TUNEN and MOL (1987).
Chloridazon-catechol dioxygenase (CCD) from Phenylobacterium DSM	Immunoglobulin G against CCD from rabbits (purified by Protein A-Sepharose CL-4B)	Activated Thiol-Sepharose 4B	SCHMITT et al. (1984).
Cholinesterase (EC 3.1.1.8) - from horse and human serum	Octaethylene glycol n-hexadecyl ether	Davisil octadecyl-bonded silica - 300 Å pore size	TORRES et al. (1988).
Chondroitinase B and C from Flavobacterium heparinum	Dermatan sulfate (non-covalent coating)	AH-Sepharose 4B with heparin or dermatan sulfate (covalently coupled)	OTOTANI and YOSIZAWA (1979).
Chorismate mutase from tubers of Solanum tuberosum L.	Cibacron Blue F3GA and tryptophan	Matrex Blue A and Tryptophanagarose from Actigel A	KUROKI and CONN (1988).
Cobalt-activated acylase (EC 3.5.1) from human liver	ε-Aminopentyl-α-hydroxyisocaproyl-DL-tyrosine ethyl ester	Sepharose 4B	SLOWINSKA and SZEWCZUK (1979).
Colipase from porcine and human pancreatic juice	Antibodies against porcine procolipase B from rabbits	CNBr-activated Sepharose 4B	RATHELOT et al. (1983a).
Colipase from porcine pancreatic extracts	Lipase	Sepharose 4B	PATTON and ANDERSSON (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Corrinoid enzyme from Clostridium thermoaceticum	Pteroylglutamic acid (folic acid)	AH-Sepharose 4B (carbodiimide technique)	WELTY and WOOD (1978).
Cu-Zn-Superoxide dismutase (SOD) from human erythrocytes	Anti-human Cu-Zn-SOD goat IgG	Sepharose 4B	ARAI et al. (1986).
2',3'-Cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.37)	Phosphohydrolase-specific antibody	CNBr-activated Sepharose 4B	DRUMMOND (1979).
Cytochromes P-450 from liver microsomes of phenobarbital treated rats	Monoclonal antibodies	CNBr-activated Sepharose 4B	FRIEDMAN et al. (1985).
Cytochrome P-450 from liver microsomes of untreated rats	Lauric acid	AH-Sepharose 4B (carbodiimide technique)	GIBSON and SCHENKMAN (1978).
Cytochrome P-450 isozymes (6) from liver microsomes of 3-methyl-cholanthrene induced animals (rats; guinea pigs and mice)	Monoclonal antibodies against cytochrome P-450	CNBr-activated Sepharose 4B	CHENG et al. (1984).
Isozymes of cytochrome P-450	Detergent-solubilized cytochrome b5	CNBr-activated Sepharose 4B	KAWATA et al. (1986).
Catalytically active cytochromes P-450	Monoclonal antibody	CNBr-activated Sepharose 4B	FRIEDMAN et al. (1985).
Cytochrome c-550 from Chromatium vinosum; cytochromes c and c2	C.vinosum flavocytochrome c-552	Affi-Gel 10	DAVIDSON et al. (1985).
Deaminases and decarboxylases:			
Acrylate decarboxylase from Porphyra tenera	Proteinaceous inhibitor of ethylene evolution in marine algae from P.tenera	Porous glass Bio-glass 1500 with succinic anhydride; phenylenediamine and succinic anhydride	WATANABE et al. (1977).
Adenosine deaminase (EC 3.5.4.4)	2',3'-O-[(2-Carboxyethyl)ethylidene] - inosine or nebularine	AH-Sepharose 4B (carbodiimide technique)	ROSEMEYER and SEELA (1979b).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Adenosine deaminase from human genetic mutant cell	Coformycin after periodate oxidation	CNBr-activated Sepharose 4B with 3,3'-iminobispropylamine	DANTON and COLEMAN (1986).
Aspartate β-decarboxylase (EC 4.1.1.12)	N-(ω-Aminohexyl)-L-aspartic acid	Sepharose 6B	CHIBATA et al. (1974b).
Cytosine deaminase from Escherichia coli	N^6-(6-Aminohexyl)pyrimidine (nucleophylic displacement of thiol group from 2-mercaptopyrimidine by hexamethylenediamine) and 5'-aminouracil	Sepharose 4B after CNBr-activation and CNBr-activated Sepharose 4B with hexamethylenediamine (carbodiimide technique)	KATSURAGI et al. (1986a).
Cytosine deaminase from Escherichia coli	2-Mercaptopyrimidine or 2-thiobarbituric acid coupled with 1,6-diaminohexane and 2-amino-4,6-dihydroxypyrimidine or 5-aminouracil and orotic acid	Sepharose 4B (activated with CNBr) and Sepharose 4B with 6-aminohexanoic acid and Sepharose 4B with 1,4-diaminobutane	KATSURAGI et al. (1986b).
L-Glutamate decarboxylase (EC 4.1.1.15) from rat brain	DL-α-Methylglutamate	CH-Sepharose 4B (carbodiimide technique)	YAMAGUCHI and MATSUMURA (1977).
Malonyl-CoA decarboxylase (EC 4.1.1.9) from Mycobacterium tuberculosis	Nicotinamide-adenine dinucleotide phosphate	NADP-agarose	KIM et al. (1979).
Methylmalonyl-CoA decarboxylase (EC 4.1.1.41) from Veillonella alcalescens	Monomeric avidin	Avidin-Sepharose	HILPERT and DIMROTH (1983).
Nicotinamide deaminase (EC 3.5.1.19) from mouse neuroblastoma	NAD^+	Sepharose 4B with hexamethylenediamine and diazotized p-aminobenzylderivative	WINTZERITH et al. (1980).
Ornithine decarboxylase (ODC)	Antibody against ODC	CNBr-activated Sepharose 4B	KRITSI et al. (1982).
Orotidine-5'-monophosphate decarboxylase (separation from orotidine-5'-monophosphate pyrophosphorylase)	2,3'-O-[1-(2-Carboxyethyl)ethylidene]-6-azauridine 5'-monophosphate	AH-Sepharose (carbodiimide technique)	ROSEMEYER and SEELA (1979a).
Orotidine-5'-phosphate decarboxylase (EC 4.1.1.23) from baker's yeast	Blue Dextran 2000 and Cibacron Blue F3GA	CNBr-activated Sepharose 4B and cross-linked Sepharose 6B	REYES and SANDQUIST (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Orotidine-5'-phosphate decarboxylase (EC 4.1.1.23) from yeast	5-[2-[N-(2-Aminoethyl)carbamyl]-ethyl]-6-azauridine 5'-monophosphate	CM Bio-Gel A with N-hydroxysuccinimide and dicyclohexylcarbodiimide	BRODY and WESTHEIMER (1979).
Oxaloacetate decarboxylase (EC 4.1.1.3) from Klebsiella aerogenes	Monomeric avidin	CNBr-activated Sepharose 4B	DIMROTH (1982).
Porphobilinogen deaminase from wheat germ	p-Chloromercuribenzoate	Affi-Gel 501	RUSSEL and POLLACK (1978).
Porphobilinogen deaminase (EC 4.3.1.8) from Euglena gracilis	Porphobilinogen	Sepharose 4B after CNBr-activation with hexamethylenediamine (carbodiimide technique)	WILLIAMS et al. (1981).
Pyruvate decarboxylase from yeast	Thiamine pyrophosphate	Sepharose 4B with 4-azobenzoyl-ε-aminocaproyl-hydrazide	KLYASHCHITSKY et al. (1980).
Dehydrogenases:			
Alanine dehydrogenase (EC 1.4.1.1) from Halobacterium salinarium	Nicotinamide adenine dinucleotide phosphate	Agarose-hexane-NADP	KERADJOPOULOS and HOLLDORF (1979).
Alcohol dehydrogenase steroid-active isozymes of horse liver	N^6-(6-Aminohexyl)-AMP	Sepharose 4B	ANDERSSON et al. (1975).
Alcohol dehydrogenase from Drosophila melanogaster	8-(6-Aminohexyl)-amino-5'AMP	Sepharose 4B after CNBr-activation	BROWN and LEE (1979).
Alcohol dehydrogenase (anodic form) from human liver	4-[3-(N-6-Aminocaproyl)-aminopropyl]-pyrazole and AMP	Sepharose 4B after CNBr-activation and Agarose-hexane-AMP (Type 2)	BOSRON et al. (1977).
Alcohol dehydrogenase from equine liver	Nicotinamide-adenine dinucleotide (NAD)	Sepharose CL-6B; Fractogel HW-65; Trisacryl GF-2000; LiChrospher Si 500 diol I or II	HEARN (1986).
Alcohol dehydrogenase isozymes (EC 1.1.1.1) from horse liver	N^6-(6-Aminohexyl)-5'AMP	Sepharose 4B after CNBr-activation or 5'-AMP-Sepharose 4B after treatment of ferrofluid (Magnetic 5'-AMP-Sepharose)	ANDERSSON and MOSBACH (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Alcohol dehydrogenase from tomato	Cibacron Blue F3GA	Blue Sepharose CL-6B	NICOLAS and CROUZET (1980).
Alcohol dehydrohenase from Bacillus stearothermophilus	NAD	Sepharose 4B with hexamethylenediamine and p-nitrobenzyl azide reduced with dithionite and diazotised with $NaNO_2$	RUNSWICK and HARRIS (1978).
Aldehyde dehydrogenase (EC 1.2.1.3) from human erytrocytes	N^6(6-Aminohexyl)-adenosine 5'-monophosphate	5'AMP-Sepharose 4B	INOUE et al. (1979).
Aldehyde dehydrogenase (EC 1.2.1.3) and alcohol dehydrogenase (EC 1.1.1.1) with coenzyme and substrate-competitive inhibitor	Nicotinamide adenine dinucleotide (NAD)	Agarose-NAD Type 3 (AGNAD)	SVANAS and WEINER (1982).
Aldehyde dehydrogenase (EC 1.2.1.3) from human brain	N^6-(6-Aminohexyl)-5'-AMP	5'-AMP Sepharose 4B	RYZLAK and PIETRUSZKO (1987).
Aldehyde dehydrogenase (membrane-bound) from rat liver microsomes	N^6-(6-Aminohexyl)-5'AMP	5'AMP-Sepharose	NAKAYASU et al. (1978).
Aspartokinase-homoserine dehydrogenase I from Escherichia coli	Green A	Matrex Gel Green A	KARSTEN et al. (1985).
Choline dehydrogenase (EC 1.1.99.1) modified with 5,5'-dithiobis(2-nitrobenzoic acid) from rat liver mitochondria	Choline	CNBr-activated Sepharose 4B with N,N-dimethyl-1,3-propanediamine and ethylene bromohydrin	TSUGE et al. (1980).
Chorismate mutase-prephenate dehydrogenase (EC 1.3.1.12) from Escherichia coli	N-Toluene-p-sulfonyl-L-p-aminophenyl alanine	CNBr-activated Sepharose 4B	SMITH et al. (1977a).
Cytoplasmic aldehyde dehydrogenase from sheep liver after modification with disulfiram	Thiol form of thiopropyl-agarose	Thiopropyl-Sepharose 6B after reduction using 2-mercaptoethanol	KITSON (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cytoplasmic glycerol phosphate dehydrogenase (EC 1.1.1.8) from rat skeletal muscle	2,4,6-Trinitrobenzenesulfonic acid	Sepharose 4B with ϵ-aminocaproic acid or hexanediamine	MC GINNIS and DE VELLIS (1978).
Cytoplasmic and mitochondrial malate dehydrogenase (EC 1.1.1.37) from Drosophila	8-(6-Aminohexyl)-amino-adenosine 5'-triphosphate	Sepharose 4B after CNBr-activation	LEE et al. (1979).
Cytosolic NAD-linked glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) from rabbit tissues	2,4,6-Trinitrobenzene	Sepharose 4B with hexamethylenediamine	OSTRO and FONDY (1977).
D-Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from Bacillus stearothermophilus and Thermus aquaticus	Nicotinamide adenine dinucleotide (NAD)	Sepharose 4B after CNBr-activation with hexamethylenediamine; p-nitrobenzyl azide; reduced with dithionite; diazotised with $NaNO_2$ in presence of HCl	HARRIS et al. (1980).
D-Lactate dehydrogenase - from Escherichia coli	Monoclonal antibody Mab1B2a (IgG 2b) - purified by Protein A-Sepharose 4B	Affi-Gel 10	SANTOS and KABACK (1986).
Diol dehydrogenase (EC 4.2.1.28) from Klebsiella pneumoniae (study of interactions with coenzyme)	Several derivatives of vitamin B12 coenzyme	CNBr-activated Sepharose 4B with hexamethylenediamine	TORAYA and FUKUI (1980).
Estradiol-17β dehydrogenase	p-Hydroxymercuribenzoate	Sepharose with ethylenediamine	NICOLAS (1974).
Formaldehyde dehydrogenase (EC 1.2.1.1) and formate dehydrogenase (EC 1.2.1.2) from pea seeds	N^6(6-Aminohexyl-)5'AMP	5'-AMP-Sepharose	UOTILA and KOIVUSALO (1979).
Glucose 6-phosphate dehydrogenase (EC 1.1.1.39) from baker's yeast	Procion brilliant red H-8BN	Sepharose 6B	FARMER and EASTERBY (1984).
Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from human erythrocytes	N^6-(6-Aminohexyl)-adenosine 2',5'-biphosphate	Sepharose 4B after CNBr-activation	MORELLI et al. (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	$NADP^+$	Sepharose 4B-200	CRANEY and GOFFREDO (1983).
Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (EC 1.1.1.49)	Triazine dyes	Matrex Gel Orange B	HEY and DEAN (1983).
Glucose-6-phosphatedehydrogenase from yeast	Dye red - brown	Silichrom SCH-2,5; S-80 with γ-aminopropyltriethoxysilane; epichlorohydrine activated dextran; thermal treatment and blocking of residual $-NH_2$ by acetylation	KADUSEVICIUS et al. (1983).
Glucose-6-phosphate dehydrogenase - folate conjugates (studies of interaction)	Folate binding protein	Sepharose	BACHAS et al. (1986).
Glucose-6-phosphate dehydrogenase from yeast	$NADP^+$ complexed to immobilized boronic acid	Sepharose 6B with 6-aminocaproyl-3-aminophenylboronic acid	BOURIOTIS et al. (1981).
Glucose-6-phosphate dehydrogenase from human erythrocytes	2'5'-ADP	2'5'-ADP-Sepharose 4B	PITTALIS et al. (1992).
Glutamate dehydrogenase (EC 1.4.1.4)	Cibacron Blue F3GA	Epichlorohydrin cross-linked Sepharose 6B	AGRAWAL and RAO (1983).
Glutamate dehydrogenase from bovine liver	N^6-(6-Aminohexyl)-AMP and 8-(6-aminohexyl)-amino-AMP	Sepharose 4B after CNBr-activation	BRODELIUS and KAPLAN (1979).
Glutamate dehydrogenase (EC 1.4.1.3) from ox brain and liver	Guanosine 5'-triphosphate after periodate oxidation	Sepharose 4B after CNBr-activation with L-glutamic acid γ-methyl ester and hydrazine hydrate	MC CARTHY et al. (1980).
Glutamate dehydrogenase (EC 1.4.1.4) from halophilic bacteria of Dead Sea	8-(6-Aminohexyl)amino derivative of $NADP^+$	Sepharose 4B after CNBr-activation	LEICHT et al. (1978).
Glutamate dehydrogenase from Acanthamoeba culbertsoni	Cibacron Blue F3GA	Sepharose 6B	SINGH et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from several lactic acid bacteria	Blue dextran	Sepharose 4B	KAWAI et al. (1980).
Glyceraldehyde-3-P-dehydrogenase (EC 1.2.1.12) from HeLa cytosol	Polyriboadenylic acid	Sepharose 4B after CNBr-activation	MILHAUD et al. (1978).
Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) from human heart	Cibacron blue F3GA	Sepharose 4B	MC GINNIS (1983).
Hexose-6-phosphate dehydrogenase from rat liver	N^6-(6-Aminohexyl)adenosine 2',5'-bisphosphate	2'5'ADP-Sepharose 4B	HINO and MINAKAMI (1982).
Histidinol dehydrogenase (EC 1.1.1.23)	Antibodies	Sepharose 4B	ANDORN and ARONOVITCH (1984).
Histidinol dehydrogenase from multienzyme conjugate of yeast	Adenosine 5'-monophosphate	AMP-agarose(type II)	KEESEY et al. (1979).
Inosine 5'-monophosphate dehydrogenase (EC 1.2.1.14) from Escherichia coli	8-(6-Aminohexyl)-AMP; N^6-(6-aminohexyl)-AMP and 8-(8-aminooctyl)-IMP	Sepharose 4B	GILBERT et al. (1979).
Inosine 5'-monophosphate dehydrogenase (EC 1.2.1.14) from Escherichia coli	Procion dyes	Sepharose 4B	LOWE et al. (1980).
Inosinic acid dehydrogenase (EC 1.2.1.14) from Aerobacter aerogenes	8-(6-Aminohexyl)-amino-guanosine 5'-monophosphate	Sepharose 4B after CNBr-activation	BRODELIUS et al. (1978).
Isocitrate dehydrogenase	N^6-(6-Aminohexyl)-adenosine 5'-monophosphate or -2',5'-bisphosphate	Sepharose 4B	BRODELIUS et al. (1974).
Isocitrate dehydrogenase from rat liver	Anti-(yeast glucose-6-phosphate dehydrogenase) IgG	CNBr-activated Sepharose 4B	STAPLETON and PORTER (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Isoenzymes (five) of lactate dehydrogenase (EC 1.1.1.27) from potato Solanum tuberosum	Cibacron blue F3GA and ATP after periodate oxidation	Sepharose CL-6B and Sepharose after CNBr-activation with adipic acid dihydrazide	JERVIS (1981).
Isozymes of human alcohol dehydrogenase	Cibacron Blue F3G-A	Blue Sepharose CL-6B	ADINOLFI and HOPKINSON (1978).
L-Fucose dehydrogenase (EC 1.1.1.122) from rabbit liver	N^6-(6-Aminohexyl)-adenosine 5'-monophosphate	5'AMP-Sepharose 4B	ENDO and HIYAMA (1979).
L-Lactate dehydrogenase from Lactobacillus casei	Cibacron blue F3G-A	Sephadex G-200	GORDON and DOELLE (1976).
L-Lactate dehydrogenase from human erythrocytes; liver and heart	8-(6-aminohexyl)amino-AMP	Sepharose 4B	BACHMAN and LEE (1976).
L-Lactate dehydrogenase from rabbit muscle; beef heart or human blood serum	p-Aminophenylpyruvic acid or p-aminophenyllactic acid	Cross-linked copolymer of acrylamide and (6-acrylamidohexanoyloxy) succinimide or copolymer of agarose; BIS and 6-acrylamido hexanoic acid	BROWN and JOYEAU (1979).
L-Lactate dehydrogenase	Oxamate	Sepharose	TROMMER and BECKER (1976).
L-Threonine dehydrogenase	NAD^+	Sepharose 4B with ε-aminohexanoic acid	LOWE et al. (1973).
Lactase dehydrogenase and phosphoglycerate kinase from rat liver	Dye tetraiodofluorescein	Affi-Gel 102 and AH-Sepharose 4B (carbodiimide technique)	TUCKER et al. (1981).
Lactate dehydrogenase isoenzyme C4 (EC 1.1.1.27)	N^6-(6-Aminohexyl)-5'-AMP	5'AMP-Sepharose	ANSARI (1981).
Lactate dehydrogenase from equine liver	N^6-(Aminohexyl)-AMP	Sepharose CL-6B; Fractogel HW-65; Trisacryl GF-2000; LiChrospher Si 500 diol I or II	HEARN (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle	Antraquinone-triazine derivatives (Cibacron Blue; Procion Blue; Remazol Brilliant Blue and Ostazin Brilliant Red)	Bead cellulose (heated to 80°C)	GEMEINER AND VISKUPIČ (1981).
Lactate dehydrogenase (LDH) or LDH isoenzymes from beef heart	N^6-(6-Aminohexyl-)5'AMP	5'AMP-Sepharose 4B	DANIELSSON et al. (1981).
Lactate dehydrogenase from bovine heart	Cibacron Blue F3GA coupled to dextran T40 or T500 after periodate oxidation with soy bean trypsin inhibitor	Sepharose CL-4B after CNBr-activation with attached trypsin	MATTIASSON and OLSSON (1986).
Lactate dehydrogenase	Heterobifunctional affinity ligand Cibacron Blue-dextran-soy bean trypsin inhibitor	Sepharose CL-4B after CNBr-activation in presence of triethylamine and 60% acetone with trypsin	MATTIASSON and OLSSON (1986).
Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase; EC 1.6.4.3) from pig heart yeast and Escherichia coli	Lipoyl chloride	Aminoalkyl glass	SCOUTEN (1974).
Lipoamide dehydrogenase isoenzymes from pig heart	NAD^+ and lipoate	Sepharose 4B with ε-aminohexanoic acid and Sepharose 4B with ethylenediamine or L-lysine	VISSER and STRATING (1975).
Malate dehydrogenase (EC 1.1.3.7) from rat liver	Aspartic acid	CH-Sepharose 4B	CREMEL et al. (1985).
Malate dehydrogenase (EC 1.1.1.37) from porcine heart	Procion Red HE3B	Matrex Gel Red A	SMITH and SUNDARAM (1983).
Malate dehydrogenase (EC 1.1.1.37) from pig heart	Remazol Brilliant Blue R	Hydroxyethyl methacrylate gel Spheron 1000 (reaction mixture heated to 50 °C)	KONEČNÝ et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Malate dehydrogenase (EC 1.1.1.37) from thermophilic and mesophilic bacteria Bacillus subtilis; Stearothermophilus and caldotenax	Nicotinamide adenine dinucleotide and N^6-(6-aminohexyl)-adenosine-5'-monophosphate;	Agarose-hexane-NAD^+ and 5'AMP-Sepharose 4B	WRIGHT and SUNDARAM (1979).
Membrane-bound; flavin-linked D-lactate dehydrogenase (D-LDH) from Escherichia coli	Monoclonal antibodies against D-LDH (purified by protein A-Sepharose)	Affi-Gel 10	SANTOS et al. (1985).
$NAD(P)^+$ -Dependent lactate dehydrogenase (EC 1.1.1.27)	N1-Carboxymethyl-nicotinamide in presence of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline after coupling to HMD-Ultrogel activated with sodium pyruvate	6-Aminohexyl agarose HMD-Ultrogel	TORREILLES et al. (1986).
NAD^+ -malic dehydrogenase from pigeon liver	Cibacron Blue F3GA	Blue Sepharose CL-6B	RAMADOSS et al. (1983).
NAD^+-specific glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from Escherichia coli	Nicotinamide adenine dinucleotide	Agarose-hexane-NAD^+ (type 1)	HILLMAN (1979).
NAD-Dependent formate dehydrogenase from Gram-negative methylotrophic bacteria	Oxidized or reduced coenzyme	AH-Sepharose (carbodiimide technique)	YAVARKOVSKAYA et al. (1979).
$NADP^+$ -Dependent isocitrate dehydrogenase (EC 1.1.1.42) from Drosophila melanogaster	Procion Brilliant Blue	Sepharose 4B (gently stirred in 10 mM NaOH)	WILLIAMSON et al. (1980).
NADP-Dependent 6-phosphogluconate dehydrogenase (EC 1.1.1.44) from lamb's liver	Procion Red HE3B	Matrex Gel Red-A	CARNE (1982).
NADP-Malate dehydrogenase (EC 1.1.1.82) from leaves of Zea mays	Cibacron Blue F3GA	Affi-Gel Blue	KAGAWA and BRUNO (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
NADP-Specific isocitrate dehydrogenase (ID - EC 1.1.1.42) from Escherichia coli	Immunoglobulin against ID from rabbits (purified by Protein A-agarose)	Protein A-agarose used for covalent coupling of IgG by use of dimethylsuberimidate	REEVES et al. (1981).
NADP-Specific glutamate dehydrogenase from Chlorella	NADP	Agarose-hexane-NADP; Type 3	YEUNG et al. (1981).
Nicotinamide adenine dinucleotide specific isocitrate dehydrogenase (EC 1.1.1.41) from Neurospora crassa	Adenosine 5'-monophosphate	Ribose-linked AMP affinity matrix	NEALON and COOK (1979).
ω-Hydroxyfatty acid dehydrogenase (wound induced) from potato tubers	NADP after periodate oxidation	Sepharose 4B after CNBr-activation with adipic acid dihydrazide	AGRAWAL and KOLATTUKUDY (1978).
Pyruvate dehydrogenase complex from Azobacter vinelandii	Glutathione-2-pyridyl disulphide	Thiol-Sepharose 4B	DE GRAAF-HESS and DE KOK (1982).
Pyruvate dehydrogenase b phosphatase from beef kidney and heart	Pyruvate dehydrogenase complex via dihydrolipoyl transacetylase core	Sepharose CL-6B with glutathione and crosslinking reagent N,N'-phenylenedimaleimide	PRATT et al. (1982).
sn-Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) from rabbit tissues	NAD attached to hexamethylenediamine through C-8 position of adenine moiety	Agarose-bound NAD-Type 3	MC LOUGHLIN et al. (1980).
Sorbitol dehydrogenase (EC 1.1.1.14) from rat liver	Nicotinamide adenine dinucleotide	AGNAD; type 1	LEISSING and MC GUINNESS (1978).
Spermidine dehydrogenase from Serratia marcescens	1,8-Diaminooctane	Sepharose 4B after CNBr-activation	OKADA et al. (1979).
Steroid-1-dehydrogenase (EC 1.3.99.4) from Nocardia opaca	N-(4-Androsten-3-on-17β-oxycarbonyl)-ε-aminocaproic acid	Sepharose 4B after CNBr-activation with adipic acid dihydrazide or 1,12-dodecanediamine or hexamethylenediamine with carbodiimide technique	ATRAT et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Succinate semialdehyde dehydrogenase (EC 1.2.1.24) from human brain	N^6-(6-Aminohexyl)-adenosine 5'-monophosphate	5'AMP-Sepharose 4B	CASH et al. (1978).
Tetrahydrofolate dehydrogenase	Pteroyllysine	Sepharose 4B with ethanolamine	PASTORE et al. (1974).
UDP-glucose dehydrogenase (EC 1.1.1.22) from calf liver	AMP and P1-(6-amino-1-hexyl)-P2-(5'-uridine)-pyrophosphate	AGAMP - Type 2 and Sepharose 4B after CNBr-activation	GEREN et al. (1977).
Xanthine dehydrogenase (EC 1.2.1.37) from rat liver	Blue dextran	Blue dextran-Sepharose	SULEIMAN and STEVENS (1987).
Xylitol dehydrogenase (EC 1.1.1.9) from Pachysolen tannophilus	β-NAD (attached through C-8)	β-NAD-agarose (N 1008)	BOLEN et al. (1986).
Deoxyuridine triphosphatase nucleotidohydrolase (dUTPase; EC 3.6.1.23) from anemic rat spleen	Cibacron Blue F3GA	Blue Sepharose	HOKARI and SAKAGISHI (1987).
Diacylglycerol lipase from bovine brain	Heparin	Heparin Sepharose 4B	FAROOQUI et al. (1984).
DNA gyrase from Escherichia coli	Novobiocin	Epoxy-activated Sepharose 6B	STAUDENBAUER and ORR (1981).
DNA topoisomerase I from mouse mammary carcinoma cells	Heparin or sulphated dermatan sulphate	Epoxy-activated Sepharose 4B with ammonia (carbodiimide technique)	ISHII et al. (1987).
Dopamine β-hydroxylase (EC 1.14.17.1) from normal human plasma	Concanavalin A	Con A-Sepharose	FRIGON and STONE (1978).
DT-Diaphorase (NAD(P)H:quinone oxidoreductase; EC 1.6.99.2) from rat liver	Dicoumarol	Sepharose 6B with divinyl sulfone; 4-aminobenzoic acid hydrazide; diazotized with $NaNO_2$ in presence of HCl	HOJEBERG et al. (1981).
DT-Diaphorase (EC 1.6.99.2) from rat liver	Dicoumarol	Sepharose 4B with bisamine propylamine and succinic anhydride	RASE et al. (1976).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Endo-β-N-acetylglucosaminidase from Bacillus alvei	Yeast mannan	CNBr-activated Sepharose 4B	MORINAGA et al. (1983).
Endo-D-galacturonase (EC 3.2.1.15) from Aspergillus niger	Sodium tri(D-galactosiduronate)	Separon H 1000 saturated with dry hydrogen chloride gas	REXOVÁ-BENKOVÁ et al. (1983).
Endopolygalacturonase (EC 3.2.1.15) from Kluyveromyces fragilis	Sodium pectate	Pectate cross-linked with epichlorohydrin	INOUE et al. (1984).
Endopolygalacturonase (EC 3.2.1.15) from Aspergillus niger	Polygalacturonic acid	Keratin coated silica gel	LOBARZEWSKI et al. (1985).
Endopolygalacturonases (EC 3.2.1.15) from Aspergillus niger or tomato	Tetra- or pentagalactosiduronic acids	Separon H 1000	REXOVÁ-BENKOVÁ et al. (1986).
Endo-1,4-β-glucanase (EC 3.2.1.4) - from Aspergillus niger	Concanavalin A	Con A-Sepharose	WOODWARD et el. (1986).
Endoglucanase from Trichoderma viride	Concanavalin A	Con A-agarose	GONG et al. (1979).
Enzyme converting β-endorphin into methionine enkephalin	Peptide from lipoprotein LPH (64-67)	Sepharose 4B with adipic acid dihydrazide	KOIDA et al. (1979).
Esterases from Drosophila mojavensis (DmE-4 and DmE-5)	Antibodies against DmE-4 and DmE-5	Protein A-Sepharose CL-4B (coupled with use of dimethyl pimelimidate dihydrochloride)	PEN et al. (1986).
Exo-cellobiohydrolases (EC 3.2.1.91) from Trichoderma reesei	p-Aminobenzyl 1-thio-β-D-cellobioside	Affi-Gel 10	VAN TILBEURGH et al. (1984).
Exopolygalacturonate lyase (EC 4.2.2.9) from Erwinia carotovora	Sodium pectate	Pectate cross-linked with epichlorohydrin partially degraded by endopolygalacturonase followed by reduction with $NaBH_4$	KEGOYA et al. (1984).
Extracellular superoxide dismutase (EC 1.15.1.1) from human blood plasma	Heparin	Heparin-Sepharose	KARLSSON and MARKLUND (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Extramitochondrial malic enzyme (L-malate:$NADP^+$ oxidoreductase; EC 1.1.1.40) from postmitochondrial supernatant of rat skeletal muscle	N^6-(6-Aminohexyl)adenosine 2',5'-bisphosphate	2'5'ADP-Sepharose 4B	SWIERCZYNSKI (1980).
Fatty acid cyclooxygenase (EC 1.14.99.1) from sheep vesicular glands	Flurbiprofen = dl-2-(2-fluoro-4-biphenylyl) propionic acid	Sepharose 4B after CNBr-activation with 3,3'-diaminodipropylamine	HEMLER et al. (1976).
Ferredoxin-$NADP^+$ oxidoreductase (EC 1.18.1.2) from cyanobacterium Anabaena sp.	N^6-(6-Aminohexyl)adenosine 2',5'-bisphosphate (coupling via N^6 position)	2'5'ADP-Sepharose 4B	SERRANO and RIVAS (1982).
Ferredoxin-$NADP^+$ oxidoreductase (EC 1.18.1.2) from nitrogen-fixing Anabaena variabilis	Cibacron Blue F3GA	Blue Sepharose	SANCHO et al. (1988).
Ferrochelatase (EC 4.99.1.1) from Rhodopseudomonas sphaeroides and Aquaspirillum intersonii	Copper phthalocyanine chromophore	Matrex Gel Blue B	DAILEY (1986).
Ferrochelatase (EC 4.99.1.1) from bovine liver mitochondrial membranes	Reactive Red 120 and Cibacron Blue F3GA	Sepharose CL-6B (coupling at 80 °C) and Blue Sepharose CL-6B	DAILEY et al. (1986).
Ferrochelatase (EC 4.99.1.1) from chicken erythrocyte mitochondria	Cibacron Blue F3GA	Blue Sepharose CL-6B	HANSON and DAILEY (1984).
Flavokinase (EC 2.7.1.26) from rat liver	N(10)-ω-Carboxybutyl-7,8-dimethylisoalloxazine	Aminoalkyl agarose Affi-Gel 102	MERRILL and MC CORMICK (1980b).
Formiminotetrahydrofolate-cyclodeaminase (EC 4.3.1.4)	Tetrahydrofolate	Sepharose 4B with hexamethylenediamine	SLAVÍK et al. (1974).
β-Fructosidase (radish)	Concanavalin A and Lens culinaris agglutinin	Con A-Sepharose; LCA-Sepharose; Con A-Ultrogel; LCA-Ultrogel	FAYE et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
α-L-Fucosidase (EC 3.2.1.51) from human liver of fucosidosis patient	Fucosamine or concanavalin A	Agarose-ε-aminohexanoylfucosamine and Con A-Sepharose 4B	ALHADEFF and ANDREWS-SMITH (1980).
α-L-Fucosidase (EC 3.2.1.51) from human spleen	Fucosamine	ε-Aminocaproyl fucosamine-agarose	CHIEN and DAWSON (1980).
α-L-Fucosidase I from almond emulsion	ε-Aminocaproyl-lacto-N-fucopentaosylamine	Sepharose 4B after CNBr-activation	YOSHIMA et al. (1979).
Fucosidase from almond emulsin	Cibacron Blue F3GA	Sepharose CL-4B	IMBER et al. (1982).
Fumarase (EC 4.2.1.2) from pig heart	2-(5'-Phenylpentyl)fumaric acid after nitration; reduction and diazotization	Sepharose 4B with tyramine (after CNBr-activation)	CHAUDHURI and THOMAS (1979).
Fumarase from pig heart	α and β Citrylhexamethylenediamine	Sepharose 4B	HIROTA and SHIMAMURA (1985).
Galactolipase (EC 3.1.1.26) from rice bran	Palmitoyl chloride	Well-dried and -defatted gauze	MATSUDA and HIRAYAMA (1979).
Galactosidases:			
α-D-Galactosidase (EC 3.2.1.22) from soybean meal	N-ε-Aminocaproyl-N-ε-aminocaproyl-α-D-galactopyranosylamine	Sepharose 4B after CNBr-activation	HARPAZ et al. (1977).
α-Galactosidase I (EC 3.2.1.22) - from mung beans	Concanavalin A and melibiose	Con A-Sepharose and Melibiose-agarose	DEY (1984).
α-Galactosidase (EC 3.2.1.22) from human spleen; placenta and plasma	N-6-Aminohexanoyl-α-D-galactopyranosylamine	Sepharose 4B with 6-aminohexanoic acid	BISHOP and DESNICK (1981).
β-D-Galactosidase (EC 3.2.1.23) from bovine liver	D-Galactose	Sepharose 6B with divinyl sulphone	DI CIOCCIO et al. (1984).
β-Galactosidase from rat liver	Ovalbumin	Sepharose 4B	DEAN (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
β-Galactosidase (EC 3.2.1.23) from rat mammary gland	p-Aminophenyl-β-D-thiogalactoside	Agarose-p-Aminophenyl-β-D-thiogalactoside	KUO and WELLS (1978).
β-Galactosidase-Protein A fusion protein (fusion of Protein A gene and Escherichia coli gene encoding β-galactosidase)	Immunoglobulin IgG	IgG-Sepharose 4B	NILSSON et al. (1985).
Acid β-D-galactosidases A2 and A3 from human liver	Concanavalin A and 6-aminohexyl 1-thio-β-D-galactopyranoside	Con A-Sepharose 4B and CNBr-activated Sepharose 4B	FROST et al. (1978).
Acid β-D-galactosidase (EC 3.2.1.23) from human liver	Concanavalin A	Con A-Sepharose 4B	MILLER (1978).
GM1-Ganglioside β-galactosidase from normal cat brain and liver	p-Aminophenyl β-D-thiogalactoside and concanavalin A	Sepharose 4B after CNBr-activation and Con A-Sepharose	ANDERSON et al. (1978).
Hybrid proteins exhibiting β-galactosidase activity	p-Aminophenyl-β-D-thiogalactoside	CH-Sepharose 4B	ULLMANN (1984).
Gentamicin acetyltransferase I (EC 2.3.1.60) - from Escherichia coli	Gentamicin C 1a	Affi-Gel 10	WILLIAMS and NORTHROP (1976).
β-1,3-Glucanase (EC 3.2.1.6) from commercial enzyme preparation "Kitalase" from Basidiomycetes aphyllophoroles	Glucans pachyman or laminaran after reaction with cyanogen bromide	AH-Sepharose CL-6B	TOTANI et al. (1983).
Glucoamylase (EC 3.2.1.3) from Aspergillus niger C	Amylopectin activated using CNBr	AH-Sepharose or aminopropyl-controlled pore glass (pore diam. 500 Å)	PASZCZYNSKI et al. (1982).
Glucocerebrosidase (EC 3.2.1.45) from extracts of human tissues	Monoclonal anti-(glucocerebrosidase) antibodies	CNBr-activated Sepharose 4B	AERTS et al. (1986).
Glucosidases:			
Glucosidase I from calf liver microsomes	N-Carboxypentyl-1-deoxynojirimycin	AH-Sepharose 4B (carbodiimide technique)	HETTKAMP et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
α-Glucosidase (EC 3.2.1.20) from crude yeast protein	3-nitro-4-(6aminohexylamido)-phenylboronic acid or m-aminophenylboronic acid	Sepharose CL-6B or Matrex Gel PBA-60	MYOHANEN et al. (1981).
β-glucosidase(EC 3.2.1.21) - from Alcaligenes faecalis	2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranoside and 1-allyloxy-2,3-epoxypropane after reductive ozonolysis	Chitosan (reductive amination)	HOLME et al. (1988).
β-Glucosidase (EC 3.2.1.21) from Candida guilliermondii	β-Glucoside of salicyl aldehyde	Sepharose 4B with adipic dihydrazide	ROTH and SRINIVASAN (1978).
β-Glucosidase from calf liver cytosol	N-(9-Carboxynonyl)-deoxynojirimycin	AH-Sepharose 4B (carbodiimide technique)	LEGLER and BIEBERICH (1988).
β-Glucosidase (EC 3.2.1.21) - from Aspergillus niger	Concanavalin A	Con A-Sepharose	WOODWARD et al. (1986).
Acid β-glucosidase (EC 3.2.1.45) from human placenta	Dextran sulphate after CNBr-activation	CNBr-activated Sepharose 4B with lysine	SHAFIT-ZAGARDO and TURNER (1981).
Acid β-glucosidase (EC 3.2.1.45) from normal human placentae and spleen of patient with type-1 Ashkenazi Jewish Gaucher disease	N-(9-Carboxynonyl)- and N-(11-carboxyundecyl)-deoxynojirimycin	AH-Sepharose (carbodiimide technique)	OSIECKI-NEWMAN et al. (1986).
Exo-oligo-1,6-glucosidase (EC 3.2.1.10) from Bacillus cereus (EOG)	Antibodies against EOG from rabbits	CNBr-activated Sepharose 4B	SUZUKI et al. (1983).
Lysosomal β-glucosidase (EC 3.2.1.45) from human placentas	Sphingosyl-1-O-β-D-glucoside (GS) or GS with 1,12-dodecanedicarboxylic acid	CH-Sepharose 4B or AH-Sepharose 4B (carbodiimide technique)	GRABOWSKI and DAGAN (1984).
Glucosylceramidase (EC 3.2.1.45) from human placenta	Glucosyl sphingosine	Epoxy-activated Sepharose 6B	STRASBERG et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
β-Glucuronidase (EC 3.2.1.31) from human placenta	Immunoglobulin G against β-glucuronidase from goats	Sepharose 2B after CNBr-activation	BROT et al. (1978).
Glutamate decarboxylase (EC 4.1.1.15) from hog brain	N^6-(Aminohexyl)carbamoyl-methyl-adenosine 5'-triphosphate	ATP-agarose	WU and MARTIN (1984).
Glutathione peroxidase (EC 1.11.1.9) - from rat liver microsomes	S-Hexylglutathione	Epoxy-activated Separose 6B	REDDY et al. (1981).
Glycineamide ribonucleotide transformylase (EC 2.1.2.2) from chicken liver	Glycineamide ribonucleotide	CNBr-activated Sepharose 4B	CAPERELLI et al. (1978).
Glyoxalase I from rabbit liver	Blue dextran	CNBr-activated Sepharose 4B	ELANGO et al. (1981).
Glyoxalase II (S-2-hydroxyacylglutathione hydrolase,EC 3.1.2.6.) from Swiss mouse liver	Oxidized glutathione (GSSG)	CNBr-activated Sepharose 4B	ORAY and NORTON (1980).
Guanine aminohydrolase from rabbit liver	9-(p-Aminoethoxyphenyl)guanine	Sepharose 4B after CNBr-activation	BERGSTROM and BIEBER (1978).
Guanine aminohydrolase (EC 3.5.4.3)	2',3'-O-[1-(2-Carboxyethyl)-ethylidene]xanthosine	AH-Sepharose 4B	ROSEMEYER and SEELA (1981).
Guanosine 5'-(β,γ-imido)triphosphate-activated adenylate cyclase from rabbit myocardial membranes	7-Succinyl-7-deacetylforskolin	Sepharose CL-4B with 1,1'-carbonyldiimidazole,6-aminohexoic acid and ethanolamine (carbodiimide technique)	PFEUFFER et al. (1985).
Guanosine triphosphate (GTP) cyclohydrolase (EC 3.5.4.16) from Escherichia coli	UTP or GTP	UTP-agarose or GTP-agarose	FERRE et al. (1986).
Guanosine triphosphate cyclohydrolase from Escherichia coli	Periodate oxidized GTP	Sepharose 4B with ε-aminocaproic acid methylester and hydrazine hydrate	YIM and BROWN (1976).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Guanosine triphosphate cyclohydrolase I. (EC 3.5.4.16)	Dihydrofolate (folate after coupling to AH-Sepharose reduced in situ)	AH-Sepharose 4B	THEN (1979).
Guanylate cyclase (GC; EC 4.6.1.2) - from rat lang	Monoclonal antibody B4 against GC	CNBr-activated Sepharose 4B	KAMISAKI et al. (1986).
Guanylate cyclase (EC 4.6.1.2) from synaptosomal soluble fraction of rat brain	Guanosine 5'-triphosphate after periodate oxidation	Sepharose 4B after CNBr-activation with adipic acid dihydrazide	NAKANE and DEGUCHI (1978).
Heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8) from Flavobacterium heparinum	Glycosaminoglycan (GAG) coupled in presence of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide	AH-Sepharose 4B previosly coated non-covalently with GAG	OTOTANI et al. (1981).
Heparinase (EC 4.2.2.7)	Antibody	CNBr-Sepharose 4B	LINHARDT et al. (1985).
Hepatic ATP citrate lyase (EC 4.1.3.8) from rats	Cibacron Blue F3G-A	Blue Sepharose	REDSHAW and LOTEN (1981).
Hexokinase (EC 2.7.1.1) from pig hearts (eluted by nucleotide substrates or inhibitors)	Procion brilliant red H-8BN	Sepharose 6B	FARMER and EASTERBY (1982).
Hexokinase isoenzymes from yeast	D-Glucosamine	Activated CH-Sepharose 4B	KOPETZKI and ENTIAN (1982).
Hexosaminidase from human placenta	Concanavalin A	Con A-Sepharose	MAHURAN and LOWDEN (1980).
β-Hexosaminidases (EC 3.2.1.30) from bovine brain	p-Aminophenyl-2-acetamido-2-deoxy-β-D-galactopyranoside	CH-Sepharose 4B	LISMAN and OVERDIJK (1978).
Histaminase (EC 1.4.3.6) from placental extract	Cadaverine	AH-Sepharose 4B with bromoacetic acid and N-hydroxylsuccinimide	LIN et al. (1981b).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Homogentisate-1,2-dioxygenase from Aspergillus niger	2-Hydroxyphenylacetate	Sepharose after CNBr-activation with benzidine diazotized with sodium nitrite in presence of HCl	SUGUMARAN and VAIDYANETHAN (1978).
Hyaluronidase from monkey testis homogenate	Concanavalin A	Con A-Sepharose	BANSAL et al. (1980).
Hyaluronidase (EC 3.2.1.35) from bull seminal-plasma	Concanavalin A and heparin	Con A-Sepharose and Sepharose 4B with 2,4,6-trichloro-1,3,5-triazine	SRIVASTAVA and FAROOQUI (1979).
Hydrogenase from Rhizobium japonicum	Procion red HE-3B dye	Reactive Red 120-agarose	STULTS et al. (1986).
Hydrolases:			
A-Adenosylhomocysteine hydrolase (EC 3.1.1.1) from rat liver	S-Adenosylhomocysteine	Affi-Gel 10	BRISKE-ANDERSON and DUERRE (1982).
Acyl-CoA hydrolase (EC 3.1.2.2) from bovine heart microsomes	Cibacron Blue 3GA	Reactive Blue 2-agarose	SANJANWALA et al. (1987).
Carboxylic-ester hydrolase (CEH - EC 3.1.1.1) - from human,dog and rat pancreatic juices	Antibodies against human pancreatic CEH	Affi-Gel 10	ABOUAKIL et al. (1988).
Cholesterol ester hydrolase (EC 3.1.1.13) from rabbit aortic lysosomes	Concanavalin A	Con A-Sepharose	DOUSSET et al. (1981).
Cytosolic epoxide hydrolase (EC 3.3.2.3)	Methoxycitronellyl thiol	Sepharose CL-6B with 1,4-butanediol diglycidyl	HAMMOCK et al. (1986).
Epoxide hydrolase (EC 3.3.2.3) from liver cytosol of normal and clofibrate-treated mice	7-Methoxycitronellylthiol	Sepharose CL-6B with 1,4-bis(2,3-epoxypropoxy)butane	PRESTWICH and HAMMOCK (1985).
Hydrolases (22) from human liver	Lectins of six different carbohydrate specifities	Sepharose 4B after CNBr-activation	FIDDLER et al. (1979).
Keratin hydrolase from psoriatic scales	Aprotinin	CNBr-activated Sepharose 4B	HIBINO (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Lactase/phlorizin hydrolase (EC 3.2.1.23/62) from human small intestine	Immunoglobulin G against lactase from rabbits (purified by Protein A-Sepharose)	Sepharose 4B after CNBr-activation	SKOVBJERG et al. (1981).
S-Acyl fatty acid synthase thioester hydrolase from duck uropygial glands	Fatty acid synthase	Sepharose CL-4B	ROGERS and KOLATTUKUDY (1984).
S-Adenosylhomocysteine hydrolase (EC 3.3.1.1)	S-Adenosylhomocysteine	S-Adenosylhomocysteine-agarose	HERSHFIELD et al. (1985).
S-Adenosylhomocysteine hydrolase (EC 3.3.1.1) from Alcaligenes faecalis	S-Adenosylhomocysteine (AdoHcy)	AdoHcy-agarose gel	MATUSZEWSKA and BORCHARDT (1987).
Vanillate hydrolase	Vanillic acid	Phenyl-Sepharose	BUSWELL et al. (1981).
α-L-Iduronidase (EC 3.2.1.76) (corrective and noncorrective forms) from human urine	Heparin	CNBr-activated Sepharose 4B	SHAPIRO et al. (1976).
Imidazoleglycerolphosphate dehydratase (EC 4.2.1.19) and histidinol phosphatase (EC 3.1.3.15) (hisB bifunctional enzyme with hisB gene codes) from Salmonella typhimurium	Anti-hisB immune IgG from rabbits	Sepharose 4B	STAPLES and HOUSTON (1980).
Indole oxygenase from leaves of Tecoma stans	5-Hydroxyindole	Epoxy-activated Sepharose 6B	KUNAPULI and VAIDYANATHAN (1983).
myo-Inositol oxygenase (EC 1.13.99.1) from rat kidney	3-O-Substituted D-galacto-hexodialdose	Sepharose CL-6B after partial acid hydrolysis treated with galactose oxidase	KOLLER and KOLLER (1984).
Iodothyronine deiodinase from rat liver microsomes	3,3',5-triiodothyronine and 2-thiouracil-6-propionic acid	Activated CH-Sepharose 4B or CNBr-activated Sepharose 4B and AH-Sepharose 4B (carbodiimide technique)	MOL et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Juvenile hormones esterase from Manduca sexta	3-(4'-Mercaptobutylthio)-1,1,1-trifluoropropan-2-one	Sepharose CL-6B	ABDEL-AAL and HAMMOCK (1985).
2-Keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) from Zymomonas mobilis	Procion Yellow MX-GR dye	Sepharose CL-4B	SCOPES (1984).
Kinases:			
Acetate kinase from Escherichia coli	AMP	Agarose-hexane adenosine 5'-phosphate (AGAMP) - type 3	SWARTZ et al. (1978).
Adenosine kinase (EC 2.7.1.20) from human placenta	N^6(6-Aminohexyl)-5'AMP	5'AMP-Sepharose 4B	ANDRES and FOX (1979).
Adenosine kinase (EC 2.7.1.20) from rat brain	N^6-(6-Aminohexyl-)5'AMP	5'AMP-Sepharose 4B	YAMADA et al. (1980).
Adenylate kinase from human heart and brain; beef heart mitochondria and fish muscle	ATP	Sepharose 4B after CNBr-activation with hexamethylenediamine treated with p-nitrobenzoyl azide; reduced with sodium dithionite and treated with $NaNO_2$ in presence of HCl	LEE et al. (1977).
Ca^{2+} -Activated; phospholipid-dependent protein kinase (protein kinase C)	N-(2-Aminoethyl)-5-isoquinolinesulfonamide	CNBr-activated Sepharose 4B	INAGAKI et al. (1985).
Calmodulin-dependent protein kinase from cytosol of rabbit skeletal muscle	Calmodulin	Affi-Gel Calmodulin	SATO et al. (1988).
cAMP-dependent protein kinase	8-(β-Hydroxyethylthio)-cAMP	Epoxy-activated Sepharose 6B	CHARLTON (1985).
Casein kinases from maize seedlings	Casein or 5'-AMP	Sepharose 4B and 5'-AMP-Sepharose 4B	DOBROWOLSKA et al. (1986).
Creatine kinase isozyme (EC 2.7.3.2) from beef heart	ATP	Agarose-hexane-adenosine 5'-triphosphate (type 3)	HALL et al. (1979).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Creatine kinase from human brain	8-(6-Aminohexylamino)-adenosine-5'-triphosphate	Sepharose 4B after CNBr-activation	LEE et al. (1977).
Creatine phosphokinase (ATP:creatine phosphotransferase; EC 2.7.3.2)	p-Mercuribenzoate	Sepharose with ethylenediamine	MADELIAN and WARREN (1975).
Cyclic nucleotide independent protein kinases from rat liver nuclei	Histones; phosvitin or casein	Sepharose CL-4B after CNBr-activation	THORNBURG et al. (1978).
Deoxycytidine kinase (EC 2.7.1.74) - from leukemic human T-lymphoblasts	Deoxycytidine 5'-adenosine 5'''-P1,P4-tetraphosphate after periodate oxidation	Adipic acid dihydrazide-Sepharose	KIM et al. (1988).
Deoxynucleoside kinases - from Lactobacillus acidophilus	Deoxynucleoside 5'-adenosine 5'''-P1,P4-tetraphosphate dNp4A (dN represents dAdo,dCyd,dGuo or dThd moiety linked through its 5'-hydroxyl to terminal phosphate of adenosine tetraposphate) after periodate oxidation	Adipic acid diydrazide-Separose	IKEDA and IVES (1985).
Enterokinase (EC 3.4. 21.9) from X native human duodenal fluid	p-Aminobenzamidine	Sepharose 4B after CNBr-activation with glycylglycine (carbodiimide technique)	GRANT et al. (1978).
Ethanolamine kinase and choline kinase (EC 2.7.1.32) from rat liver	Choline	Epoxy-activated Sepharose 6B	BROPHY and VANCE (1976).
Galactokinase from Trigonella foenum-graecum	Galactosamine	CH-Sepharose (carbodiimide technique)	FOGLIETTI (1976).
Glycerol kinase - from Escherichia coli	Heparin	Heparin-agarose	KEE et al. (1988).
Glycogen synthase kinase from rat skeletal muscle	Cibaron Blue F3GA	Affi-Gel Blue gel	HEGAZY et al. (1987).
Guanosine 3':5'-monophosphate-dependent protein kinase from bovine lung	8-2-Aminoethylthio-cGMP	CNBr activated Sepharose 4B	LINCOLN et al. (1977).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Hexokinase from yeast	ATP complexed to immobilized boronic acid	Sepharose 6B with 6-aminocaproyl-3-aminophenylboronic acid	BOURIOTIS et al. (1981).
Hexokinase from Saccharomyces cerevisiae	D-Glucosamine	Activated CH-Sepharose 4B	KOPETZKI and ENTIAN (1982).
Hexokinase (EC 2.7.1.1) from human placenta	D(+)Glucosamine hyrochloride and Cibaron Blue F3GA	Activated CH-Sepharose 4B and Matrex Blue A	MAGNANI et al. (1988).
Hexokinase isozymes (EC 2.7.1.1) from Novikoff hepatoma	Cibacron Blue 3GA	Blue 2-Agarose	RADOJKOVIC and URETA (1987).
Inositol-1,4,5-triphosphate 3-kinase from guinea pig peritoneal macrophages	Calmodulin	Affi-Gel Calmodulin	KIMURA et al. (1987).
Insulin receptor/kinase from human placenta	Wheat germ lectin and insulin	CNBr-activated Sepharose and Affi-Gel 10	PIKE et al. (1986).
Isoenzymes of creatine kinase (EC 2.7.3.2) from chicken breast muscle and heart (CK) (quantitation)	Antibodies against homogenous homomeric isoenzymes of CK (purified by isoenzymes of CK coupled to CNBr-activated Sepharose 4B)	CNBr-activated Sepharose 4B	PERRIARD et al. (1978).
Malate thiokinase (EC 6.2.1.x) from Pseudomonas MA	Adenosine 3',5'-diphosphate	Agarose-hexane-adenosine 3'5'-diphosphate	ELWELL and HERSH (1979).
Modulator-deficient myosin light-chain kinase from rabbit skeletal muscle	Rabbit skeletal modulator protein	Sepharose 4B after CNBr-activation	YAZAWA and YAGI (1978).
Myosin light-chain kinases (I and II) from bovine carotid artery	Brain calmodulin	CNBr-activated Sepharose 4B	BHALLA et al. (1982).
Nicotinamide adenine dinucleotide kinase (EC 2.7.1.23) from yeast	Nicotinamide adenine dinucleotide	Agarose-hexane-NAD (AGNAD; Type 1)	TSENG et al. (1979).
Phosphatidylinositol kinase (EC 2.7.1.67) from bovine brain myelin	Phosphatidylinositol	Epoxy-activated Sepharose	SALTIEL et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) from rat liver	Guanosine triphosphate after periodate oxidation	CNBr-activated Sepharose 4B with adipic acid dihydrazide	IYNEDJIAN (1979).
Phosphofructokinase (EC 2.7.1.11) - from turtle white muscle	Adenosine 5'-diphosphate (ADP) - attached through N^6-amino group	ADP-agarose	BROOKS and STOREY (1988).
Phosphofructokinase (EC 2.7.1.11) from Bacillus subtilis	Blue dextran 2,000	Sepharose 4B	KAWAI et al. (1982).
Phosphoglycerate kinase (EC 2.7.2.3) from human erythrocytes	Procion Red HE3B	Matrex Gel Red A	CHEN-MAROTEL et al. (1983).
Phosphorylase kinase isozymes from rabbit skeletal muscle	Calmodulin	Sepharose 4B with divinyl sulfone	SHARMA et al. (1980).
Protamine kinase	8-(6-Aminohexyl)amino cyclic AMP	Sepharose 4B	JERGIL and MOSBACH (1974).
Protein kinase C from rabbit brain	N-(2-Aminoethyl)-5-isoquinolinesulfonamide	CNBr-activated Sepharose 4B	INAGAKI et al. (1987).
Protein kinase C from rabbit reticulocyte lysates	Heparin	Heparin-Sepharose	PELAEZ et al. (1987).
Protein kinase C from bovine brains	Substrate protamine	Protamine-agarose	WALTON et al. (1987).
Pyridoxal kinase (EC 2.7.1.35) from rat brain	Pyridoxal hydrochloride	AH-Sepharose	CASH et al. (1980).
Pyrimidine nucleoside monophosphate kinase (EC 2.7.4.9) from rat bone marrow cells	Cibacron Blue F3GA	Sepharose 6B	SEAGRAVE and REYES (1985).
Pyruvate kinase (EC 2.7.1.40) from Candida guilliermondii	Blue dextran	Sepharose 4B	KAWAI et al. (1983a).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Pyruvate kinase (EC 2.7.1.40) from Salmonella typhimurium	Cibacron Blue FGA	Blue Sepharose CL-6B	GARCIA-OLALLA and GARRIDO-PERTIERRA (1987).
Rous sarcoma virus src kinase	1. Casein; 2. L-tyrosine	Casein-agarose; L-Tyrosine-agarose	FUKAMI and LIPMANN (1985).
Thiamine pyrophosphokinase (EC 2.7.6.2) from brewer's yeast	Thiamine pyrophosphate	Sepharose 4B after CNBr-activation with N-4-nitrobenzoyl-ε-aminocaproylhydrazide reduced by sodium dithionite and with $NaNO_2$ in presence of HCl	CHERNIKEVICH et al. (1981).
Thymidine kinase from Morris hepatoma or rat liver	Glycoprotein inhibitor from rat kidney extract	CNBr-activated Sepharose 4B	MADHAV et al. (1980).
L-Kynurenine 3-hydroxylase (EC 1.14.1.2) from rat liver	NADP	NADP-Adipic acid dihydrazide-agarose	NISHIMOTO et al. (1979).
Laccase (EC 1.10.3.2) from Pholiota mutabilis	1) Concanavalin A 2) Syringic aldehyde	1) Con A-Sepharose 2) AH-Sepharose 4B	LEONOWICZ and MALINOWSKA (1982).
Lactase from human small intestine	Concanavalin A and immunoglobulin G against human lactase from rabbits	Con A-Sepharose 4B and Sepharose 4B after CNBr-activation	LAU (1987).
β-Lactamase I from Bacillus cereus and Pseudomonas maltophilia	m-Aminophenylboronic acid	Affi-Gel 10 or Succinimide activated CH-Sepharose 4B	CARTWRIGHT and WALEY (1984).
β-Lactamase from Streptomyces albus G	Cephalosporin C	CNBr-activated Sepharose	DUEZ et al. (1981).
Lipases:			
Lipase (EC 3.1.1.3)	Colipase	Ultrogel AcA22	ALESSANDRI et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Lipase (glycerol ester hydrolase; EC 3.1.1.3) from Chromobacterium viscosum and Candida cylindracea	Silicone oil DC QF-1 or silicone grease	Glass beads (coated)	ISOBE and SUGIURA (1977).
Lipase - from human skim milk	Antibodies against human pancreatic carboxylic-ester hydrolase	Affi-Gel 10	ABOUAKIL et al. (1988).
Lipase from porcine pancreas	Sodium taurodeoxycholate	Glass micro beads	MOMSEN and BROCKMAN (1978).
Avian lipoprotein lipase	Monoclonal antibody	Sepharose 4B	GERSHENWALD et al. (1985).
Bile salt-activated lipase from human milk whey	Concanavalin A and sodium cholate	Sepharose 4B with 3,3'-diamino-dipropylamine (carbodiimide technique)	WANG (1980).
Lipolytic acyl-hydrolase from Phaseolus vulgaris leaves	Palmitoyl chloride	Well-dried and -defatted gauze	MATSUDA et al. (1979).
Lipoxygenase-2 (EC 1.13.1.13)	Linoleic acid	Aminoethyl Sepharose	ANDRAWIS et al. (1982).
Lipoxygenase-2 (EC 1.13.11.12) from soybean	Antibodies against lipoxygenase-1 from rabbits	CNBr-activated Sepharose 4B	VERNOOY-GERRITSEN et al. (1982).
5-Lipoxygenase from porcine leukocytes	Monoclonal anti-5-lipoxygenase antibody	Affi-Gel 10	RIENDEAU et al. (1989).
Luciferases (EC 1.14.14.3) from Vibrio harvevi or fischeri or Photobacterium phosphoreum	2,2-Diphenylpropylamine	Sepharose 6B with 1,4-bis(2,3-epoxypropoxy)butane and sodium borohydride	BALDWIN et al. (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Luciferase from firefly abdomen	Blue Dextran	CNBr-activated Sepharose 4B or silica with γ-glycidoxypropyltrimethoxysilane	RAJGOPAL and VIJAYALAKSHMI (1982).
Luciferase from firefly lanterns	Triazine dyes	Sepharose 4B	RAJGOPAL and VIJAYALAKSHMI (1983).
Bacterial luciferase from Vibrio harveyi	Cibacron Blue F3GA	Blue Sepharose CL-6B	TSAI (1985).
Lysozymes:			
Lysozyme (EC 3.2.1.17)	Anti-lysozyme monoclonal antibody	Carbodiimidazole-activated Fractogel HW65F; Trisacryl GF2000 or CNBr-activated Sepharose 4B	HEARN and DAVIES (1990).
Lysozyme (EC 3.2.1.17)	Heparin	Ultrogel A4R	BOSCHETTI et al. (1981).
Lysozyme (EC 3.2.1.17) from human saliva	Chitin	Chitin	VASSTRAND and JENSEN (1980).
Lysozyme (EC 3.2.1.17) from hen egg-white	Secondary amine derivative of β (1 ⇒ 4)linked threemer of N-acetyl-D-glucosamine	Cellulofine GC 700-m gel (45-105 micrometer diameter) after CNBr-activation	YAMASAKI and ETO (1981).
Lysozymes (EC 3.2.1.17) from human parotid salina and urine	Anti-(human leukemic lysozyme) IgG from sheep	Epoxy-activated Sepharose 6B	MAC KAY et al. (1982).
Active Kyn 62-lysozyme (tryptophan 62 replaced by kynurenine)	Chitin	Chitin-coated cellulose	YAMASAKI et al. (1979).
Bacteriophag T4 lysozyme from T4 infected Escherichia coli	C6 Muropeptide	Affi-Gel 202	SZEWCZYK et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Malic enzyme (L-malate:$NADP^+$ oxidoreductase; EC 1.1.1.40) from pigeon liver	N^6-(6-Aminohexyl)-adenosine 2',5'-bisphosphate	Agarose-N^6-(6-aminohexyl)-adenosine 2',5'-bisphosphate	CHANG and CHANG (1982).
Malolactic enzyme from Leuconostoc oenos	Nicotinamide adenine dinucleotide (NAD)	Agarose-hexane-NAD (AGNAD)	SPETTOLI et al. (1984).
Maltase (EC 3.2.1.20) from rat kidney brush border membrane	Inhibitor of maltase Tris	Epoxy-activated Sepharose 6B	REISS and SACKTOR (1981).
Manganese superoxide dismutase from bullfrog liver mitochondria	Cibacron Blue F3GA	DEAE Affi-Gel Blue	ABE and OKAZAKI (1987).
β-Mannanase (EC 3.2.1.78) from lucerne seed	Mannan or glucomannan	Sepharose with hexamethylenediamine	MC CLEARY (1978).
1,2-α-Mannosidase from Aspergillus oryzae	Baker's yeast mannan	Mannan gel prepared by use of epichlorohydrin	TANIMOTO et al. (1986).
α-D-Mannosidase (EC.3.2.1.24) from jack bean meal	Benzidine	Sepharose 4B after CNBr-activation	WAGH (1978).
Acidic α-D-mannosidase (EC 3.2.1.24) from bovine kidney	Concanavalin A and D-mannosylamine	Con A-Sepharose and CH Sepharose 4B (carbodiimide technique)	PHILLIPS et al. (1977).
Mannuronan C-5-epimerase	Alginate (substrat)	Epoxy-activated Sepharose 6B	SKJAK-BRAEK and LARSEN (1982).
Mitochondrial type II DNA topoisomerase (enzyme) - from trypanosomatid Crithidia fasciculata	Inhibitor novobiocin	Epoxy-activated Sepharose 6B	MELENDY and RAY (1989).
Murein transglycosylase (EC 3.2.1.-) from Escherichia coli	Cibacron Blue F3GA and poly(U)	Blue Sepharose CL-6B and poly(U)-Sepharose 4B	KUSSER and SCHWARZ (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Mutarotase (EC 5.1.3.3) from hog kidney	Phloretin	Sepharose 4B with epichlorohydrin; concentrated ammonia solution; p-nitrobenzoyl azide reduced with dithionite and diazotized with sodium nitrite in presence of HCl	TOYODA et al. (1982).
Myeloperoxidase - from normal or myeloid leukaemia leukocytes	L-Thyroxine (T4) (coupled via its phenolic group)	Epoxy-activated Sepharose 6B	VAN ZYL et al. (1988).
Neuraminidase from influenza virus	p-Aminophenyloxamic acid	Copolymers of N-vinylpyrrolidone and acryloylchloride onto surface of γ-aminopropylsilylated porous glass	IVANOV et al. (1985).
Nicotinamide nucleotide transhydrogenase (mitochondrial)	$NADP^+$ (bound at the C8 atom in the adenine moiety)	Agarose-hexane-$NADP^+$	CARLENOR et al. (1985).
Nucleases:			
Deoxyribonuclease (deoxyribonucleate oligonucleotidohydrolase; EC 3.1.4.5) from bovine pancreats	Single-stranded (heat-denatured) calf thymus DNA	Sepharose 4B	SCHABORT (1972).
Deoxyribonuclease (EC 3.1.4.5) from ovine and bovine pancreas	Concanavalin A	Con A-agarose	WADANO et al. (1979).
DNA Endonuclease from Escherichia coli	Calf thymus DNA	Cellulose- according ALBERTS and HERRICK (1971)	LJUNGQUIST (1977).
DNase I from porcine pancreas	Calf thymus double stranded DNA (dDNA) (under non-digestive conditions)	Sepharose	TANAKA et al. (1980).
Endonuclease (EC 3.1.4.21) specific for apurinic sites from Bacillus stearothermophilus	DNA	Sepharose 2B or 4B after CNBr-activation	BIBOR and VERLY (1978).
Endonucleases from Escherichia coli and Bacillus globiggii	Blue dextran and folic acid	Sepharose 4B and Epoxy-activated Sepharose 6B or AH-Sepharose 4B	MIROSHNICHENKO et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Exonuclease A5	Cytidine-2'(3')5'-diphosphate	Silochrom with heterocyclic base	BANNIKOVA et al. (1983).
Guanine-specific ribonuclease from Fusarium moniliforme	Guanosine 5'-monophosphate after periodate oxidation (after coupling stabilitation with $NaBH_4$ reduction)	AH-Sepharose 4B	YOSHIDA et al. (1980b).
Guanyloribonuclease from Streptomyces aureofaciens	2',3'-O-2-Carboxyethylideneinosine 2'(3')-phosphorothioate with ethyl chloroformate or 5'-O-(2-aminoethylamidocarbonyl-methyl) inosine 2'(3')-phosphorothioate	AH-Sepharose 4B or Sepharose 4B after CNBr-activation	KOIS et al. (1980).
Pyrimidine base-specific ribonuclease from bull semen	Periodate-oxidized ATP	Adipoyldihydrazo-Sepharose CL-4B	KRIETSCH et al. (1983).
Restriction endonucleases from B.amyloliquefaciens; P.alcalifaciens; X.holicola or B.globiggi	Cibacron Blue F3GA	Cibacron Blue F3GA-agarose	BAKSI et al. (1978).
Restriction endonucleases from unfractionated bacterial extracts	Pyran and Cibacron Blue F3GA	Sepharose 4B after CNBr-activation	GEORGE and CHIRIKJIAN (1978).
Ribonuclease H I (hybrid nuclease,EC 3.1.26.4) from calf thymus	Immunoglobulin G against calf thymus ribonuclease H I from rabbit	Sepharose 4B after CNBr-activation	BUSEN (1982).
Ribonucleases C and U from human urine (EC 3.1.27.5)	Poly(G)	CNBr-activated Sepharose 4B	CRANSTON et al. (1980).
Ribonuclease from bovine seminal plasma	DNA	Cellulose	MURTI and PANDIT (1983).
Ribonucleases from chicken liver	5'-Amino-5'-deoxyuridine 2'(3')-phosphate	Sepharose 4B	MIURA et al. (1984).
Ribonuclease U2 (EC 3.1.27.4) from Ustilago sphaerogena	5'-AMP-dialdehyde (prepared by periodate oxidation)	AH-Sepharose 4B (after coupling reduction by $NaBH_4$)	UCHIDA and SHIBATA (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Ribonucleases from human urine; pancreas; lung; leukocyte; kidney; liver; spleen; stomach and semen (organ-specific differences)	Concanavalin A; Ricinus communis agglutinin; Aleuria aurantia lectin; lentil lectin and wheat germ agglutinin	Sepharose	YAMASHITA et al. (1986).
RNase A	5'-Uridinetriphosphate-hexane	5'-UTP-agarose	SMITH et al. (1978).
Single-strand-specific nuclease from germinating pea seeds	Concanavalin A	Sepharose after CNBr-activation	NASEEM and HADI (1987).
Site-specific endonuclease EcoRI from Escherichia coli RY13	Antibodies against endonuclease EcoRI from rabbits	Sepharose 4B after CNBr-activation	ERUSLANOV et al. (1980).
5'-Nucleotidase (EC 3.1.3.5) from rat liver	Monoclonal antibody against rat liver 5'-nucleotidase	Cellulose with m-nitrobenzyloxymethyl pyridinium chloride; reduced with dithionite and diazotized with nitrous acid	BAILYES et al. (1982).
5'-Nucleotidase (EC 3.1.3.5) from various sources	N^6-(6-Aminohexyl-) 5'AMP and Lens culinaris lectin	5'-AMP-Sepharose and CNBr-activated Sepharose 4B	DIECKHOFF et al. (1985).
5'Nucleotidase (or 5'-ribonucleotide phosphohydrolase; EC 3.1.3.5) - from bovine liver plasma membranes	Concanavalin A and AMP	Concanavalin A-Ultrogel and 5'AMP-Sepharose 4B	HARB et al. (1983).
Nucleotide pyrophosphatase from rat liver plasma membranes and of endoplasmatic reticulum	Adenosine 5'-monophosphate (coupling via phosphate)	AH-Sepharose 4B (carbodiimide technique)	BISCHOFF et al. (1975).
Ornithine transcarbamylase (EC 2.1.3.3)	δ-N-(phosphonacetyl)-L-ornithine	Sepharose 4B	DE MARTINIS et al. (1981).
Oxidases:			
Aldehyde oxidase (EC 1.2.3.1)	Anti-aldehyde oxidase-antibody	CNBr-activated Sepharose	ANDRES (1976).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Amine oxidase (EC 1.4.3.6) from bovine serum	1,6-diamino hexane	Sepharose 4B	SVENSON and HYNNING (1981).
Amine oxidase from bovine plasma	Hexamethylenediamine and concanavalin A	AH-Sepharose 4B and Con A-Sepharose	TURINI et al. (1982).
Cytochrome c oxidase from Micrococcus luteus	Yeast cytochrome c	CH-Sepharose 4B (carbodiimide technique)	ARTZATBANOV et al. (1987).
Cytochrome-c oxidase - from bovine heart mitochondria	Horse heart cytochrome c and yeast cytochrome c	CNBr-activated Sepharose 4B and Activated thiol-Sepharose 4B or Affi-Gel 102 with succinimidyl-4-(p-maleimidophenyl)butyrate	BROGER et al. (1986).
D-amino acid oxidase apoenzyme (EC 1.4.3.3)	Concanavalin A	Blue Sepharose CL-6B	LEONIL et al. (1985).
Diamine oxidase (DAO - EC 1.4.3.6) from Lens culinaris	Anti-DAO-antibodies	CNBr-activated Sepharose 4B	FEDERICO et al. (1985).
Galactose oxidase (EC 1.1.3.9) - from Dactylium dendroides	Melibiose	Melibiose-polyacrylamide	KELLEHER et al. (1988).
Galactose oxidase (EC 1.1.3.9) from Dactylium dendroides	D-Galactose	Sepharose 4B	MENDONCA and ZANCAN (1987).
Monoamine oxidase A and B (EC 1.4.3.4)	Monoclonal antibody	Sepharose 4B after CNBr-activation	DENNEY et al. (1982).
Putrescine oxidase (EC 1.4.3.4) from Micrococcus rubens	1,12-Diaminododecane	Sepharose 4B after CNBr-activation	OKADA et al. (1979).
Pyridoxamine (pyridoxine) 5'-phosphate oxidase (EC 1.4.3.5) from rabbit liver	Pyridoxal 5'-phosphate	N'-(ω-Aminohexyl)-agarose (resulting imine reduced with sodium borohydride)	BOWERS-KOMRO et al. (1986).
Sulphydryl oxidase from bovine milk	L-Cysteine	Controlled-pore glass beads (pore diam. 200nm) with 3-aminopropyltriethoxysilane and succinic anhydride	SLIWKOWSKI et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Urate oxidase from rat liver	8-Aminoxanthine	Sepharose 4B with 1,4-bis(2,3-epoxypropoxy)butane	WATANABE and SUGA (1978).
Xanthine oxidase from bovine milk	Folate	Sepharose 4B after CNBr-activation with hexamethylenediamine (carbodiimide technique)	GARDLIK et al. (1987).
Pectinesterase (EC 3.1.1.11) and D-galacturonase (EC 3.2.1.15) from tomatoes (separation)	Pectic acid	Pectic acid cross-linked by epichlorohydrin	REXOVÁ-BENKOVÁ et al. (1977).
Penicillin acylase	Phenyl chloride	Eupergit 'C' with ethylenediamine or hexamethylenediamine	BIHARI and BUCHHOLZ (1984).
Penicillinase	Methicillin	Sepharose 4B with epichlorohydrin; phloroglucinol; divinylsulphone and ethylenediamine	COOMBE and GEORGE (1976).
Peptidylarginine deiminase (EC 3.5.3.15) from rabbit skeletal muscle	Soybean trypsin inhibitor (Kunitz)	Sepharose 4B after CNBr-activation	TAKAHARA et al. (1986).
Peroxidase (EC 1.11.1.7) from Arachis hypogaea	Anti-cationic peroxidase fraction IgGs	CNBr-activated Sepharose 4B	CHIBBAR and VAN HUYSTEE (1984).
Peroxidase (EC 1.11.1.7) from mycelium of Trametes versicolor	Vanillic aldehyde	γ-Aminopropyltriethoxysilane glass	LOBARZEWSKI (1981).
Peroxidase from hog thyroid microsomes	Monoclonal antibody against peroxidase	CNBr-activated Sepharose 4B	NAKAGAWA et al. (1985).
Peroxidase from rat intestines	Concanavalin A	Con A-Sepharose	VALOTI et al. (1988).
Phenol sulphotransferase (EC 2.8.2.1)	p-Hydroxyphenylacetic acid	Sepharose 4B with 3,3'diamino-dipropylamine and succinic anhydride	BORCHARDT and SCHASTEEN (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
L-Phenylalanine ammonialyse (EC 4.3.1.5) from cut-injured sweet potato tissues	L-Phenylalanine	Sepharose 4B after CNBr-activation	TANAKA and URITANI (1977).
Phenylalanine ammonia lyase (EC 4.3.1.5)	L-Phenylalanine	CNBr-activated Sepharose 4B	USSUF and NAIR (1980).
Phenylalanine hydroxylase (EC 1.14.16.1) from monkey liver (Mocaca irus)	2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine; 5-formyl-6,7-dimethyl-5,6,7,8-tetrahydropterin; 5-formyl or 5-methyl-tetrahydrofolate	CH-Sepharose or AH-Sepharose	COTTON and JENNINGS (1978).
Phosphatases:			
Acid phosphatase in 3 different form based on varied sialic acid content from rat liver	Sialic acid binding lectin carcinoscorpin	Sepharose 4B after CNBr-activation	MOHAN et al. (1981).
Acid phosphatase (EC 3.1.3.2) from cotyledons of germinating soybean seeds	Procion Red HE3B	Procion Red HE3B-agarose	ULLAH and GIBSON (1988).
Acid phosphatase isoenzyme (EC 3.1.3.2) from human liver	Concanavalin A	Sepharose 4B after CNBr-activation	SAINI and VAN ETTEN (1978).
Acid phosphatase (EC 3.1.3.2) from human prostatic tissue	L(+)-Tartrate	AH-Sepharose 4B (carbodiimide technique)	VIHKO et al. (1978).
Alkaline phosphatase	Monoclonal antibody	CNBr-Sepharose	HSU et al. (1985).
Alkaline phosphatase (EC 3.1.3.1) from calf intestine	Procion Red HE-3B (via chlorine group)	Sepharose 4B	KIRCHBERGER et al. (1987).
Alkaline phosphatase isoenzymes (EC 3.1.3.1) - from human liver and bone	Wheat germ lectin (WGL)	WGL-Sepharose 4B	GONCHOROFF et al. (1989).
Alkaline phosphatase (AP) isoenzymes (EC 3.1.3.1) from rat osteosarcoma; calvaria; kidney and placenta	Monoclonal antibody against rat osteosarcoma AP	Protein A-Sepharose CL-4B conjugated with dimethylpimelimidate dihydrochloride	NAIR et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Alkaline phosphatases (EC 3.1.3.1) (desialylated with neuramidase EC 3.2.1.18) from bovine kidney or human placenta	Diazotized 4-aminobenzylphosphonic acid	Sepharose 4B after periodate oxidation with L-histidine and sodium cyanoborohydride added in three portions	MOSSNER et al. (1980).
Alkaline phosphatase three isoenzymes (EC 3.1.3.1.) from human placenta (I),intestine (II); liver,bone and kidney (III)	Concanavalin A; lentil lectin,wheat germ lectin and Helix pomatia lectin	Con A-Sepharose 4B,Lentil Lectin-Sepharose 4B,Wheat germ Lectin-Sepharose 6MB,Helix pomatia Lectin-Sepharose 6MB	LEHMANN (1980).
Alkaline phosphatase from bovine intestine	Diazotized 4-aminobenzylphosphonic acid	Sepharose 6B with L-histidine	LANDT et al. (1978).
Alkaline phosphatase isoenzymes (EC 3.1.3.1) from rat	Concanavalin A; phytohemagglutinin-L (Phaseolus vulgaris; L-PHA); peak lectin (Pisum sativum; PSA); wheat germ agglutinin (Triticum vulgaris	WGA); Con A-Sepharose; L-PHA-agarose; PSA-agarose; WGA-agarose,	
Alkaline phosphatase from human liver	p-Aminobenzylphosphonic acid after diazotization with sodium nitrite	Sepharose 4B with tyramine hydrochloride	SEARGEANT and STINSON (1979).
Alkaline phosphatase from human placentas	Cibacron Blue 3GA dye	Sepharose 4B	YASMIN and QADRI (1984).
Fructose 1,6-bisphosphatase (EC 3.1.3.11) and fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) from rabbit liver	Cibacron Blue F3GA	Blue Sepharose CL-6B	KIDO et al. (1980).
Glucose-6-phosphatase (membrane-bound; EC 3.1.3.9) from human placenta and liver	Glucose-6-phosphate	Epoxy-activated Sepharose 6B	RECZEK and VILLEE (1982).
Human intestinal alkaline phosphatase (ALP - EC 3.1.3.1) from meconium and adult intestinal samples	Monoclonal antibody against intestinal-type ALP	CNBr-activated Sepharose 4B	VOCKLEY and HARRIS (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Multiple forms (sugar-chain heterogeneity) of alkaline phosphatases (EC 3.1.3.1) - from human liver; bone; kidney; duodeum; term placenta; hepatoma and malignant mesothelioma of omentum	Concanavalin A (Con A); pea lectin (Pisum sativum,PSA); wheat germ agglutinin (WGA); phytohemagglutinin E (Phaseolus vulgaris; PHA-E)	Con A-Sepharose; PSA-; WGA- or PHA-E-agarose	KOYAMA et al. (1987).
Organophosphate-hydrolysing (FNT) phosphatase from bacterium Alcaligenes	Cibacron 3GA	Sepharose CL-6B	PAI (1983).
Phosphoprotein phosphatase(s) (EC 3.1.3.16) from rat liver	Protamine sulphate	Sepharose 4B after CNBr-activation	TINANJI and PAHLMAN (1978).
Phosphorylase phosphatase (EC 3.1.3.17) from rabbit liver	Hexamethylenediamine	Sepharose	BRANDT et al. (1975).
3'-Phosphate RNA cyclase - from cell-free extracts of HeLa cells	Polyadenylic acid (Poly(A)) and heparin	Poly(A)-agarose and Heparin-Ultrogel	REINBERG et al. (1985).
Phosphodiesterases:			
Activator-dependent cAMP phosphodiesterase from bovine brain	Ca^{2+} -Dependent activator protein	CNBr-activated Sepharose 4B	KLEE and KRINKS (1978).
Calcium-dependent cyclic nucletide phosphodiesterase from bovine brain	Calcium-dependent regulatory protein	CNBr-activated Sepharose 4B	KINCAID and VAUGHAN (1979).
Calcium/calmodulin-dependent forms of 3',5'-cyclic-nucleotide phosphodiesterase (EC 3.1.4.17) from bovine heart and brain	Monoclonal antibody against phosphodiesterase	Ultrogel AcA34 with glutaraldehyde	HANSEN and BEAVO (1982).
Calmodulin-stimulated cyclic nucleotide phosphodiesterase - from bovine brain or heart	Monoclonal antibodies ACAP1 or ACC-1 (purified by Protein A-Sepharose,calmodulin-Sepharose or calmodulin-Affi-Gel 15)	CNBr-activated Sepharose 4B; Affi-Gel 10 or Protein A-Sepharose	HANSEN et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Calmodulin-stimulated phosphodiesterase - from bovine brain	Calmodulin fragment 1 - 77	Sepharose 4B after CNBr-activation	DRAETTA and KLEE (1988).
cAMP-Specific phosphodiesterase isoenzyme from rat heart cytosol	cAMP-Specific phosphodiesterase inhibitor	AH-Sepharose	FOUGIER et al. (1986).
Cyclic 3',5'nucleotide phosphodiesterases (EC 3.1.4.17) from bovine heart	Succinylated trimethylpapaveroline	Sepharose 4B with diaminodipropylamine	MOHINDRU et al. (1978).
Cyclic adenosine monophosphate phosphodiesterase from human platelets	N-Cyclohexyl-N-(2-hydroxybutyl)-5-[6-(1,2,3,4-tetrahydro-2-oxoquinolyloxy)]-butyramide	Epoxy-activated Sepharose 6B	UMEKAWA et al. (1984).
Cyclic nucleotide phosphodiesterase (CNP) - from Lactuca cotyledons	Cibacron Blue F3GA dye and immunoglobulin G against CNP	Affi-Gel Blue and CNBr-activated Sepharose 4B	CHIATANTE et al. (1988).
Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) (PDE I) - from Neurospora crassa	Monoclonal antibodies against PDE I	CNBr-activated Sepharose 4B	ULLOA et al. (1988).
Cyclic nucleotide phosphodiesterase from rat heart	(2-Oxo-2,5-dihydro-4-furyl) 4-phenylacetic acid	AH-Sepharose 4B	PRIGENT et al. (1980).
Insulin- and glucagon-stimulated 'dense-vesicle' cyclic AMP phosphodiesterase from rat liver	6-Ethylamine guanine or Cibacron Blue F3GA	Sepharose after CNBr-activation or Affi-Gel Blue	PYNE et al. (1987).
Phosphodiesterase from bovine brain	[3-(2-Pyridylthio)propionyl] calmodulin (desorption with reducing agent)	Activated thiol-Sepharose 4B	KINCAID and VAUGHAN (1983).
Putative hormone-sensitive cyclic AMP phosphodiesterase from rat adipose tissue	N-(2-Isothiocyanate)-ethyl -derivative of cilostamide	Aminoethyl-agarose	DEGERMAN et al. (1987).
Sphingomyelinase (sphingomyelin phosphodiesterase; EC 3.1.4.12) from human placenta	Concanavalin A	Con A-Sepharose	CALLAHAN et al. (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Phosphoenolpyruvate carboxylase (PEP-C; EC 4.1.1.31) from crude extract of Sorgum leaves	Antibodies against PEP-C from rabbits	Ulrogel ACA 22 with glutaraldehyde	VIDAL et al. (1982).
Phosphoglucose isomerase (EC 5.3.1.9.) from Drosophila melanogaster	8-(6-Aminohexylamino)-adenosine-5'-triphosphate	Sepharose 4B after CNBr-activation	LEE et al. (1979).
Phosphoglycerate mutase (EC 2.7.5.3) from Leuconostoc dextranicum	Blue dextran	Sepharose 4B	KAWAI et al. (1983b).
Phospholipase A2 (EC 3.1.1.4) from porcine pancreas; Crotalus adamanteus; rat mitochondria and platelets	10-O-p-Toluenesulfonyldecane-1-O-phosphocholine	AH-Sepharose 4B	AARSMAN et al. (1984).
Phospholipase A2 (EC 3.1.1.4) from Agkistrodon halys blomhoffii venom and porcine pancreas	Phospholipid	Organo-silica support Silochrom	EVSTRATOVA et al. (1982).
Phospholipase A2 from bovine ileum smooth muscle	1-(12'-Phthalimidododecanoyl)-2-hexadecyl-sn-glycero-3-phosphocoline after hydrazinolysis	Affi-Gel 10	TAHIR and HIDER (1983).
Phospholipase A2 from venom of Naja naja oxiane and Agkistrodon halys blomhoffii	sn-Glycero-3-phosphocholine	Silica Silakril	OSTAPENKO et al. (1984).
Phospholipase C (phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3) from Clostridium perfringens	Egg-yolk lipoprotein	Sepharose 4B	TAKAHASHI et al. (1974).
Phosphorylases:			
Chloroplast α-1,4 glucan phosphorylase (EC 2.4.1.1) from spinach leaves	Starch	Sepharose	STEUP (1981).
Glycogen phosphorylase b from smooth muscle	Glycogen after periodate oxidation (after coupling stabilization with $NaBH_4$)	Sepharose 4B after CNBr-activation with succinic acid dihydrazide	VIKTOROVA et al. (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Latent and spontaneously active forms of phosphorylase and phosphatase	Heparin	Heparin-Sepharose CL-6B	ERDODI et al. (1985).
Maltodextrin phosphorylase (EC 2.4.1.1) from Escherichia coli	Glycogen	Sepharose 4B after CNBr-activation	SCHACHTELE et al. (1978).
Nicotinamide riboside phosphorylase (EC 2.4.2.1) from beef liver	Dye Green A	Matrex Gel Green A	IMAI and ANDERSON (1987).
Phosphorylase a (EC 2.4.1.1) from human platelets	N^6-(6-Aminohexyl)-adenosine 5'-monophosphate	5'AMP-Sepharose 4B	GERGELY et al. (1979).
Phytase (myo-inositol-hexakisphosphate phosphorylase; EC 3.1.3.8) from cotyledons of germinating soybean seeds	Procion Red HE3B	Procion Red HE3B-agarose	GIBSON and ULLAH (1988).
Polynucleotide phosphorylase (EC 2.7.7.8)	Heparin	CNBr-activated Sepharose 4B	BAUER and BUKI (1981).
Purine nucleoside phosphorylase (EC 2.4.2.1) from human red cells	6-Hydroxy-9-p-aminobenzylpurine	Sepharose CL-6B with trichlore-s-triazine	OSBORNE (1980).
Purine nucleoside phosphorylase (EC 2.4.2.1.) from human granulocytes	Formycin B after periodate oxidation	Sepharose 4B after CNBr-activation with 3,3-iminobispropylamine	WIGINTON et al. (1980).
Uridine 5'-diphosphate glucose pyrophosphorylase (EC 2.7.7.9) from Physarum polycephalum	Uridine 5'-triphosphate	Aminoethyl polyacrylamide beads P-150 with m-aminobenzeneboronic acid	MAESTAS et al. (1980).
Phosphotransacetylase (EC 2.3.1.8) from Clostridium kluyveri	C8-(6-Aminohexyl)-amino-desulfo-CoA	Sepharose 4B	SMITH and KAPLAN (1979).
Polymerases:			
Bacteriophage T7 DNA polymerase from T7-infected Escherichia coli	Thioredoxin-specific antibodies (purified by Affi-Gel TM10 with thioredoxin-S2)	CNBr-activated Sepharose 4B	NORDSTROM et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Deoxyribonucleic acid polymerase (DNA nucleotidyltransferase; EC 2.7.7.7) from cytoplasm of microplasmodia of myxomycete Physarum polycephalum	Double-stranded salmon sperm DNA	Cellulose APX -prepared according Alberts and Herrick (1971) and irradiation with ultraviolet lamp	BAER and SCHIEBEL (1978).
Deoxyribonucleic acid polymerase (EC 2.7.7.7) induced by Bacillus subtilis bacteriophage PBS2 from extracts of infected cells	Denatured salmon sperm DNA	Cellulose CF11 -prepared according Alberts and Herrick (1971)	HITZEMAN and PRICE (1978) (1978).
DNA α-Polymerase from calf thymus	Monoclonal immunoglobulin G (IgG) against human KB cell DNA α-polymerase	Affi-Gel 10	WAHL et al. (1984).
DNA Polymerase α(DNAp) from KB cells	Monoclonal antibodies against DNAp (purified by Protein A-Sepharose)	Sepharose 4B	WONG et al. (1986).
DNA-dependent RNA polymerase III (EC 2.7.7.6) from Drosophila hydei pupae	Heparin and calf thymus DNA	Sepharose 4B after CNBr-activation and 4% agarose (for electrophoresis,mixed with DNA at 50 °C,solified by pouring onto ice-cooled glass dish and cut into pieces)	GUNDELFINGER et al. (1980).
DNA-dependent RNA polymerase III (zinc metalloenzyme) from Saccharomyces cerevisiae	Heat denatured calf thymus DNA	CNBr-activated Sepharose 4B	WANDZILAK and BENSON (1978).
DNA-Dependent RNA polymerase (class A,B and C; EC 2.7.7.6) from chicken embryos	Chicken embryo total tRNA after periodate oxidation	Sepharose 4B after CNBr-activation with hydrazine (after coupling reduction with $NaBH_4$)	WITTIG and WITTIG (1978).
DNA-polymerase I from Escherichia coli	Adenosine 5'-phosphate	Agarose-hexane-adenosine 5'-phosphate (Type 2 and 3)	TSANG LEE and WHYTE (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Eukaryotic DNA-dependent DNA polymerase α-primase - from cherry salmon (Oncorhynchus masou) testes	5-(E)-(4-Aminostyryl)ara UTP	Affi-Gel 10	IZUTA and SANEYOSHI (1988).
Poly(A)polymerases from rat liver nuclei	Chromosomal RNA	CNBr-activated Sepharose	ANTONIADES and ANTONOGLOU (1978).
Poly(A)polymerase (EC 2.7.7.19) from rat liver nuclei	ATP after periodate oxidation	Sepharose 4B with adipic acid dihydrazide	GREZ and NIESSING (1977).
Poly(adenosine diphosphate ribose)polymerase from Ehrlich ascites tumor cells	Single-stranded calf thymus DNA	4% agarose (for electrophoresis,mixed with DNA at 50 °C, solified by pouring onto ice-cooled glass dish and cut into pieces)	KRISTENSEN and HOLTLUND (1978).
RNA Polymerase II from rat livers (study of DNA-binding property)	Calf thymus double-stranded DNA	Sephadex G-25 (fine)	OKAI et al. (1982).
RNA polymerases from yeast	Heparin	Bio-Gel A-15m after CNBr-activation	HAMMOND and HOLLAND (1983).
RNA Polymerase II A (EC 2.7.7.6) - from wheat germ	Heparin	Heparin-Ultrogel	JOB et al. (1982).
RNA-Dependent DNA polymerase (reverse transcriptase,EC 2.7.7.7) from avian myeloblastosis virus	Polycitidylic acid	Sepharose 4B after CNBr-activation	MODAK and MARCUS (1977).
RNA-Dependent DNA polymerase (reverse transcriptase; EC 2.7.7.7) from avian sarcoma virus B77	Polyuridilic acid	Whatman cellulose CF11	HIZI and JOKLIK (1977).
RNA-Polymerase II (EC 2.7.7.6) from calf thymus or wheat germ	O-[-[[(Aminoethyl)-amino]carbonyl]-pent-1-yl]-α-amanitin	CNBr-activated Sepharose 4B	LUTTER and FAULSTICH (1984).
Soybean DNA-dependent RNA polymerase I	Plasmid pHFK 206 containing soybean rDNA insert	CNBr-activated Sepharose 4B	GROSSMANN et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Procolipase from porcine pancreas	Antibodies against porcine procolipase B from rabbits (purified by porcine colipase coupled to CNBr-activated Sepharose 4B)	CNBr-activated Sepharose 4B	RATHELOT et al. (1983b).
Phenylalanine hydroxylase (EC 1.14.16.1) from rat liver	2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine dihydrochloride	Sepharose 4B with 1,4-bis(2,3-epoxypropoxy)butane	WEBBER et al. (1980).
Prolyl hydroxylase (EC 1.14.11.2) from 13-day old chick embryos	Poly-L-proline	Bio-Gel A-5m	KEDERSHA and BERG (1981).
Prolyl hydroxylase (EC 1.14.11.2) from tissue of chick embryos or human placentas	Poly (L-proline)	Sepharose 4B	KIVIRIKKO and MYLLYLA (1987).
Propionyl-CoA carboxylase from human liver	Monomeric avidin (biotin-pretreated)	Sepharose CL-4B after CNBr-activation	GRAVEL et al. (1980).
Protein disulphide-isomerase (PDI; EC 5.3.4.1) (removal of residual albumin) from human placenta and rat liver	Human or rat serum albumin	CNBr-activated Sepharose 4B	KAETZEL et al. (1987).
Protein methylase II (EC 2.1.1.24) from calf brain	S-Adenosyl-L-homocysteine	Sepharose 4B-1,6-diaminohexane with O-bromoacetyl-N-hydroxysuccinimide	KIM et al. (1978).
Proteinases and their zymogens:			
Acid proteases from hen's egg yolk	Pepstatin	DADPA-Pepstatin-agarose (diaminodipropylamine spacer)	WOUTERS and STOCKX (1981).
Acid proteinase from Aspergillus oryzea	ε-Aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester	Separon H 1000 with epichlorohydrin	TURKOVÁ et al. (1981).
Acid proteinase from Aspergillus niger	Histidine	Sepharose 4B with epichlorohydrin or 1,4-butanediol diglycidyl ether	KANOUN et al. (1986).
Acid protease from raw shovu (fermented soy sauce)	Pepstatin	Sepharose 6B with $NH_2(CH_2)_2NH_2$	FUKUSHIMA et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Acid proteinase (isorenin) from rat brain	Hydroxysuccinimidate ester of pepstatin A (pepstatin A with N-hydroxysuccinimide by use of carbodiimide technique)	AH-Sepharose (unreacted aminohexyl groups reacted with CH_3COONa in presence of carbodiimide)	HACKENTHAL et al. (1978).
Acrosin from rabbit sperm	Proflavin	Sepharose 4B after CNBr-activation with 6-aminocaproic acid coupled by carbodiimide technique	BRANTNER et al. (1976).
Actinidin (cysteine proteinase) from kiwifruit Actinidia chinensis	2-Pyridyl disulphide derivative of mercaptohydroxypropyl ether	Activated thiopropyl-Sepharose 6B	BROCKLEHURST et al. (1981).
Alkaline proteinase (EC 3.4.21.14) from commercial technical preparations	Antibody	Glycidyl methacrylate copolymer with 1,6-diaminohexane activated with glutaraldehyde	KÁŠ et al. (1986).
Alkinase A (alkaline metalloendopeptidase from Streptomyces violaceorectus)	Carbobenzoxyglycyl-L-leucine	AH-Sepharose	INOUYE et al. (1984).
α-Chymotrypsin	Benzyloxycarbonyl-glycyl-D-phenyl-alanine or -D-leucine	Spheron 300 with hexamethylenediamine	TURKOVÁ et al. (1976a).
α-Chymotrypsin	D-Tryptophan methyl ester	Trisacryl-GF-2000 with p-nitrophenylchloroformate or N-hydroxysuccinimidechloroformate and with ε-aminocaproic acid	MIRON and WILCHEK (1985).
α-Chymotrypsin from porcine pancreatic extract	α-Chymotrypsin	Sepharose 4B after CNBr-activation	CHIANCONE and GATTONI (1986).
α-Thrombin (human) (determination of anionic binding region)	Fibrinogen (after coupling converted to fibrin with α-thrombin)	Sepharose 4B after CNBr-activation	BERLINER et al. (1985).
Aminopeptidase from brain membrane of cat	Concanavalin A	Con A-Sepharose	ZHERNOSEKOV et al. (1985).
Aminopeptidase (EC 3.4.11.7) from pig kidney	Amastatin	AH-Sepharose 4B	TOBE et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Aminopeptidase from Aspergillus flavus	D-Leucine	Spheron 300 with hexamethylenediamine	TURKOVÁ et al. (1976b).
Aminopeptidase II from protoplast supernatant of yeast	Bestatin	AH-Sepharose 4B	KNUVER and ROHM (1982).
Aminopeptidases from Aspergillus oryzae and Bacillus thuringiensis	H-Thr(But)-Phe-Pro-OH	Sepharose 4B with phenylenediamine or AH-Sepharose 4B	LYUBLINSKAYA et al. (1984a).
Aminopeptidases I (closely resembles aminopeptidase EC 3.4.11.12) and II from brewer's yeast	(2S	3S)-3-Amino-2-hydroxy-4-phenylbutanoyl-Val-Gly; AH-Sepharose 4B,	
Aminopeptidases from uterine secretions from pig ovariectomized gilts injected with progesterone; allantoic and amniotic fluids	L-Leucylglycine	Sepharose 6B after CNBr-activation with hexamethylenediamine (carbodiimide technique)	BASHA et al. (1978).
Aminopeptidase N (detergent form) - rabbit intestinal	Concanavalin A and antibodies against impurities from goat	Concanavalin A-Ultrogel and Indubiose AC A 22 activated with glutaraldehyde	FERACCI and MAROUX (1980).
Aminopeptidase A (EC 3.4.11.7) from pig kidney cortex	Immunoglobulin G against pig intestinal aminopeptidase A from rabbits	CNBr-activated Sepharose 4B	DANIELSEN et al. (1980).
Aminopeptidase (EC 3.4.11.2) from rat brush-border membrane	Concanavalin A and Ricinus communis lectin	Sepharose 4B	ERICKSON and KIM (1983).
Angiotensin-converting enzyme (ACE) (EC 3.4.15.1) from rat lung and corpus striatum	Concanavalin A and monoclonal antibody against rat lung ACE (purified by Protein A-Sepharose)	Sepharose CL-4B and CDI Activated Fractogel TSK (Reacti-Gel HW-65F)	STRITTMATTER et al. (1985).
Angiotensin I converting enzyme (EC 3.4.15.1) from human plasma (= Dipeptidyl carboxypeptidase)	Captopril (D-(3-mercapto-2-methylpropanoyl)-L-proline) with N-hydroxysuccinimide	AH-Sepharose 4B (carbodiimide technique)	TAIRA et al. (1985).
Angiotensin converting enzyme (dipeptidyl carboxypeptidase; EC 3.4.15.1) from rabbit lung tissue	N-[1(S)-Carboxy-5-aminopentyl]-L-Phe-Gly	Epoxy-activated Sepharose 6B with 6-[N-(p-aminobenzoyl)amino]caproic acid and N-hydroxysuccinimide	PANTOLIANO et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Angiotensin I-converting enzyme (EC 3.4.15.1) from human plasma	L-Cysteinyl(benzyl)-L-proline or L-cysteinyl-L-proline	Sepharose CL-6B with trichloro-s-triazine or Sepharose CL-4B after CNBr-activation	HARRIS et al. (1981).
Angiotensin converting enzyme (EC 3.4.15.1) from pig brain	Lisinopril = N-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline	Sepharose CL-4B with 1,4-bis (2,3-epoxypropoxy)butane (in presence of $NaBH_4$),6-[N-(p-aminobenzoyl) amino]-caproic acid and N-hydroxysuccinimide ester	HOOPER and TURNER (1987).
Angiotensin-converting enzyme (EC 3.4.15.1) from human lung and kidney	N-[1(S)-Carboxy-5-aminopentyl]glycylglycine	Epoxy-activated Sepharose 6B with N-(p-aminobenzoyl)-6-aminocaproic acid and N-hydroxysuccinimide	EHLERS et al. (1986).
Anhydrotrypsin	Tryptic digest of salmine	Sepharose 4B after CNBr-activation	ISHII et al. (1983).
β-Kallikrein (EC 3.4.21.35) from porcine pancreas	Pancreatic trypsin inhibitor Type I-P	CNBr-activated Sepharose 4B	BIZZOZERO and DUTLER (1987).
Ca^{2+} -Activated neutral protease from chicken skeletal muscle	α - Casein	Activated CH-Sepharose 4B	KUBOTA et al. (1981).
Capillary injury-related protease from lung lymph of sheep	Cibacron Blue 3GA; trypsin inhibitor aprotinin and p-aminobenzamidine	Reactive Blue 2-agarose; Aprotinin-agarose and p-Aminobenzamidine-agarose	ORLOWSKI et al. (1987).
Carboxylic proteinases (EC 3.4.4.1) from swine; horse; Aspergillus awamori; and carnivorous plant Nepenthes	Bacitracin (polypeptide antibiotic)	CNBr-activated Sepharose 4B or 6B	STEPANOV et al. (1978).
Carboxypeptidase (EC 3.4.12.2) from crayfish Astacus fluviatilis	Potato carboxypeptidase inhibitor	CNBr-activated Sepharose 4B	ZWILLING et al. (1979).
Carboxypeptidase N (EC 3.4.2.2) from rat serum	IgG fraction of rabbit antiserum to rat carboxypeptidase N	CNBr-activated Sepharose 4B	SIMONIANOVA and PETAKOVA (1979).
Carboxypeptidase Y from Saccharomyces cerevisiae (EC 3.4.16.1)	Gly-Gly-p-aminobenzylsuccinic acid	Spheron with epichlorohydrin	TURKOVÁ et al. (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Carboxypeptidase from wheat seeds	D,L-(p-Aminobenzyl)succinic acid	Sepharose 4B with C-aminocaproic acid and N-hydroxysuccinimide	SARBAKANOVA et al. (1987).
Carboxypeptidase N (arginine carboxypeptidase; EC 3.4.12.7) from human plasma	p-Aminobenzoyl-L-arginine	Sepharose 6B with epichlorohydrin and azodianiline (carbodiimide technique)	PLUMMER and HURWITZ (1978).
Carboxypeptidase II from malted barley	D,L-(p-Aminobenzyl)succinic acid	Sepharose 4B with ε-aminocaproic acid activated by N-hydroxysuccinimide in presence of N,N'-dicyclohexylcarbodiimide	BREDDAM et al. (1985).
Carboxypeptidases A and B (EC 3.4.17.2) from mammalian pancreatic acetone powders	DL-(p-Aminobenzyl)succinic acid	Sepharose 4B with ε-aminocaproic acid and N-hydroxysuccinimide	BAZZONE et al. (1979).
Carboxypeptidase Y from Saccharomyces cerevisiae (EC 3.4.16.1)	Histidine	Sepharose 4B with epichlorohydrin or 1,4-butanediol diglycidyl ether	KANOUN et al. (1986).
Carboxypeptidase A (EC 3.4.12.2) and its chemically modified derivatives	p-Amino-D,L-benzylsuccinic acid	Sepharose 4B with ε-aminocaproic acid and N-hydroxysuccinimide	CUENI et al. (1980).
Cathepsin D (EC 3.4.2.3.5; two types) from rat spleen	Pepstatin with N-hydroxysuccinimide and concanavalin A	AH-Sepharose 4B and Con A-Sepharose 4B	YAMAMOTO et al. (1979).
Cathepsin D (EC 3.4.23.5) from bovine brain	Pepstatin A	AH-Sepharose 4B	WHITAKER and SEYER (1979).
Cathepsin D (EC3.4.23.5) - from human erythrocyte-free leukocytes	Pepstatin	Diaminodipropylamine-pepstatin-Sepharose CL-6B	VON CLAUSBRUCH and TSCHESCHE (1988).
Cathepsin L (cysteine proteinase EC 3.4.22.15) from rat brain	Concanavalin A	CNBr-activated Sepharose 4B	MARKS and BERG (1987).
Cathepsin D from human gastric mucosa	Casein; Z-Phe-Phe and hydroxybenzotriazole esters of pepstatin A	Sepharose 4B and Sepharose 4B with hexamethylenediamine	POHL et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cathepsin D (EC 3.4.23.5) from human brain	Pepstatin	CNBr-activated Sepharose 4B with hexamethylenediamine	AZARYAN et al. (1983).
Cathepsin D from pig myometrium	Concanavalin A and pepstatin	Con A-Sepharose and Sepharose CL-4B	AFTING and BECKER (1981).
Cathepsins D-I and D-II from unfertilized sea urchin egg Tetrapygus niger	Hemoglobin	Hemoglobin-agarose	SANCHEZ-CHIANG et al. (1986).
Chymopapain A (EC 3.4.4.11) from dried latex of papaya	Glutahione-2-pyridyl disulphide	Sepharose 2B	BAINES et al. (1986).
Chymopapain S (EC 3.4.22.6) from papaya latex	Glutathione-2-pyridyl disulphide	Activated thiol-Sepharose 4B	KHAN and POLGAR (1983).
Chymosin (rennin - EC 3.4.23.4)	Pepstatin with N-hydroxysuccinimide	Aminohexylagarose	KOBAYASHI and MURAKAMI (1978).
Chymosin from calf and kid abomassa	Histidine	Sepharose 4B with epichlorohydrin or 1,4-butanediol diglycidyl ether	KANOUN et al. (1986).
Chymotrypsin B from bovine pancreas	Bowman-Birk trypsin inhibitor	Sepharose 4B after CNBr-activation	WILIMOWSKA-PELC et al. (1983).
Chymotrypsin (EC 3.4.21.1)	Trypsin inhibitor from potatos	Bead cellulose with benzoquinone	KUČERA (1981).
Chymotrypsin (EC 3.4.21.1) - bovine	Gly-Gly-L-Leu-L-phenylalaninal	Sepharose CL-4B with 1,4-butanediol diglycidyl ether and Gly-Gly	NISHIKATA (1983).
Chymotrypsin and trypsin from dilute solutions and pancreatic tissue	Soybean trypsin inhibitor	CNBr-activated Sepharose 4B	AMNEUS et al. (1979).
Chymotrypsin and potential intracellular target proteins from fibroblasts of patients having Bloom syndrom	Anticarcinogenic Bowman Birk inhibitor (soybean-derived)	CNBr-activated Sepharose	BILLINGS et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Chymotrypsin from fluorescein-labeled conjugates of α-chymotrypsin and pancreas trypsin inhibitor (Trasylol)	Soy-bean trypsin inhibitor	CNBr-activated Sepharose 4B	KASCHE and BUCHTMANN (1981).
Clostridiopeptidase (EC 3.4.24.3)	Collagen	Collagen incorporated into a 5% polyacrylamide gel	EVANS (1985).
Clostridium histolyticum collagenase (clostridiopeptidase A; EC 3.4.24.3)	Alkali-treated elastin	Alkali-treated elastin	SERAFINI-FRACASSINI et al. (1975).
Clostripain (clostridiopeptidase B; EC 3.4.22.8) from Clostridium histolyticum	Butylenediamine	Sepharose 4B	KULA et al. (1976).
Clostripain	L-Arginine; p-aminophenylmercuric acid;poly(L-arginine)	CH-Sepharose 4B; Sepharose 4B,	EMOND and KEIL (1977).
Clottable fibrinogen from plasma	Thrombin-activated fibrinogen	Sepharose 6B	STEMBERGER and HORMANN (1975).
Collagenase from human skin fibroblasts and rheumatoid synovium	L-Propyl-L-leucylglycinehydroxamic acid	Activated CH-Sepharose 4B	MOORE and SPILBURG (1986).
Collagenase (EC 3.4.24.7) from human polymorphonuclear leukocytes	Wheat germ agglutinin	Wheat germ lectin-agarose	CALLAWAY et al. (1986).
Collagenases from Clostridium histolyticum	Reactive Red 120 dye	Agarose	BOND and VAN WART (1984).
Collagenase (EC 3.4.24.3) from Clostridium histolyticum	Heparin	Heparin-Sepharose CL-6B	BICSAK and HARPER (1985).
Collagenase from Clostridium histolyticum (EC 3.4.24.3)	L-Arginine; soybean trypsin inhibitor	CH-Sepharose 4B; Sepharose 4B	EMOD et al. (1981).
Cuticle-degrading proteases from entomopathogen Metarhizium anisopliae	Soya bean trypsin inhibitor	CNBr-activated Sepharose 4B	LEGER et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cytotoxic protease from peripheral blood lymphocytes	Soybean trypsin inhibitor	Sepharose 4B after CNBr-activation	HATCHER et al. (1978).
D-alanine carboxypeptidase from membranes of Bacillus subtilis	6-Aminopenicillanic acid	Sepharose with 3,3'-diaminopropylamine and succinic anhydride	BLUMBERG and STROMINGER (1974).
D-Alanine carboxypeptidase from Escherichia coli	p-Aminobenzylpenicillin	Sepharose 4B	GORECKI et al. (1975).
DD-Carboxypeptidases transpeptidases L and H (membrane-bound penicillin-binding proteins; EC 3.4.17.8 and EC 2.3.2.10) from Proteus mirabilis	Ampicillin	Succinylaminododecyl-cellulose	SCHILF and MARTIN (1980).
Different amphiphilic forms of microvillus aminopeptidase (EC 3.4.11.2) from pig small intestine	Antibodies against aminopeptidase-M from rabbits	Sepharose 4B	SJOSTOM et al. (1978).
Dipeptidase (dehydropeptidase I; EC 3.4.13.11) from rat lung and rat and hog kidney	(Z)-7-(2-Aminoethylthio)-2-(2,2-dimethylcyclopropane carboxamido)-2-heptanoic acid	Affi-Gel 10	HITCHCOCK et al. (1987).
Dipeptidyl aminopeptidase (EC 3.4.14.1) from beef spleen	Glycyl-D-phenylalanine; benzoyl-L-arginine or L-phenylalanine and p-aminophenylmercury(II) acetate	Sepharose 4B with hexamethylenediamine and Sepharose 4B	METRIONE (1978).
Dipeptidyl peptidase IV (EC 3.4.14.-) from pig small intestinal brush border membrane	Antibodies against kidney dipeptidyl peptidase IV from rabbits	Sepharose 4B after CNBr-activation	SVENSSON et al. (1978).
Dipeptidyl-aminopeptidase IV (DAP-IV) from human kidney	Antibodies against DAP-IV from rabbits	CNBr-activated Sepharose 4B	HAMA et al. (1982).
Dipeptidyl carboxypeptidase (EC 3.4.15.1) from rabbit testes	N-α-[1-(S)carboxy-3-phenylpropyl]-L-lysyl-L-proline	Sepharose CL-4B with 1,4-butanediol diglycidyl ether	EL-DORRY et al. (1982).
Dipeptidyl peptidase IV (EC 3.4.14.-) from rat brush-border membrane	Concanavalin A and Ricinus communis lectin	Sepharose 4B	ERICKSON and KIM (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Elastase-like enzyme (EC 3.4.21.11) from bovine granulocyte	L-Alanyl-L-alanyl-L-alanine	CH-Sepharose 4B activated with N-hydroxysuccinimide	MAROSSY et al. (1980).
Elastase (EC 3.4.21.11) from pancreatic powder Pancreatin	L-Alanyl-L-alanyl-L-alanine	Acivated CH-Sepharose 4B	KATAGIRI et al. (1978).
Elastase from human leukocytes	Suc-L-Tyr-D-Leu-D-Val-pNA	AH-Sepharose	OKADA et al. (1982).
Elastase from Pseudomonas aeruginosa	2-(N-Hydroxycarboxamido)-3-phenylpropanoyl-L-alanylglycine	Affi-Gel 101	NISHINO and POWERS (1980).
Elastase from human leukocytes	L-Trialanine p-nitroanilide	Sepharose CL-6B with 1,4-butanediol diglycidyl ether	ANDERSSON et al. (1980).
Elastase-like proteinase from human leukocytes	Suc-L-Tyr-D-Leu-D-Val-pNA	AH-Sepharose	NAGAMATSU et al. (1984).
Elastase-type enzyme from human arterial wall	Lima-bean trypsin inhibitor	CNBr-activated Sepharose 4B	BELLON et al. (1980).
Endogenous-protease-free isopeptidase (cleaves chromatin protein A24) from calf thymus	Calf thymus histone fractions	CNBr-activated Sepharose 4B	KANDA et al. (1984).
Endopeptidase-24.11 (enkephalinase) (EC 3.4.24.11) from pig brain	Monoclonal antibody against microvillar endopeptidase-24.11	CNBr-activated Sepharose 4B	RELTON et al. (1983).
Enterokinase (membrane bound serine proteinase; EC 3.4.21.9)	Enterokinase inhibitor from kidney bean	Affi-Gel 10	JACOB and PATTABIRAMAN (1984).
Enteropeptidase (porcine; EC 3.4.21.9) free of intestial aminopeptidase activity	Concanavalin A	Con A-Sepharose	BARNS and ELMSLIE (1976).
Enzymatically active glandular kallikrein (EC 3.4.21.8) from human plasma	Anti-human urinary kallikrein γ-globulins (IgG)	CNBr-activated Sepharose 4B	GEIGER et al. (1980).
Fibrinolytic proteinase from human spleen	Suc-L-Tyr-D-Leu-D-Val-pNA	AH-Sepharose	OKADA et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Fibronectin or thrombin	Heparin in presence of $NaCNBH_3$	Sepharose 4B with epichlorohydrin; and ammonia or hydrazine hydrate	SASAKI et al. (1987).
Ficin (EC 3.4.22.3)	p-Aminophenylmercury(II) acetate	Sepharose 4B-200	ANDERSON and HALL (1974).
Folylpolyglutamate endopeptidase from chicken intestine	γ-Hexaglutamate with two additional minor components corresponding to 3-5 glutamate residues	Sepharose 4B	ROSENBERG and SAINI (1980).
Formylmethionine aminopeptidase (EC 3.4.11.-) from rat liver	N-Formylbestatin with soluble carbodiimide (EDC)	AH-Sepharose 4B (remaining amino groups masked after coupling with acetic anhydride)	SUDA et al. (1980).
Fully active ficin (EC 3.4.22.3) from Ficus glabrata	Glutathione-2-pyridyl disulphide	Sepharose 2B	MALTHOUSE and BROCKLEHURST (1976).
Glu-plasminogen	Lysine	Lysine-Sepharose 4B	BOK and MANGEL (1985).
Guanidinobenzoatase (proteolytic enzyme capable of degrading fibronectin which present on surface of tumour cells) - from mouse Ehrlich ascites plasma	Agmatine (decarboxylation product of arginine)	Activated CH-Sepharose 4B	STEVEN et al. (1986).
Heat-stable protease from Pseudomonas fluorescens T16	Carbobenzoxy-D-phenylalanine	Carbobenzoxy-D-Phe-triethylene tetramine-Sepharose 4B	PATEL et al. (1983).
Hemisphaericin (SH-proteinase) - from Bromelia hemisphaerica	p-Chloromercurobenzoic acid	Sepharose 4B after CNBr-activation with ethylenediamine	OCHOA et al. (1987).
High-molecular-weight renin (EC 3.4.99.19)	Antibodies against renin	CNBr-activated Sepharose 4B	IKEMOTO et al. (1983).
Human cathepsin B	Semicarbazone of Gly-Phe-glycinal	Activated CH-Sepharose 4B	RICH et al. (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Human leukocyte elastase and chymotrypsin-like cathepsin G from purulent sputum	Elastin	Elastin-Sepharose 4B (3,3'-diaminodipropylamine succinic acid spacer arm)	VISCARELLO et al. (1983).
Human neutrophil collagenase from granulocytes	Inhibitor Trasylol	Sepharose 4B	CHRISTNER et al. (1982).
Intracellular serine protease from Bacillus subtilis	Gramicidin-S	Sepharose 4B after CNBr-activation	ISOTOVA et al. (1978).
Kallikrein (EC 3.4.21.8) from tissue of rat spleen	Monoclonal antibodies	Affi-Gel 10	CHAO et al. (1984).
Kallikrein and elastase from hog pancreas powder	p-Aminobenzamidine	Sepharose 4B with ε-aminocaproic acid	HONDA et al. (1986).
Kallikrein (EC 3.4.21.8) from human plasma	Soya-bean trypsin inhibitor and rabbit anti-(human immunoglobulins) immunoglobulin (immunoadsorption of contaminating proteins)	Sepharose 4B	NAGASE and BARRETT (1981).
Kallikrein (serine proteinase; EC 3.4.21.8) from rat stomach	p-Aminobenzamidine	Sepharose 4B after CNBr-activation with ε-aminohexanoic acid (carbodiimide technique)	UCHIDA et al. (1980).
Kallikrein (EC 3.4.21.8) from rat serum	Aprotinin (trypsin inhibitor Trasylol)	Agarose	SCHELL and ARSENE (1982).
L-Leucine aminopeptidase (EC 3.4.11.1) from human liver	Anti-leucine aminopeptidase antibodies	Formyl-cellulofine gel	KOHNO et al. (1986).
Leucine aminopeptidase from Aspergillus oryzae; Bacillus thuringiensis; Trichoderma koningii	H-Thr(But)-Phe-Pro-OH	Aminosilochrom with glycine	LYUBLINSKAYA et al. (1984b).
Metallo carboxypeptidase from Streptomyces griseus K-1 (Pronase)	p-Amino-DL-benzylsuccinic acid	Sepharose 4B	NARAHASHI et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Metallo-proteinase from Aspergillus oryzae (Biodiastase powder)	Talopeptin (6-deoxy-α-L-talopyranosyloxyphospho-L-leucyl-L-tryptophan) from Streptomyces rishiriensis and mozunensis	Sepharose 4B with hexamethylenediamine	KASAI et al. (1984).
Metalloproteinase from human rheumatoid synovial fibroblasts	Green A dye and gelatin	Matrex Gel Green A and Affi-Gel 10	OKADA et al. (1986).
Milk-clotting enzyme (rennet) from Endothia parasitica	N-Isobutyrylpepstatin	Sepharose 4B with hexamethylenediamine	KOBAYASHI et al. (1982).
Multiple proteases from Streptomyces moderatus Fraction IV	L-Alanyl-L-alanyl-L-alanine	Activated CH-Sepharose 4B	CHANDRASEKARAN and DHAR (1987).
Neutral metalloendopeptidase from Streptomyces mauvecolor	Carbobenzoxyglycyl-D-leucine	AH-Sepharose	INOUYE et al. (1985).
Neutral metallo-endopeptidase that degrades B-chain of insulin; glucagon and ACTH from rat kidney microsomes	Insulin B-chain	CNBr-activated Sepharose 4B	VARANDANI and SHROYER (1977).
Neutral proteinase (EC 3.4.24 group) from Streptomyces naraensis	Carbobenzoxy-L-phenylalanine or carbobenzoxy-L-leucine	AH-Sepharose 4B	HIRAMATSU (1982).
Neutral protease from Bacillus stearothermophilus	Bacitracin	Sorbsil C200 epoxy silica	VAN DEN BÜRG èt al. (1989).
Papain	Gly-Gly-Tyr(Bz)-Arg or Gly-Gly-Tyr-Arg	Sepharose 4B after CNBr-activation	FUNK et al. (1979).
Papain from dried papaya latex	Glutathione-2-pyridyl disulphide	Sepharose 2B	BROCKLEHURST et al. (1974).
Papain from crude papain	ε-Aminocaproyl-L-leucyl-D-phenylalanine	Sepharose 4B after CNBr-activation	SYU et al. (1983).
Papaya peptidase A from comercial dried papaya powder	Glycyl-glycyl-L-(O-benzyl)tyrosyl-L-arginine	CNBr-activated Sepharose 4B	SCHACK and KAARSHOLM (1984).
Pepsin (chicken)	Pepsin inhibitor from Ascaris lumbricoides	Sepharose 4B	KEILOVÁ et al. (1975).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Pepsin (EC 3.4.23.1) and gastricsin (EC 3.4.23.3) from human gastric juice (separation)	Val-D-Leu-Pro-Phe-Phe-Val-D-Leu	Sepharose 4B	KUČEROVÁ et al. (1986).
Pepsin (EC 3.4.23.1)	ε-Aminocaproyl-L-Phe-D-Phe-OMe	Separon with epichlorohydrin	TURKOVÁ et al. (1982).
Pepsin (human; porcine and chicken); gastricsin (human) and cathepsin D (bovine)	Val-D-Leu-Pro-Phe-Phe-Val-D-Leu	Sepharose 4B with 6-aminohexanoic acid and N-hydroxybenztriazol	POHL et al. (1984).
Pepsin from crude porcine pepsin	ε-2,4-Dinitrophenyl-aminocapronylhydrazide	Sepharose 4B after CNBr-activation	LAVRENOVA et al. (1979).
Pepsinogens (EC 3.4.4.1) from stomachs of mice	N-Hydroxysuccinyl-pepstatin	AH-Sepharose 4B	ESUMI et al. (1978).
Plasminogen (precursor of EC 3.4.21.7) from human platelet-poor plasma	Lysine	Lysine-Sepharose 4B and Epoxy-activated Sepharose 6B	TRAAS et al. (1984).
Plasminogen-activating enzyme (66K; HPA66) from human melanoma cells	Monoclonal IgG1 antibody against HPA66 (purified by Protein A-Sepharose)	CNBr-activated Sepharose	NIELSEN et al. (1983).
Plasminogen activator (serine proteinase)	4-Aminobenzamidine	4-Aminobenzamidine aminododecyl cellulose	DANO et al. (1980).
Plasminogen (zymogen of the proteolytic enzyme plasmin - EC 3.4.21.7) from human plasma	L-Lysine monohydrochloride	Sepharose 4B	DEUTSCH and MERTZ (1970).
Prekallikrein from human plasma	Antikallikrein IgY	CNBr-activated Sepharose CL-4B	BURGER et al. (1986).
Procollagenase from rabbit uterine fibroblast	Heparin	Heparin-Sepharose 6B	ISHIBASHI et al. (1987).
Prolidase (EC 3.4.13.9) from human erythrocyte	Monoclonal mouse IgG against human prolidase	CNBr-activated Sepharose 4B	ENDO et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Protease B from Streptomyces griseus	Gly-Gly-L-Leu-L-phenylalaninal	Sepharose CL-4B with 1,4-butanediol diglycidyl ether and Gly-Gly	NISHIKATA (1983).
Protease from rat liver mitochondria	Cytochrome c	Bio-Gel P-100 with glutaraldehyde	SUBRAMANIAN et al. (1978).
Proteinase B (EC 3.4.22.9) from commercial baker's yeast and from wild type strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis	3-Nitro-Tyr41-proteinase B inhibitor from yeast	Activated CH-Sepharose 4B	KOMINAMI et al. (1981).
Proteolytic enzymes from wheat leaves	Hemoglobin	Sepharose 4B	FRITH et al. (1978).
Pteroyl oligo-γ-L-glutamyl endopeptidase from chick intestine	Synthetic γ-oligoglutamyl peptides	Sepharose 4B	SAINI and ROSENBERG (1974).
Renin (angiotensin I-forming enzyme - EC 3.4.99.19) from human kidney	Hemoglobin and N-hydroxysuccinyl-pepstatin	Sepharose 4B or Bio-Gel A-5m and Sepharose with hexamethylenediamine	YOKOSAWA et al. (1980).
Renin (aspartic proteinase EC 3.4.23.15) from human kidney	Concanavalin A and inhibitor ES-305: bis[(1-naphthyl)methyl] acetylhistidylstatine 2(S)-methylbutylamide	Con A-Sepharose and Epoxy-acivated Sepharose 6B	TAKADA et al. (1987).
Renin (EC 3.99.19) (aspartyl protease) from human kidney cortex	Aminohexyl pepstatin and synthetic octapeptide renin inhibitor H-77 with reduced peptide bond between Leu(5)-Leu(6)	Activated CH-Sepharose	SHINAGAWA et al. (1986).
Renin (EC 3.4.23.15) from human kidney	Pepstatin and (D-leu6)octapeptide or antibody against renin	AH-Sepharose and CNBr-activated Sepharose 4B	SLATER and STROUT (1981).
Renin and cathepsin D from rat kidney	N-Hydroxysuccinyl-pepstatin and immunoglobulins G against rat spleen cathepsin D and rat kidney renin (removed contamination)	Sepharose with hexamethylenediamine and Sepharose	FIGUEIREDO et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Serine proteinase (I) from Escherichia coli	Bacitracin	Sepharose 4B	SHAGINYAN et al. (1980).
Streptomyces griseus trypsin from Pronase	p-Aminobenzamidine	Sepharose CL-4B with N-chloroacetylglycylglycine or N-chloroacetyl-6-aminohexanoic acid	SHIMURA and KASAI (1984).
Subtilisin DY from culture medium of Bacillus subtilis	Bacitracin (cyclic nonapeptide)	Bead cellulose activated with 2,4,6-trichlorotriazine	TURKOVÁ et al. (1987).
Sulfhydryl-protease from beans	Mercury derivative of methacrylanilide (Hg-MAA)	Hydroxyalkyl methacrylate gel with 15% MAA	TURKOVÁ et al. (1975).
Thermolysin	Phosphoramidone [N-(α-L--rhamnopyranosyloxyhydroxyphosphinyl)-L--leucyl-L-tryptophan]	AH-Sepharose 4B	KOMIYAMA et al. (1975).
Thermolysin	Glycyl-D-phenylalanine	Sepharose 4B with triethylenetetramine	WALSH et al. (1974).
Thermostable neutral protease from Bacillus stearothermophilus	Chloroacetyl-D-phenylalanine	Sepharose 4B with triethylenetetramine	SIDLER and ZUBER (1980).
Thiolsubtilisin (active site ser of subtilisin converted to cys)	p-Chloromercuribenzoate	Affi-Gel 501	PHILIP et al. (1979).
Thrombin	L-Arginin methyl ester	Polystyrenes grafted with sulphonate groups	FISCHER et al. (1986).
Thrombin (human)	N-t-Butyl-N'-phenylpropenamide	p-N-Hydroxyethylsulfamoyl polystyrene from polystyrene Biobeads S-X2	KHAMLICHI et al. (1988).
Thrombin (serine proteinase; EC 3.4.21.5) from human blood serum	L-Lysine; L-arginine; gramicidin S; or bacitracin	γ-Chloropropyl- or γ-glycidohydroxypropyl-derivatives of Silochrom S-80	GAIDA et al. (1988).
Thrombin from activated human prothrombin complex concentrate	L-Arginyl methyl ester	Chlorosulphonated polystyrene	YU et al. (1986b).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Tonin (selective serine endopeptidase)	Antibody against tonin	CNBr-activated Sepharose 4B	IKEDA et al. (1981).
Tryase (serine proteinase) from rat liver	Soya-bean trypsin inhibitor (Kunitz)	CNBr-activated Sepharose 4B	SAKLATVALA et al. (1981).
Trypsin	Soybean trypsin inhibitor	Toyopearl HW-55 with epichlorohydrin; concentrated ammonia solution and succinic anhydride	MATSUMOTO et al. (1982).
Trypsin	Casein	Casein precipitated with trichloroacetic acid	ŠAFAŘÍK (1984).
Trypsin (bovine)	p-Aminobenzamidine or soya bean inhibitor	Sepharose CL-6B; Fractogel HW-65; Trisacryl GF-2000; LiChrospher Si 500 diol I or II	HEARN (1986).
Trypsin (EC 3.4.21.4)	p-Aminobenzamidine	Spheron with 1,6-diamino hexane	TURKOVÁ and SEIFERTOVÁ (1978).
Trypsin (EC 3.4.21.4)	Ovomucoid	Spheron	TURKOVÁ and SEIFERTOVÁ (1978).
Trypsin (EC 3.4.21.4) bovine and from Streptomyces griseus (Pronase E)	L-Argininal semicarbazone (unmasked with HCl/formalin)	Sepharose 4B after CNBr-activation with glycylglycine and N-hydroxysuccinimide	NISHIKATA et al. (1981).
Trypsin from bovine crude preparation	Soybean trypsin inhibitor	Sepharose CL-6B activated with 1,1'-carbonyldiimidazole	HEARN et al. (1979).
Trypsin-family proteases	Leucyl-argininal	Sepharose 6B with 1,4-butanediol-diglycidyl ether or Sepharose 4B with 6-aminohexanoic acid or with glycylglycine	ISHII and KASAI (1981).
Trypsin-like protease from Streptomyces griseus (Pronase)	Double-headed inhibitor from Phaseolus vulgaris	AH-Sepharose	MOSOLOV et al. (1978).
Tryptic serine protease tryptase from dog mastocytoma cells	Benzamidine	Benzamidine-Sepharose	CAUGHEY et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Urokinase (proteolytic enzyme EC 3.4.21.31)	Pyroglutamyl-lysyl-leucyl-argininal	CH-Sepharose 4B (carbodiimide technique)	SOMENO et al. (1985).
Urokinase (EC 3.4.21.31)	Monoclonal antibodies against urokinase	Affi-Gel 10	HERION and BOLLEN (1983).
Urokinase-related proteins from human epidermoid carcinoma	Immunoglobulin G against urokinase (purified by Protein A-Sepharose and urokinase-Sepharose)	CNBr-activated Sepharose CL-4B	WUN et al. (1982).
Protocatechuate 3,4-dioxygenase from Pseudomonas fluorescens	Protocatechuate	AH-Sepharose 4B (carbodiimide technique)	PUJAR and RIBBONS (1983).
Pullulanase (EC 3.2.1.41) from Aerobacter aerogenes	CNBr-activated Schardinger α-dextran	AH-Sepharose 4B	ENEVOLDSEN et al. (1977).
Pyridine dinucleotide transhydrogenase (EC 1.6.1.1.) from bovine heart mitochondria	Nicotinamide adenine dinucleotide	Agarose-hexane-NAD (AGNAD; type 1)	WU et al. (1982).
Pyridine nucleotide transhydrogenase from Azotobacter vinelandii	N^6-(6-Aminohexyl) adenosine 2',5'-bisphosphate	2'5'ADP-Sepharose 4B	VOORDOUW et al. (1980).
Reductases:			
Adrenodoxin reductase (EC 1.6.7.1) from bovine adrenal cortex mitochondria	N^6-(6-Aminohexyl)-adenosine-2',5'-bisphosphate	2',5'-ADP-Sepharose 4B	MONTELIUS et al. (1979).
Aldehyde reductase (EC 1.1.1.2) from rat liver	$NADP^+$,N^6-(6-aminohexyl)adenosine 2',5'-bisphosphate and Procion Red HE3B	Sepharose 4B with adipic acid dihydrazide; 2'5'ADP-Sepharose 4B and Sepharose 4B	TURNER and HRYSZKO (1980).
Aldose reductase (EC 1.1.1.21) from Pachysolen tannophilus	β-NAD (attached through C-8 and through ribose hydroxyls)	β-NAD-agarose (N 1008 and N 6130)	BOLEN et al. (1986).
Aldose reductase (EC 1.1.1.21) from bovine lens	Procion Red HE3B	Matrex Gel Red A	INAGAKI et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cytochrome c reductase	Cytochrome c from Saccharomyces cerevisiae	Activated thiol-Sepharose 4B	BILL et al. (1982).
Cytochrome-b5 reductase	Detergent-solubilized cytochrome b5	CNBr-activated Sepharose 4B	KAWATA et al. (1986).
δ1-Pyrroline-5-carboxylate reductase (EC 1.5.1.2) from baker's yeast	AMP	5'AMP-Sepharose 4B	MATSUZAWA and ISHIGURO (1980).
Dihydrofolate reductase (EC 1.5.1.3) from rat liver	Anti-(yeast glucose-6-phosphate dehydrogenase) IgG	CNBr-activated Sepharose 4B	STAPLETON and PORTER (1985).
Dihydrofolate reductase (EC 1.5.1.3) from soybean seedlings	Methotrexate and Cibacron Blue F3GA	AH-Sepharose 4B (carbodiimide technique) and Blue Sepharose CL-6B	RATNAM et al. (1987).
Dihydrofolate reductase (EC 1.5.1.4) from Walker 256 carcinoma	Cibacron Blue F3GA	Agarose 4B	JOHNSON et al. (1980).
Dihydropterin reductase from beef kidney	Cibacron Blue F3GA in presence of tetrahydropterin	Blue Sepharose CL-6B	CHAUVIN et al. (1979).
Dihydropteridine reductase (EC 1.6.99.7) from bovine liver and adrenal medula	5'-AMP	5'-AMP-Sepharose 4B	AKSNES et al. (1979).
Expressed P-450 reductase from Escherichia coli proteins	N^6-(6-Aminohexyl)adenosine 2',5'-biphosphate	2',5'ADP-Sepharose	PORTER et al. (1987).
Ferredoxin-thioredoxin reductase from spinach and corn leaves	Ferredoxin	CNBr-activated Sepharose 4B	DROUX et al. (1987).
Glutathione reductase (EC 1.6.4.2) from Escherichia coli	N^6-(6-Aminohexyl)adenosine 2',5'-bisphosphate and 8-(6-aminohexyl)-aminophospho-adenosine diphosphoribose	N^6-2',5'-ADP-Sepharose and CNBr-activated Sepharose 4B	MATA et al. (1984).
Glutathione reductase (EC 1.6.4.2) from Euglena gracilis z	Cibacron Blue F3GA	Blue Sepharose	SHIGEOKA et al. (1987).
Glutathione reductase from calf liver	N^6-(6-Aminohexyl)adenosine 2',5'-bisphosphate	2',5'-ADP-Sepharose 4B	CARLBERG and MANNERVIK (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Menadione reductase (EC 1.6.99.2) from rat liver	4-(p-Iminomethylaniline)-5-methoxy-1,2-benzoquinone	CNBr-activated Sepharose 4B	TITOVETS (1982).
NAD(P)H-nitrate reductase (EC 1.6.6.2) from Ankistrodesmus braunii	Blue dextran 2 and β-nicotinamide adenine dinucleotide	Blue dextran 2-Sepharose CL-6B and NAD-Agaroses (N-1008; N-6130; N-9505)	MARQUEZ et al. (1982).
NADH-cytochrome b5 reductase from microsomes of bovine liver	(N^6-Hexane)-adenosine 5'-diphosphate	ADP-agarose (AGADP; Type 2)	SCHAFER and HULTQUIST (1980).
NADH: Hydroxypyruvate reductase from unicellular green algae Chlamydomonas reinhardtii	Cibacron Blue F3GA	Affi-Gel Blue	HUSIC and TOLBERT (1987).
NADH:Nitrate reductase (EC 1.6.6.1) from barley roots	Blue dextran	CNBr-activated Sepharose 4B	OJI et al. (1982).
NADPH Cytochrome P-450 reductase from human liver	N^6-(6-Aminohexyl)adenosine 2',5'-bis-phosphate	2',5'ADP agarose	SCHWARTZMAN et al. (1987).
NADPH-adrenodoxin reductase from bovine adrenocortical mitochondria	Bovine adrenodoxin	Sepharose 6B after CNBr-activation	SUGIYAMA and YAMANO (1975).
NADPH-cytochrome c (P-450) reductase (EC 1.6.2.4) from house flies	Nicotinamide adenine dinucleotide phosphate (NADP)	Agarose-hexane-NADP	MAYER and DURRANT (1979).
NADPH-Cytochrome P-450 reductase from rat liver	NADP after periodate oxidation	Sepharose 4B with adipic acid dihydrazide	DIGNAM and STROBEL (1977).
NADPH-Cytochrome P-450 reductase (EC 1.6.2.4) from rabbit liver microsomes	N^6-(6-Aminohexyl)-adenosine 2',5'-bisphosphate	Sepharose 4B after CNBr-activation	FRENCH and COON (1979).
Nitrate reductase from Chlorella vulgaris	Cibacron Blue F3GA	Blue Sepharose CL-6B	RAMADOSS et al. (1983).
Nitrate reductase (EC 1.6.6.2) from Ankistrodesmus braunii	Blue Dextran	Sepharose 4B	DE LA ROSA et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Nitrite reductase (EC 1.7.7.1) from spinach leaves	Ferredoxin	Sepharose 4B	IDA (1977).
Phage-T4-induced ribonucleotide reductase	p-Aminophenyl ester of dATP	Sepharose 4B	BERGLUND and ECKSTEIN (1972).
Respiratory nitrate reductase (EC 1.7.99.4) from Klebsiella aerogenes (study of vectorial orientation)	Antibodies against nitrate reductase	Protein A-Sepharose CL-4B	VAN'T RIET et al. (1981).
Ribonucleotide reductase (EC 1.17.4.1) from Escherichia coli	p-Aminophenyl esters of γ-phosphate of dTTP or dATP	Sepharose 4B	VON DOBELN (1977).
Thioredoxin reductase (EC 1.6.4.5) from rabbit liver (only after removing of low-molecular-weight metabolites from lysates)	N^6-(6-Aminohexyl)adenosine 2,5'-bisphosphate	2',5'ADP-Sepharose	HUNT et al. (1983).
UDP-N-Acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158) from Escherichia coli	NADP (hexamethylenediamine attached at adenosine C8)	AGNADP TYPE 3	ANWAR and VLAOVIC (1979).
Reverse transcriptase of Rauscher murine leukemia virus	Poly(U)	Poly(U)-Sepharose 4B	SARNGADHARAN et al. (1980).
Ribonucleic acid-dependent nucleoside triphosphate phosphohydrolases (ATPases) - from rat liver nuclei	Heparin	Heparin-Ultrogel	BLANCHARD and RICHARDSON (1983).
Ribulose 1,5-bisphosphate carboxylase/oxygenase from Rhodopseudomonas sphaeroides	Dye Green A	Matrex Gel Green A	JOUANNEAU and TABITA (1987).
Salicylate hydroxylase (EC 1.14.13.1) from Pseudomonas	p-Aminosalicylate	Activated CH-Sepharose 4B	YOU and ROE (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Sialidase (EC 3.2.1.18) from human liver	Equine submandibular gland mucin	Sepharose 4B	MICHALSKI et al. (1982).
Staphylocoagulase	Bovine prothrombin	CNBr-activated Sepharose 4B	IGARASHI et al. (1979).
Steroid alcohol sulphotransferase (EC 2.8.2.-) from human adrenal tissue	Dehydroepiandrosterone-17-(O-carboxymethyl)oxime	AH-Sepharose 4B	ADAMS and MC DONALD (1979).
Synthases and synthetases:			
(2'-5')An synthetase [(2'-5')An = oligonucleotides consisting of 5'-adenylic acid residues joined by 2'-5'phosphodiester linkage] from rabbit reticulocyte lysates	Polyriboinosinic acid polyribocytidylic acid [Poly(rI).poly(rC)] and heparin	Poly(rI),poly(rC)-agarose and Heparin-agarose	WU and ESLAMI (1983).
Adenylosuccinate synthetase (EC 6.3.4.4) from tumor cells	Hadacidin	Sepharose 4B with 1,6-diaminohexane	MATSUDA et al. (1980).
Aminoacyl-tRNA synthetase complexes (EC 6.1.1.-) specific for isoleucine, leucine,methionine,lysine,arginine,glutamic acid; and glutathione - from sheep liver and spleen and from rabbit reticulocytes and liver	Heparin and unfractionated Escherichia coli tRNA or brewers' yeast tRNA after periodate oxidation	Heparin-Ultrogel A4R and Sepharose 4B after CNBr-activation with hydrazine (after coupling reduction with natrium borohydride)	KELLERMANN et al. (1982).
Aminoacyl-tRNA synthetases (EC 6.1.1.-) from yellow lupin seeds	Heparin	Heparin-Sepharose CL-6B	JOACHIMIAK et al. (1981).
Aminoacyl-tRNA synthetase	AMP	Agarose-hexyl-adenosine-5'-phosphate	FROMANT et al. (1981).
Arginyl-tRNA synthetase and aspartyl-tRNA synthetase from baker's yeast	Blue - Dextran	CNBr-activated Sepharose 4B	DROCOURT et al. (1980).
Asparagine synthetase (EC 6.3.1.1) from rat liver	Cibacron Blue F3GA	Reactive Blue 2-Agarose	HONGO and SATO (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Carnosine synthetase (EC 6.3.2.11) from avian muscle	Cibacron Blue F3GA	Reactive Blue 2-agarose	WOOD and JOHNSON (1981).
Citrate synthase (EC 4.1.3.7) from pig heart	1-N^6-Etheno-Coenzyme A-SH	Sepharose 4B after CNBr-activation with ε-aminocaproic acid (carbodiimide technique)	LIMBACH and SCHMIDT (1984).
Citrate synthase (EC 4.1.3.7) and succinic thiokinase (EC 6.2.1.4)	N^6-[N-(6-Aminohexyl)carbamoylmethyl]-S-(5-thio-2-nitrobenzoic acid)-Con A	Sepharose 4B	RIEKE et al. (1979).
Estrogen synthetase (= cytochrome P-450ES or aromatase) from human placenta microsomes	Testosterone-17-O-hemisuccinate	AH-Sepharose 4B (carbodiimide technique)	HAGERMAN (1987).
Farnesylpyrophosphate synthetase (EC 2.5.1.1) from chicken and pig livers and yeast	O-(6-Amino-1-hexyl)-P-geranylmethyl phosphonophosphatate	Affi-Gel 10	BARTLETT et al. (1985).
Fatty acid synthetase from Mycobacterium smegmatis	$NADP^+$	$NADP^+$-agarose (AGNADP,type 4)	WOOD et al. (1978).
γ-Glutamylcysteine synthetase (probe for active site)	Cystamine	Activated CH-Sepharose 4B	SEELING and MEISTER (1982).
Glutamate synthase (EC 2.6.1.53) from Escherichia coli	N^6-(6-Aminohexyl)adenosine 2',5'-bisphosphate	2',5'-ADP-Sepharose	SCHMIDT and JERVIS (1980).
Glutamine synthetase (EC 6.3.1.2) from Rhodospirillum rubrum	ADP	ADP-hexane-agarose	SOLIMAN et al. (1981).
Glutamine synthetase (EC 6.3.1.2) with adenylylated subunits	Monoclonal antibodies (anti-AMP)	Affi-Gel 10	CHUNG and RHEE (1984).
Glycogen synthase from rat liver	Glucosamine-6-P	Affi-Gel 10	HUANG et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Gramicidin S synthetase from Bacillus brevis	Ornithine	Sepharose 4B after CNBr-activation with 3,3-diaminodipropylamine (carbodiimide technique)	HORI et al. (1978).
Guanosine monophosphate (GMP) synthetase (EC 6.3.5.2) from Yoshida sarcoma ascites cells	Xanthosine monophosphate (XMP) with ribose moiety after periodate oxidation	AH-Sepharose 4B (after coupling with $NaBH_4$)	HIRAI et al. (1987).
Histidyl-tRNA synthetase (EC 6.1.1.21) from Neurospora crassa	Cibacron Blue F3GA and single-stranded DNA	Affi-Gel Blue and 4% agarose	CHEN and SOMBERG (1980).
Lactose synthetase from human milk	α-Lactalbumin	Sepharose 6B	ANDREWS (1970).
Leucyl-tRNA and lysyl-tRNA synthetase (EC 6.1.1.-) - from sheep liver	tRNA from Escherichia coli after periodate oxidation (after coupling reduction with natriumborohydride)	Sepharose 4B after CNBr-activation with hydrazine	CIRAKOGLU and WALLER (1985b).
Lysyl-tRNA synthetases (EC 6.1.1.-) - from Saccharomyces cerevisiae	Heparin and tRNA from Escherichia coli after periodate oxidation	Heparin-Ultrogel A4R and Sepharose 4B after CNBr-activation with hydrazine (after coupling reduction with natrium borohydride)	CIRAKOGLU and WALLER (1985a).
Methionyl-tRNA synthetase from yellow lupin seeds	Spermine	CNBr-activated Sepharose 4B	JOACHIMIAK et al. (1979).
myo-Inositol-1-phosphate synthase (EC 5.5.1.4) from Lemna gibba	NAD^+	Sepharose 4B with 6-aminohexanoic acid	OGUNYEMI et al. (1978).
Phenylalanyl-transfer RNA synthetase from soybean cotyledons	tRNA after periodate oxidation	CNBr-activated Sepharose 4B with hydrazine	SWAMY and PILLAY (1979).
Phenylalanyl-tRNA synthetase (EC 6.1.1.20) from Thermus thermophilus HB8	Heparin	Heparin-Sepharose	ANKILOVA et al. (1988).
Phosphatidylinositol synthase (EC 2.7.8.11) from rat brain	Oxidized derivative of CDP-diacylglycerol	Sepharose 4B with adipic acid dihydrazide	GHALAYINI and EICHBERG (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Porphobilinogen (PBG) synthase (EC 4.2.1.24) from bovine liver	Subunits of octameric PBG synthase	CNBr-activated Sepharose 4B	GURNE et al. (1977).
Prostacyclin (PGI2) synthase from bovine aorta	Monoclonal antibodies against PGI2 synthase	Affi-Gel 10	DE WITT and SMITH (1983).
Seryl-tRNA synthetase from rat liver	Seryl-tRNA	Sepharose 4B	BEFORT et al. (1974).
Spermide synthase from rat prostate	S-Adenosyl(5')-3-thiopropylamine	AH-Sepharose 4B with O-bromoacetyl-N-hydroxysuccinimide (carbodiimide technique)	SAMEJIMA and YAMANOHA (1982).
Spermine synthase from bovine brain	Spermine	CH-Sepharose 4B	PAJULA et al. (1978).
Succinyl-CoA synthetase from rat liver	Periodate-oxidized guanosine 5'-diphosphate	Sepharose 4B with adipic acid dihydrazide	BALL and NISHIMURA (1980).
Thromboxane synthase from porcine lung	Cibacron Blue 3GA and monoclonal antibodies against thromboxane synthase (TS2) from ascites fluid (purified by Protein A-Sepharose CL-4B)	Blue 2-agarose and Affi-Gel 10	SHEN and TAI (1986).
Thymidylate synthase (EC 2.1.1.45) from Escherichia coli; calf thymus and experimental tumors	Tetrahydromethotrexate	Sepharose 4B with ethylenediamine	SLAVÍK and SLAVÍKOVÁ (1980).
Thymidylate synthase (EC 2.1.1.45) from human colon adenocarcinoma	Cibacron Blue F3GA	Affi-Gel Blue	RADPARVAR et al. (1988).
Thymidylate synthase (EC 2.1.1.45) from mouse and calf thymus glands	10-Formyl-5,8-dideazafolic acid	CNBr-activated Sepharose 4B with ethylenediamine (carbodiimide technique)	RODE et al. (1986).
Trehalose phosphate synthetase from Mycobacterium smegmatis	Heparin	Sepharose 4B	ELBEIN and MITCHELL (1975).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Tryptophan synthase (EC 4.2.1.20) from Escherichia coli	N-Aminohexanoyl-L-tryptophanol phosphate	Sepharose 6B with divinyl sulfone; adipic dihydrazide and terephthaloyl bis(N-hydroxysuccinimide) ester	GSCHWIND et al. (1979).
Tryptophanyl-tRNA synthetase from Escherichia coli	Blue-dextran	CNBr-activated Sepharose 4B	DROCOURT et al. (1979).
Tyrosyl-tRNA synthetase from baker's yeast	Tyrosine	Copolymer of malic acid anhydride and butanediol divinyl ether with 1,6-diaminohexane	BEIKIRCH et al. (1974).
Uridine-5'-monophosphate synthase from Ehrlich ascites carcinoma	5-[2-[N-(2-Aminoethyl)carbamyl]-ethyl]-6-azauridine 5'-monophosphate	CM Bio-Gel A with N-hydroxysuccinimide and dicyclohexylcarbodiimide	MC CLARD et al. (1980).
Uroporphyrinogen I synthase (EC 4.3.1.8) from Chorella regularis	Porphobilinogen	AH-Sepharose 4B	SHIOI et al. (1980).
Valyl-tRNA synthetase from Euglena gracilis cytoplasma	Blue-Dextran	Sepharose 4B	SARANTOGLOU et al. (1980).
Testicular hyaluronidase (EC 3.2.1.35)	Dermatan sulphate	AH-Sepharose	LYON and PHELPS (1981).
Threonine dehydratase (EC 4.2.1.16) from Salmonella typhimurium	N^6-(6-Aminohexyl)-adenosine 5'-monophosphate	5'AMP-Sepharose 4B	BHADRA and DATTA (1978).
Thyroid peroxidase from calf thyroid glands	Glycyl-leucyl-tyrosine	Sepharose 4B	YAMAMOTO and DEGROOT (1982).
Toluene dioxygenase from Pseudomonas putida	p-Toluic acid	AH-Sepharose 4B	SUBRAMANIAN et al. (1979).
Topoisomerase II (DNA gyrase) - from crude extracts of Escherichia coli	Novobiocin (drug; potent inhibitor of DNA replication in Escherichia coli)	Epoxy-activated Sepharose 6B	STAUDENBAUER and ORR (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Transferases:			
Acetyltransferase from Escherichia coli (involved in gentamicin inactivation)	Getamicin C1 or kanamicin A	Indubiose 4A	LE GOFFIC and MOREAU (1973).
Adenine phosphoribosyltransferase (EC 2.4.2.7) from normal human erythrocytes	Oxidized GMP (with sodium periodate and after coupling treated with $NaBH_4$)	CNBr-activated Sepharose 4B with 3,3'-iminobispropylamine	HOLDEN et al. (1979).
Adenosine diphosphoribosyltransferase (EC 2.4.2.30) from calf thymus	3-Aminobenzamidine	AH-Sepharose 4B (carbodiimide technique)	BUKI et al. (1987).
ADP-ribosyltransferase (EC 2.4.2.30) from human placenta	4-[[3-(Aminocarbonyl)phenyl]amino]-4-oxobutanoic acid	Sepharose 4B with 3,3'-iminodipropaneamine	BURTSCHER et al. (1986).
Alanine aminotransferase (EC 2.6.1.2) from porcine heart	Copper(II) salt of L-lysine treated by EDTA	CNBr-activated Sepharose 4B	MASUDA et al. (1986).
α-N-Acetylgalactosaminide $\alpha 2 \Rightarrow 6$ sialyltransferase (EC 2.4.99.1) from porcine submaxillary glands	Cytidine 5'-diphosphate-hexanolamine	Sepharose 4B after CNBr-activation	SADLER et al. (1979).
Anthranilate phosphoribosyltransferase	Anthranilic acid	Sepharose 2B with hexamethylenediamine and succinic anhydride	MARCUS (1974).
Arginyl-tRNA:protein arginyltransferase (EC 2.3.2.8)	1. Heparin 2. Angiotensin II	1. Hydrazine-derivatized Sepharose 4B 2. p-Aminobenzamidoalkyl Sepharose 4B	KATO and NOZAWA (1984).
Arylamine N-acetyltransferase (EC 2.3.1.5) - from chicken liver	Monoclonal antibody 78 against N-acetyltransferase from chicken liver	CNBr-activated Sepharose 4B	DEGUCHI et al. (1988).
Aspartate aminotransferase from chicken hearts	Pyromellitic acid and Cibacron Blue F3GA	Sepharose 4B with diaminopropanol and Blue-Sepharose	VANSTEELANDT et al. (1981).
Aspartate aminotransferase (EC 1.1.3.7) from rat liver	Aspartatic acid	CH-Sepharose 4B	CREMEL et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
β 1,3-Galactosyltranferase from swine trachea mucosa	Asialo Cowper's gland mucin	Sepharose 4B after CNBr-activation	MENDICINO et al. (1982).
β-Galactosyltransferase from Ehrlich ascites tumor cells and bovine milk	N-Acetyl-D-glucosamine (GlcNAc)	β-GlcNAc-Synsorb beads	ELICES and GOLDSTEIN (1987).
Catechol-O-methyltransferase (EC 2.1.1.6) from rat heart and brain	3,4-Dimethoxy-5-hydroxyphenylethylamine	Sepharose 4B after CNBr-activation with twice diaminopropylamine and succinic anhydride (carbodiimide technique)	BORCHARDT and CHENG (1978).
Chloramphenicol acetyltransferase (EC 2.3.1.28) from Escherichia coli	Chloramphenicol	CNBr-activated Sepharose 4B with 6-aminohexanoic acid (carbodiimide technique)	PACKMAN and SHAW (1981).
Choline acetyltransferase (EC 2.3.1.6) from rat and cow brain	Coenzyme A	Sepharose after CNBr-activation	RYAN and MC CLURE (1979).
Choline acetyltransferase (EC 2.3.1.6)	N-(10-Carboxy)-decamethylene-4-(1-naphthylvinyl)-pyridinium chloride	Sepharose 4B-200 with 3,3'-diaminodipropylamine	COZZARI and HARTMAN (1983).
Cholinephosphate cytidyltransferase (EC 2.7.7.15) from rat liver	Glycerolphosphorylcholine	Epoxy-activated Sepharose 6B	CHOY and VANCE (1976).
Collagen glucosyltransferase from chick embryos	Citrate-soluble rat skin collagen and UDP glucuronic acid	Sepharose 4B after CNBr-activation and AH-Sepharose 4B	MYLLYLA et al. (1977).
Collagen galactosyltransferase from chick embryos	Concanavalin A or citrate-soluble rat skin collagen	Con A-Sepharose 4B or Sepharose 4B after CNBr-activation	RISTELI (1978).
Cyclodextrin glucosyltransferase (EC 2.4.1.19) from Bacillus macerans	Physically modified starch	Maize starch gelatinized; frozen at -20 °C; kept at this temperature for 24 hours and then allowed to thaw	GOTTVALDOVÁ et al. (1988).
DNA Methyltransferase from HeLA cells (determination of DNA methylation reaction)	DNA or double-stranded DNA from Micrococcus luteus	Cellulose or Epoxy-activated Sepharose 6B	HUBSCHER et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Furanocoumarin O-methyltransferases from Ruta graveolens	S-Adenosyl-L-homocysteine	AH-Sepharose 4B	SHARMA and BROWN (1979).
Galactosyl phosphate transferase from Salmonella anatum	N1-(Uridine-5'-phosphoryl)- or N1-(uridine-5'-pyrophosphoryl)-1,6-diaminohexanes	CNBr-activated Sepharose 4B	KUSOV et al. (1979).
Galactosyltransferase (EC 2.4.1.22) from Golgi membranes of lactating sheep mammary gland	UDP-hexanolamine and α-lactalbumin	Sepharose 4B after CNBr-activation	SMITH and BREW (1977).
Galactosyltransferase from human serum	p-Aminophenyl-N-acetyl-β-D-glucosamine	CNBr-activated Sepharose	BERGER et al. (1976).
γ-Glutamyl transferase (EC 2.3.2.2) from ascites hepatoma AH-66 cells and human primary hepatoma	Phaseolus vulgaris erythroagglutinating lectin	E-PHA agarose	TANIGUCHI et al. (1985).
γ-Glutamyltransferase (EC 2.3.2.2)	Phenyl boronate	Phenyl boronate MATREX PBA-60	COOK and PETERS (1985).
GDP-Fucose:GM1 α1⇒2 fucosyltransferase (chemical carcinogen-induced enzyme) from rat liver and hepatoma cells	Guanosinediphosphate (GDP)-hexanolamine	Sepharose after CNBr-activation	HOLMES and HAKOMORI (1987).
Glucosyltransferases (EC 2.4.1.-) from Candida bogoriensis	13-Hydroxydocosanoic acid; 13-sophorosyloxydocosanoic acid or 13-sophorosyloxydocosanoic acid-6',6"-diacetate	ω-Aminohexylagarose	BREITHAUPT and LIGHT (1982).
Glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14) from Bacillus subtilis	N^6-(aminohexyl)-ATP	ATP-agarose AGATP type II	WONG and SWITZER (1979).
Glutathione S-transferases A and C from rat liver cytosol	Cholic acid	AH-Sepharose 4B	PATTINSON (1981b).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glutathione S-transferases from bovine liver	Orange A	Matrex Gel Orange A	ASAOKA (1984).
Glutathione S-transferases (EC 2.5.1.18) from rat liver	Reduced glutathione	Chloroacetyl derivative of Sepharose CL-4B with lysine or aliphatic diamine of various carbon chain lengths	INOUE et al. (1981).
Glutathione S-transferase K from rat liver	S-Hexylglutathione and glutathione	Epoxy-activated Sepharose 6B	HAYES (1986).
Glutathione transferase (EC 2.5.1.18) from rat liver microsomes	Reduced glutathione	Epoxy-activated Sepharose 6B	DIERICKX (1986).
Glycoprotein sialyltransferase (EC 2.4.99.1) from human liver	Cytidine diphosphate-hexanolamine	CNBr-activated Sepharose 4B	ALHADEFF and HOLZINGER (1982).
Human strand transferase (catalyzes ATP-dependent homologous pairing and strand exchange of duplex linear DNA and single - stranded circular DNA) - from human tumor (leukemic T-lymphoblast) cell line	Z-DNA = left-handed conformation of DNA double helix containing alternating purine and pyridine residues (brominated ^{3}H-labeled poly(dG-dC))	Agarose column matrix with coupling via biotin-avidin	FISHEL et al. (1988).
Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) from Saccharomyces cerevisiae	Guanosine monophosphate (GMP) after periodate oxidation (after coupling adding of solid $NaBH_4$)	AH-Sepharose	SCHMIDT et al. (1979).
Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) from normal human erythrocytes	Guanosine 5'monophosphate oxidized with sodium periodate (after coupling treated with $NaBH_4$)	CNBr-activated Sepharose with 3,3'-iminobispropylamine	HOLDEN and KELLEY (1978).
Indoethylamine N-methyltransferases from Phalaris tuberosa	S-Adenosylhomocysteine	Agarose A-0.5m or Sepharose 4B with diaminodipropylamine	MACK and SLAYTOR (1978).
L-Lysine 6-aminotransferase (EC 2.6.1.36) - from Flavobacterium lutescence	L-Lysylacetamidododecyl	Sepharose 6B after CNBr-activation with α,ω-diaminoalkane and with O-bromoacetyl-N-hydroysuccinimide	YAGI et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
L-Phosphoserine aminotransferase (EC 2.6.1.52) from bovine liver	Cibacron Blue F3GA	Affi-Gel Blue	LUND et al. (1987).
Lecithin:cholesterol acyltransferase from human plasma	Plasma lipoproteins	Sepharose 4B with dodecylamine	LACKO and CHEN (1977).
mRNA Guanylyltransferase from Hela cell nuclei	Cibacron Blue F3GA dye and guanosine 5'-triphosphate after periodate oxidation	Affi-Gel Blue and GTP coupled to agarose with adipic acid dihydrazide	VENKATESAN et.al. (1980).
NAD^+ chemical analogues containing 2'-deoxyribose or 3'-deoxyribose (synthesized enzymatically with oxidized nicotinamide mononucletide (NMN^+)-adenyl transferase EC 2.7.7.18)	m-Aminophenylboronic acid	Affi-Gel 601	ALVAREZ-GONZALES (1988).
Nucleoside phosphotransferase from barley	Deoxyadenosine-3'-(4-aminophenylphosphate)	Sepharose CL-6B with carbonylimidazole; ethylenediamine and succinic anhydride (carbodiimide technique)	IKEDA et al. (1984).
Oligosaccharyltransferase solubilized from hen oviduct membranes	Tetradecapeptide of Asn-X-Ser/Thr sequences (Thr-His-Thr-Asn-Ile-Ser-Glu-Ser-His-Pro-Asn-Ala-Thr-Phe)	CNBr-activated Sepharose 4B	AUBERT et al. (1982).
Ornithine aminotransferase (OAT; EC 2.6.1.13) from human liver	Antibodies against rat kidney OAT from rabbits (purified with rat kidney OAT immobilized to CNBr-activated Sepharose 4B)	CNBr-activated Sepharose 4B	LEAH et al. (1988).
Orotate phosphoribosyltransferase from Escherichia coli K-12	Orotidine-5'-monophosphate after periodate oxidation	Sepharose 4B after CNBr-activation with adipic acid dihydrazide	DODIN (1981).
S-Adenosyl-L-methionine:O-diphenol-O-methyl-transferases (EC 2.1.1.6) - from tobacco plants Nicotiana tabacum L.	Adenosine	Adenosine-agarose (adenosine Type 3)	DUMAS et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
S-Adenosylmethionine:5- and 8-hydroxyfuranocoumarin O-methyltransferases from Ruta graveolens	5-(3-Carboxypropanamido)xanthotoxin	AH-Sepharose 4B (carbodiimide technique)	SHARMA et al. (1979).
Serine acetyltransferase (EC 2.3.1.30) from Salmonella typhimurium	Immunoglobulin G against O-acetylserine sulfhydrylase (EC 4.2.99.8) from rabbits (purified by Protein A-Sepharose CL-4B)	Sepharose 4B after CNBr-activation	BAECKER and WEDDING (1980).
Serine hydroxymethyltransferase (EC 2.1.2.1) from rat liver	Copper(II)salt of L-lysine after elution of Cu(II)ions with EDTA	CNBr-activated Sepharose 4B	MASUDA et al. (1987).
Sialyltransferase from human teratocarcinoma cells	Heparin	Heparin-Sepharose	SCHWARTING et al. (1987).
Terminal deoxynucleotidyltransferase from calf thymus	Monoclonal antibody against terminal transferase with N-hydroxysuccinimidobiotin	Controlled pore glass CPG/460 Glycophase G after periiodate oxidation with avidin (+ $NaBH_4$)	FULLER et al. (1985).
tRNA Methyltransferase (EC 2.1.1)	tRNA (immobilized via their oxidized 3'-termini)	Aminohydroxybutylcellulose	GAMBARYAN et al. (1980).
Tyrosine aminotransferase (EC 2.6.1.5) from rat liver	Pyridoxamine-5'-phosphoric acid	AH-Sepharose 4B with succinic anhydride (carbodiimide technique)	DONNER et al. (1978).
UDP-Acetylgalactosamine-protein N-acetylgalactosamine transferase (membrane-bound,EC 2.4.1.41) from ascites hepatoma AH66	Apomucin (deglycosylated bovine submaxillary mucin)	Sepharose 4B after CNBr-activation	SUGIURA et al. (1982).
Uridine diphosphate glucuronyltransferase (EC 2.4.1.17) from phenobarbibal-treated rat liver	P1-(6-Aminohexyl)-P2-(5'-uridine)-pyrophosphate (or UDP-hexanolamine)	CNBr-activated Sepharose 4B	BURCHELL (1978).
Xylosyltransferase (UDP-D-xylose:core protein β-D-xylosyltransferase)	Proteoglycan (Smith degraded) or Core protein from cartilage proteoglycan	Sepharose 4B	SCHWARTZ and RODEN (1974).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Transglutaminase (EC 2.3.2.13) from guinea pig liver (GPLT)	Monoclonal antibody against GPLT	Affi-Gel 10	IKURA et al. (1985).
Transketolase (EC 2.2.1.1) from rat liver (TK)	Antibodies against TK from rabbit (purified by TK-Sepharose)	CNBr-activated Sepharose 4B	PAOLETTI and ALDINUCCI (1986).
Trehalose (EC 3.2.1.28) from pig kidney	Concanavalin A and Tris(hydroxymethyl)aminomethane	Con A-Sepharose and Epoxy-activated Sepharose 6B	YONEYAMA (1987).
T4 RNA Ligase from bacteriophage T4-infected Escherichia coli	Dextran Blue	Sepharose 4B	MEI-HAO et al. (1982).
t-RNA Methylases (EC 2.1.1.) from rat nefrome	t-RNA after periodate oxidation (via oxidized 3'-end)	Aminooxybutylcellulose	NEDOSPASOV et al. (1978).
Tryptophan 5-monooxygenase (tryptophan hydroxylase; EC 1.14.16.4) from rat brain-stem	2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine	Affi-Gel 202	NAKATA and FUJISAWA (1982).
Tryptophan 5-monooxygenase (EC 1.14.16.4) from rabbit brain	2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine	Affi-Gel 202	NAKATA and FUJISAWA (1981).
Tyrosinase from melanosomes	L-Tyrosine ethyl ester or L-dopa	Sepharose 4B	JIMBOW et al. (1981).
Tyrosine hydroxylase (EC 1.14.16.2) from rat brain and bovine adrenal medulla	Denatured DNA	DNA-cellulose	NELSON and KAUFMAN (1987).
Tyrosine hydroxylase (EC 1.14.16.2) from soluble fraction of bovine adrenal medulla	Heparin	Heparin-Sepharose CL-6B	OKA et al. (1981).
Ubiquitin-activated enzyme	Ubiquitin	Activated CH-Sepharose	CIECHANOVER et al. (1982).
Ubiquitin-protein ligase system from reticulocyte-rich blood of rabbits	Ubiquitin	Activated CH-Sepharose	HERSHKO et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
UDP-N-Acetylglucosamine: α-D-mannoside β1-2 N-acetylglucosaminyltransferase 11 (EC 2.4.1.143) from rat liver	5-Mercuri-UDP-N-acetylglucosamine	Thiopropyl-Sepharose 6B reduced with dithiothreiol	BENDIAK and SCHACHTER (1987).
Uracil-DNA glycosylase (EC 3.2.2.-) from rat liver	Poly (U)	Poly (U) - agarose; Type 6	COLSON and VERLY (1983).
Urease (EC 3.5.1.5) - from jack bean Canivalia ensiformis	Urea	Epoxy-activated Separose 6B	MENDES et al. (1988).
Uricase from bovine kidney	Urate or xanthine	Sepharose 4B with 1,4-bis(2,3-epoxypropoxy)butane in presence of sodium borohydride	BATISTA-VIERA et al. (1977).
Uronosyl C-5 epimerase from mouse mastocytoma	Concanavalin A; heparan sulfate; O-desulfated heparin and Cibacron blue	Con A-Sepharose; Sepharose 4B after CNBr-activation; Sepharose 4B with hexamethylenediamine (carbodiimide technique) and Sephadex G-200	MALSTROM et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
ENZYME SUBUNITS AND MODIFIED DERIVATIVES			
15 Kilodalton fragment of human plasma fibronectin from chymotryptic 120 kilodalton fragment cleaved with pepsin	Monoclonal antibodies against inhibiting attachment of fibronectin to cell	CNBr-activated Sepharose 4B	PIERSCHBACHER et al. (1981).
α and β Subunits of (Na^+; K^+) ATP phosphohydrolase (EC 3.6.1.3) from dog kidney	Concanavalin A and wheat germ agglutinin (WGA)	Con A-Sepharose 4B and WGA-Sepharose 6MB	OMORI et al. (1983).
Binary and ternary α2-macroglobulin-chymotrypsin complexes	D-Tryptophan methyl ester	Activated CH-Sepharose 4B	POCHON and BIETH (1982).
Catalase-dextran conjugate	Concanavalin A	Con A-Sepharose	MARSHALL and HUMPHREYS (1977).
Catalytic α-subunit of (Na^+; K^+)-ATPase (EC 3.6.1.3) from rat hepatic microsomes	Anticatalytic mouse monoclonal antibody	CNBr-activated Sepharose 4B	HUBERT et al. (1986).
Complementable fractions and complemented enzyme of β-galactosidase from Escherichia coli	p-Aminophenyl-β-D-thiogalactopyranoside	Sepharose 4B with 3;3'-diaminodipropylamine and succinic anhydride	MARINKOVIC and MARINKOVIC (1977).
ε-Subunit from F1-ATPase of Escherichia coli (removal)	Monoclonal anti-ε antibody	Sepharose CL-4B after CNBr-activation	DUNN (1986).
Functionally active human plasmin light (B) chain derivative from partially reduced and alkylated plasmin	L-Lysine monohydride	Sepharose 4B after CNBr-activation	SUMMARIA and ROBBINS (1976).
Heavy meromyosin subfragment-1 reacted with thiol reagents	ATP after periodate oxidation	Sepharose 4B with adipic acid dihydrazide	LAMED et al. (1976).
Heme-containing subunit of Chromatium vinosum flavocytochrome c-552	Cytochrome c	CNBr-activated Sepharose 4B	DAVIDSON et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Holoenzyme form of cyclic AMP-dependent protein kinase from porcine muscle	8-(6-Aminohexyl)-amino-ATP or 3-aminopyridine- NAD^+	Sepharose 4B after CNBr-activation	TAYLOR et al. (1976).
Homologous and heterologous complexes between catalytic and regulatory components of adenylate cyclase	Hydroxysuccinimide ester of 7-succinyl-7-deacetylforskolin	Sepharose CL-4B with 1;1'-carbonyldiimidazole and ethylenediamine	PFEUFFER et al. (1983).
Peripheral portion of membrane-bound proton-translocating ATPase (F1-ATPase) from Escherichia coli (study of activation and inhibition)	Monoclonal antibodies against subunit of F1-ATPase	Sepharose CL-4B after CNBr-activation	DUNN and TOZER (1987).
Protein component C5 of RNase P from crude extract of Escherichia coli	M1 RNA (catalytic subunit of Escherichia coli RNase P) dephosphorylated and labelled at 5'end with [γ-^{32}P]ATP and polynucleotide kinase after periodate oxidation	Agarose-adipic acid dihydrazide	VIOQUE and ALTMAN (1986).
Protein kinase subunits from bovine heart or rabbit skeletal muscle	8-(β-Hydroxyethylthio)-cAMP	Epoxy-acrivated Sepharose 6B	WEBER et al. (1979).
Regulatory subunit of cAMP-dependent protein kinase I from pigeon breast muscle	8-(2-Oxoethylthio)-cAMP	Epoxy-activated Sepharose 4B	GRIVENNIKOV et al. (1984).
Regulatory subunits RI and RII of protein kinase with intracellularly bound cAMP - from glucagon-stimulated rat hepatocytes	Antibodies against RI or RII - from rabbits	Protein A-Sepharose CL-4B	EKANGER et al. (1985).
SH-Modified (with thimerosal) myosin subfragment-1 (A1) isozyme	Adenosine 5'-diphosphate (Mg^{2+} in elution buffer is indispen- sable)	Agarose-hexane-ADP (AGADP TYPE 4)	EMOTO et al. (1988).
Subunits A and B of β-N-acetylhexosaminidases from boar epididymis	2-Acetamido-N-(6-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine	CNBr-activated Sepharose 4B	PARKES et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Subunits of phenylalanyl-tRNA synthetase from Escherichia coli MRE-600	t-RNAPhe after periodate oxidation	Sepharose 4B after CNBr-activation	ZYKOVA et al. (1982).
GLYCOPROTEINS AND SACCHARIDES			
2-Deoxy-2-dansylamido-D-glucose and sterically related sugars	Concanavalin A	Con A-Sepharose	BESSLER and SCHINDLER (1979).
A α -chain from S-caboxamidomethylated human fibrinogen	Concanavalin A	Concanavalin A-Sepharose 4B	BOGLI et al. (1988).
Acetylated methylmannose polysaccharide from Streptomyces griseus	Palmitoyl chloride	Aminoethyl-Bio-Gel P-150	KARI and GRAY (1979).
Adipocyte intrinsic membrane glycoproteins	Concanavalin A	Concanavalin A-agarose	CARTER-SU et al. (1980).
Alkaline phosphatase with sugar-chain analysis (EC 3.1.3.1) from serum of patient with osteosarcoma	Concanavalin A (Con A); pea lectin (Pisum sativum;PSA); wheat germ agglutinin (WGA); phytohemagglutinin E (Phaseolus vulgaris; PHA-E)	Con A- Sepharose; PSA-; WGA- or PHA-E-agarose	KUWANA et al. (1989).
α-1-Acid glycoprotein from human serum proteins	Cibacron Blue F3G-A	Sephadex G-100	BIRKENMEIER and KOPPERSCHLAGER (1982).
α1-Acid glycoprotein (orosomucoid) (AG) from human cancerous tissue	Antibodies against human AG from rabbit	CNBr-activated Sepharose 4B	OCHI et al. (1982b).
α1-Acid glycoprotein (orosomucoid) from normal and inflam- matory human sera	Concanavalin A	Con A-Sepharose 4B	NICOLLET et al. (1981).
α2-HS-glycoprotein from human plasma	Cibacron Blue F3GA and Procion Red HE3B	Affi-Gel Blue and Sepharose CL-6B	ARNAUD et al. (1983).
Antigenic fractions of human seminal plasma	Concanavalin A	Con A-Sepharose 4B	MAZZINI et al. (1979).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Antitumor acidic glycoprotein (SAGP) from Streptococcus pyogenes	Antibodies against SAGP	CNBr-activated Sepharose 4B	YOSHIDA et al. (1989).
Arabinogalactan protein from style canal of Gladiolus	Galactose-binding lectin from Tridacna maxima (tridacmin)	CNBr- activated Sepharose 4B	GLEESON et al. (1979).
Asialoglycoprotein Ib and asialoglycocalicin from human platelets	Peanut agglutinin	Sepharose 4B with polyacrylic hydrazide	CLEMETSON et al. (1981).
Asparagine-linked carbohydrate moieties from human prostatic acid phosphatase (EC 3.1.3.2) digested with Pronase P	Concanavalin A	Concanavalin A-agarose 4B	RISLEY and VAN ETTEN (1987).
Axon plasma membrane proteins	Concanavalin A	Con A-Sepharo- se	MARQUIS et al. (1980).
Basement-membrane glycoprotein laminin from Engelbreth-Holm-Swarm sarcoma	Glycosaminoglycans after periodate oxidation stopped by addition of D-mannitol	Sepharose 4B after CNBr-activation with adipic acid hydrazide (stabilization by reduction with $NaBH_4$)	DEL ROSSO et al. (1981).
Carbohydrate-rich glycoprotein carcinoembryonic antigen (CEA) from human tumour tissue	Antibodies against CEA from rabbits and concanavalin A	CNBr-activated Sepharose 4B and Con A-Sepharose	ASHMAN and DE YOUNG (1977).
Cardiac sarcolemmal vesicles from rabbit	Wheat germ lectin	Wheat germ lectin-Sepharose 6MB	MAS-OLIVA et al. (1980).
Cartilage components aggregable proteoglycans; chondroitinase degraded proteoglycans and link protein proteoglycans and protein-keratan sulphate cores	Hyaluronate	Cellulose with ε-aminocaproic acid	KALPAXIS (1985).
Cell surface glycoproteins from acute lymphoblastic leukemic cells	Biotinylated concanavalin A	CNBr-activated Sepharose 4B with streptavidin	COOK and BUCKIE (1990).
Cercarial glycoproteins of Schistosoma mansoni	Concanavalin A	Con A-Sepharose	STRAND et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Chondroitin and heparan sulphate proteoglycans from model mouse cells	Platelet factor 4 from human platelets and fibronectin from human plasma	CNBr-activated Sepharose 4B	WIGHTMAN et al. (1986).
Collagen-binding fragment of adhesive glycoprotein fibronectin from chicken embryonic fibroblasts	Gelatin or native type I collagen	CNBr-activated Sepharose 4B	HAHN and YAMADA (1979).
Colony-stimulating factor 1 from human pancreatic carcinoma cells	Concanavalin A	Con A-Sepharose 4B	SHIEH et al. (1987).
Echis carinatus venom (glycoproteins) (fractionation)	Concanavalin A; wheat germ lectin (WGL); lentil lectin and Helix pomatia lectin	Con A-Sepharose; WGL-Sepharose 6MB; Lentil Lectin-Sepharose 4B and Helix pomatia lectin-Sepharose 6MB	FOŘTOVÁ et al. (1983).
Epithelial membrane glycoprotein from milk-fat-globule membrane of human breast milk	Concanavalin A	Sepharose 4B after CNBr-activation	IMAM et al. (1981).
Equine infectious anemia virus glycoproteins	Lens culinaris (lentil) lectin	Lentil lectin-Sepharose	MONTELARO et al. (1983).
Erythropoietin from human urine	Wheat germ agglutinin and phytohemagglutinin	Sepharose 4B after CNBr-activation	SPIVAK et al. (1977).
Escherichia coli heat-labile enterotoxin from human source	Wheat-germ agglutinin (WGA)	WGA Ultrogel column	GRASSER-REGALLET et al. (1986).
Fibrinogen (human glycoprotein)	Lens culinaris agglutinin	CNBr-activated Sepharose 4B	TERCERO and DIAZ-MAURINO (1988).
Fibronectin (component of extracellular matrix; glycoprotein) from human placental fetal tissue	Gelatin	Gelatin-Sepharose 4B	LAINE et al. (1987).
Fibronectin (glycoprotein) from human plasma	Gelatin	Sepharose CL-4B after CNBr-activation	REGNAULT et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Fibronectin from human blood plasma	Gelatin or Physiogel	Sepharose 4B after CNBr-activation	MORGENTHALER et al. (1984).
Fibronectin or thrombin	Heparin in presence of NaCNBH3	Sepharose 4B with epichlorohydrin; and ammonia or hydrazine hydrate	SASAKI et al. (1987).
Follitropin from ovine pituitary	Concanavalin A	Con A-Sepharose	SAIRAM (1979).
Fuco-oligosaccharides and glycopeptides from human and rat brain glycoproteins	Lectin from Lotus tetragonolobus	Fucosylex (lectin coupled to Sepharose 4B)	SUSZ and DAWSON (1979).
Fucogalactan and mannofucogalactan from fruit bodies of Ganoderma applanatum	Concanavalin A	Sepharose 4B after CNBr-activation	USUI et al. (1981b).
Fucosyl glycoproteins from human erythrocyte membrane	Aleuria aurantia lectin	Sepharose 4B	YAZAWA et al. (1984).
Galactose-terminal glycoproteins from Trypanosoma cruzi	Peanut agglutinin (PNA)	PNA-Sepharose	MARCIPAR et al. (1982).
Gastric-glycoproteins having blood-group II activity from sheep stomachs	Antibodies against blood-group I activity	Sepharose 4B after CNBr-activation	WOOD et al. (1981).
Glucose-containing oligosaccharide	Monoclonal antibodies against human urinary glucose-containing tetrasaccharide (Glc)4 coupled to keyhole limpet hemocyanin	CNBr-activated Sepharose 4B or non-covalently bound to Protein A-Sepharose 4B	LUNDBLAD et al. (1984).
Glycated albumin from human plasma of diabetic patients	Cibacron Blue F3GA and m-aminophenyl boronic acid	Affi-Gel Blue and GLYCO-GEL B kit	DUCROCQ et al. (1987).
Glycated albumin from human sera	m-Aminophenylboronic acid	GLYCO-GEL test kit	ZOPPI et al. (1985).
Glycated hemoglobin from blood of uremic patients	m-Aminophenylboronic acid	GLYCO-GEL Test Kit	BANNON et al. (1984).
Glycated hemoglobin from plasma of nonazotemic and azotemic patients	Aminophenylboronic acid	Glyc-Affin System (boronate-agarose)	SCOTT et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glycocalicin/glycoprotein I from human platelets	Wheat germ agglutinin and thrombin	WGA-agarose and Sepharose 4B	MOROI et al. (1982).
Glycoconjugates-neutral fractions contained mannose from bovine tracheal gland serous cells	Concanavalin A	Con A-Sepharose	PAUL et al. (1988).
Glycophorins from human erythroleukemic cells	Lens culinaris lectin	Agarose-conjugated lectin	SILVER et al. (1987).
Glycoprotein gp118 of varicella-zoster virus	Cibacron blue dye	Cibacron blue dye-Sepharose	FRIEDRICHS and GROSE (1984).
Glycoprotein gp118 of varicella-zoster virus	Anti-gp118 murine monoclonal antibody	CNBr-activated Sepharose 4B	FRIEDRICHS and GROSE (1984).
Glycoprotein with gastric antisecretory activity (gastric inhibitor) from human urine	Gastric inhibitor	CNBr-activated Sepharose 4B	CARREA et al. (1979).
Glycoproteins from bovine herpesvirus 1	Wheat germ lectin (WGL) and concanavalin A	WGL-Sepharose 6MB and Con A-Sepharose 6MB	BADIA and QUEROL (1988).
Glycoproteins from tumour cellular membranes	Lentil-lectin	Lentil-lectin-Sepharose 4B	BERNARD et al. (1984).
Glycoproteins from human colonic adenocarcinoma	Ricinus communis agglutinin and concanavalin A	CNBr-activated Sepharose 4B and Con A-Sepharose	TSAO and KIM (1978).
Glycoproteins in central-nervous-system myelin from rat; bovine and human brains	Concanavalin A; Ricinus communis agglutinin-60; wheat germ and soy-bean agglutinins and fucose- binding protein from Lotus tetragonolobus	Con A-Sepharose and agarose-linked lectins	QUARLES et al. (1979).
Glycosaminoglycans (heparan sulphate fractions)	Thrombin	Sepharose 4B	FRANSSON and HAVSMARK (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glycosylated albumin -from blood of diabetic patient	Cibacron Blue F3GA	Blue Trisacryl	IBERG and FLUCKIGER (1986).
Glycosylated haemoglobin and plasma glycoprotein (assays)	Boronic acid	Glyc-Affin System (boronate agarose)	WILLEY et al. (1984).
Glycosylated haemoglobins from haemolysate of erythrocytes (normal and diabetic samples)	m-Aminophenyl boronic acid	GLYCO-GEL B	GOULD et al. (1982).
Glycosylated hemoglobin in newborn infants	m-Aminophenylboronic acid	Agarose	ABRAHAM et al. (1983).
Glycosylated hemoglobins (from normal and diabetic subjects)	m-Aminophenylboronic acid	Glyc-Affin System (boronate agarose)	ELSEWEIDY et al. (1983).
Gonadotrophins (follicle-stimulating and luteinizing hormones) from bull seminal plasma	Concanavalin A	Con A-Sepharose	SAIRAN et al. (1980).
Grass pollen allergens	Concanavalin A	Con A-Sepharose 4B	KARLSTAM and NILSSON (1982).
Heparan sulfate proteoglycans	Lipoprotein lipase coupled in presence of fully acetylated heparin	CNBr-activated Sepharose 4B	KLINGER et al. (1985).
Herpes-specific membrane glycoproteins from infected cells	Concanavalin A	Con A-Sepharose	POLIQUIN and SHORE (1980).
Heterogalactan from fruit bodies of Fomitopsis pinicola	Concanavalin A	Sepharose CL-4B after CNBr-activation	USUI et al. (1981a).
High-molecular-weight form of epidermal growth factor from human urine	Concanavalin A; wheat germ lectin and p-aminophenyl- boronic acid	Con A-Sepharose; Wheat germ lectin-agarose and phenylboronate-agarose PBA-30	MOUNT et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Human choriogonadotropin like factor from extracts of microorganisms	Concanavalin A	Con A-Sepharose	MARUO et al. (1979).
Human plasma membrane glycoprotein from human red blood cells	Monoclonal antibody (purified by Protein A-Sepharose)	CNBr-activated Sepharose 4B	MC ILLHINNEY and PATEL (1985).
Human red blood cell membrane glycoprotein	Lens culinaris hemagglutinin	Sepharose 4B with epichlorohydrin; concentrated ammonia solution; succinic anhydride and N-hydroxysuccinimide	MATSUMOTO et al. (1980).
Immunoglobulin A from human myeloma	Concanavalin A	Con A- Sepharose 4B	RAY et al. (1981).
Insulin receptor from human placental membranes	Wheat germ agglutinin and insulin	CNBr-activated Sepharose 4B and CNBr-activated Sepharose 4B with 3;3'-diaminodipropylamine and succinic anhydride	FUJITA-YAMAGUCHI et al. (1983).
L-Fucose-containing oligosaccharides	Aleuria aurantia lectin	Sepharose 4B after CNBr-activation	YAMASHITA et al. (1985).
Laminin from mouse EHS sarcoma tumor	Griffonia simplicifolia I lectin	CNBr-activated Sepharose 4B	SHIBATA et al. (1982).
Lysozymes from normal rat liver	Ricinus communis agglutinins I + II	CNBr-activated Sepharose 4B	KAMRATH et al. (1984).
Macrophage surface glycoproteins from detergent-solubilized murine membranes	Lens culinaris lectin and rat anti-mouse monoclonal antibodies	Sepharose CL-4B after CNBr-activation	SPRINGER (1981).
Major component of pregnancy-specific β1-glycoproein (SP1) from human pregnancy sera	Antibodies against SP1 and against human serum from rabbits	CNBr-activated Sepharose 4B	SORENSEN and TRENTEMOLLER (1983).
Membrane glycoproteins from human spermatozoa	Lens culinaris agglutinin	Lens culinaris agglutinin-Sepharose 4B	KALLAJOKI et al. (1985).
Membrane platelet glycoprotein glycocalicin from human blood	m-Aminophenyl boronic acid	Matrex Gel PBA-60	DE CRISTOFARO et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Mullerian inhibiting substance (a large glycoprotein secreted by the fetal and neonatal testis)	Wheat germ lectin and Green A dye	WGL-Sepharose 6MB and Matrex Gel Green A	BUDZIK et al. (1983).
Mycobacterial polymethyl polysaccharides	p-Nitrophenyl palmitate	Aminoalkylsilyl silicate	HINDSGAUL and BALLOU (1984).
Nucleotide sugars and oligosaccharides containing terminal α- D-galactopyranosyl and 2-acetamido-2-deoxy-α-D-galactopyranosyl residues	Bandeiraea simplicifolia I isolectins	CNBr-activated Sepharose 4B	BLAKE and GOLDSTEIN (1980).
Oligosaccharides and sugar nucleotides containing terminal α- linked D-galactosyl and N-acetyl-D-galactosaminyl residues	Lectin from Bandeiraea simplicifolia I	CNBr-activated Sepharose 4B	BLAKE and GOLDSTEIN (1982).
Oligosaccharides containg terminal α-linked galactose residues from rabbit erythrocytes	Isolectins from seeds of Griffonia simplicifolia (bound via its high mannose type to Con A-Sepharose)	Con A-Sepharose	WANG et al. (1988).
Oligosaccharides from urine of GM1-gangliosidosis patients	Datura stramonium lectin	CNBr-activated Sepharose 4B	YAMASHITA et al. (1987).
Oligosaccharides liberated from human immunoglobulin G	Lectins: Aleuria aurantia lectin; RCA 120 and E4-phytohemagglutinin (E4- PHA)	Sepharose 4B after CNBr-activation; RCA 120-agarose and E4-PHA-agarose	HARADA et al. (1987a).
Oligosaccharides with target antigen from human urine and human milk	Monoclonal antibodies (purified with Protein A-Sepharose)	IgG noncovalently bound or crosslinked with dimethyl suberimidate to Protein A-Sepharose CL-4B	ZOPF et al. (1987).
Ovalbumin subfractions (eight)	Concanavalin A and wheat germ agglutinin	Con A-Sepharose and Sepharose 4B	KATO et al. (1984).
Phosphatidylcholine; integral membrane proteins and glycoproteins	Deoxycholate	Bio-Gel A-5m with poly(L-lysine) hydrobromide	CRESSWELL (1979).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Phosphorylated high mannose-type oligosaccharides from human fibroblast secretions	Phosphomannosyl receptor	Affi-Gel 10	FISCHER et al. (1982).
Plasma glycoprotein kininogen (investigation of kininogen heterogeneity)	Concanavalin A and Ricinus communis agglutinin	Con A-Sepharose and CNBr-activated Sepharose 4B	TURPEINEN et al. (1981).
Plasma lysosomal enzymes (seven) from plasma; urine and fibroblasts of patients with mucolipidosis	Concanavalin A	Con A- Sepharose	DEN TANDT (1980).
Pregnancy-specific β1-glycoprotein (SP1) from maternal serum	Monoclonal antibodies against SP1	CNBr-activated Sepharose	HEIKINHEIMO et al. (1983).
Product of GDP-L-Fuc:N-acetyl-β-D-glucosaminide α1 $\Rightarrow$ 6 fucosyltransferase (quantitative method for enzyme activity)	Lentil lectin	Lentil lectin-Sepharose	VOYNOW et al. (1988).
Prolactin from human amniotic fluid (from women at 14-18 weeks pregnancy)	Concanavalin A	Con A-Sepharose	SHETH et al. (1984).
Proteins containing D-galactopyranosyl residues from human serum	Lectin from Viscum album L	Sepharose 4B after CNBr-activation	ZISKA and FRANZ (1979).
Proteoglycans from tissue culture substratum adhesion sites of human dermal fibroblast	Platelet factor-4 and human plasma fibronectin	Sepharose 4B	KENT et al. (1986).
Pyrogens (lipopolysaccharides) from Escherichia coli	Histamine	Sepharose CL-4B with hexamethylenediamine and glutaraldehyde	MINOBE et al. (1983).
Rhodopsin from bovine rod outer segments disk membranes	Concanavalin A	Con A-Sepharose	LITMAN (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Sciatin (glycoprotein from chicken sciatic nerves)	Concanavalin A	Glycosylex-A (Con A-agarose)	MARKELONIS and OH (1981).
Sialylated oligosaccharides; glycopeptides and glycoproteins	Elderberry (Sambucus nigra L.) bark lectin	Sepharose 4B after CNBr-activation	SHIBUYA et al. (1987).
Soluble glycoproteins from sheep thyroid plasma membrane	Concanavalin A	Sepharose CL-6B activated with 1;1'-carbonyldiimidazole	HEARN et al. (1979).
Subcellular markers from post-mitochondrial supernatant from rat liver	Concanavalin A	Agarose-bound concanavalin A	SEHMI et al. (1986).
Sulfated glycoproteins from human gastric juice	Lysine	Lysine-Sepharose 4B	HOTTA and GOSO (1981).
Surface glycoproteins from Schistosoma mansoni adult worms (characterization)	Lectins	Con A-Sepharose 4B; Lentil lectin-Sepharose 4B; Wheat germ lectin-Sepharose 6MB; Helix pomatia lectin-Sepharose 6MB; soybean agglutinin and 2 Ricinus communis agglutinins bound to agarose	HAYUNGA and SUMNER (1986).
Thrombospondin (component of extracellular matrix; glycoprotein) from platelet concentrates	Gelatin and heparin	Gelatin-Sepharose 4B and Heparin-Sepharose	SANTORO and FRAZIER (1987).
Triantennary oligosaccharides with C-2;6-linked structures and bi-and triantennary oligosaccharides with N-acetyllactosamine repeating structures in their outer chain moieties	Datura stramonium lectin	CNBr-activated Sepharose 4B	YAMASHITA et al. (1987).
Tumor associated glycoproteins from tumor tissue and ascitic tumor cells	D-Galactose specific mistletoe lectin I	CNBr- activated Sepharose 4B	WALZEL et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Varicella-zoster virus glycoprotein gpl/gp3	Monoclonal antibody against gpl/gp3 (purified by Protein A-Sepharose CL-4B)	CNBr- activated Sepharose CL-4B	WROBLEWSKA et al. (1985).
Vitronectin (glycoprotein also called S-protein;serum spreading factor or epibolin) - from human plasma	Heparin	Sepharose 4B after CNBr-activation	YATOHGO et al. (1988).
HORMONES			
Cartilage-derived growth factor from bovine; human and chick cartilage extracts	Heparin	Heparin-Sepharose	SULLIVAN and KLAGSBRUN (1985).
Endogenous growth factor with melanoma growth stimulatory activity (MGSA) from human malignant melanoma cells	Monoclonal antibody against MGSA	CNBr-activated Sepharose 4B	THOMAS and RICHMOND (1988).
Epidermal growth factor-urogastrone from mouse submaximillary glands	Solubilized membrane receptor	Sepharose CL-4B with concanavalin A	NEXO et al. (1979).
Estradiol-17β from plasma	Antibodies against estradiol-17-β 6(O-carboxymethyl)oxime/ovalbumin conjugate	Microcellulose after CNBr-activation	GASKELL and BROWNSEY (1983).
Fibroblast growth factor from bovine hypothalamus	Heparin and copper	Heparin-Sepharose and Chelating Sepharose saturated with copper (II)chloride	SHING (1988).
Growth factors from fetal calf mandible and adult bovine femur	Heparin	Heparin-Sepharose	HAUSCHKA et al. (1986).
Growth hormone (GH) from chicken pituitary tissue	Monoclonal mouse anti-chicken GH antibody	CNBr-activated Sepharose 4B	BERGHMAN et al. (1988).
Growth hormone (hGH) from human plasma	Antibodies against hGH from rabbits	CNBr-activated Sepharose 4B	STOLAR et al. (1984).
Human B cell growth factor from heparinized human blood	Concanavalin A	Con A-Sepharose	DUGAS et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Human tumor calcitonin (polypeptide hormone)	Antibody or concanavalin A	CNBr-activated Sepharose 4B	BAYLIN et al. (1981).
Inhibin (hormone) from bovine follicular fluid	Procion Red He3B and concanavalin A	Matrex gel red A and Con A-Sepharose	JANSEN et al. (1981).
Insulin with cleavable 3-[4-(N-biotinoyl-6-aminocaproyloxy)phenyl] propionic acid N-hydroxysuccinimide ester	Avidin	CM- Sepharose with N-hydroxy-succinimide ester	MOUTON et al. (1982).
Insulin-like growth factor I from human plasma	Human amniotic fluid somatomedin binding protein	CNBr-activated Sepharose 4B	POVOA et al. (1986).
Oestradiol-17β and testosterone (measurement)	Antibodies against 17β-oestradiol-11β-succinyl-bovine serum albumin and against testosterone	CNBr-activated Sepharose 4B	WEBB et al. (1985).
Oestradiol-17β from biological fluids	Antibodies against 6- oxo-oestradiol-17β; carboxymethyloxime and bovine serum albumin conjugate	Sepharose 6B after CNBr-activation	GLENCROSS et al. (1981).
On-resin biotinylated (with N-hydroxysuccinimidobiotin) chemically synthesized protein hormone interleukin 153 residue [Asp 205]-interleukin-1β)	Avidin	Avidin (monomeric)-agarose	LOBL et al. (1988).
Somatostatin (tetradecapeptide hormone) and higher molecular weight somatostatin-like forms from mouse hypothalamus	Antibodies against somatostatin	Activated CH-Sepharose 4B	LAUBER et al. (1979).
Thyrotropin	Anti-thyrotropin antibodies	CNBr-activated Sepharose 4B	PEKONEN et al. (1980).
Thyrotropin-releasing hormone-like material (TRH) in human peripheral blood (measurements)	Anti-TRH immunoglobin	Sepharose 4B	MALLIK et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
INHIBITORS			
Acid-resistant trypsin inhibitor from human urine	Trypsin	CNBr-activated Sepharose	WACHTER and HOCHSTRASSER (1981).
α-1-Protease inhibitor (of serine proteinases in human plasma)	Anhydrochymotrypsin	CNBr-activated Sepharose 4B	DRECHSEL et al. (1984).
α-1-Proteinase inhibitor from human serum proteins	Cibacron Blue F3G-A	Sephadex G-100	BIRKENMEIER and KOPPERSCHLAGER (1982).
α-Amylase/trypsin plant double-headed inhibitor from ragi	Trypsin	CNBr-activated Sepharose 4B	SHIVARAJ and PATTABIRAMAN (1981).
α-Antitrypsin and antithrombin from human plasma	Heparin and concanavalin A	CNBr-activated Sepharose 4B and Con A-Sepharose	D'SOUZA and ANANTHAKRISHNAN (1979).
α-Cysteine proteinase inhibitor from human plasma	S-Carboxymethyl papain	Sepharose 4B after CNBr-activation	GOUNARIS et al. (1984).
α2-Antiplasmin from human plasma	Fragment from elastase-digested plasminogen constituting three N-terminal triple-loop structures	CNBr-activated Sepharose 4B	WIMAN (1980).
α2-Thiol proteinase inhibitor (α2TPI) from human serum	Antibodies against α2TPI	CNBr-activated Chromogel A-2	SASAKI et al. (1977).
Antithrombin III from human plasma	Heparin	Sepharose after CNBr-activation with lysine	HATTON et al. (1977).
Antithrombin from plasma of female mice	4-Aminophenethyl heparin	Sepharose CL-6B with trichloro-s-triazine and aminophenethylamine diazotized by HNO_2	FINLAY et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Avidin and its fluorescent and iodinated derivatives	2-Iminobiotin	Sepharose 4B with hexamethylenediamine (carbodiimide technique)	HENEY and ORR (1981).
Catalase inhibitor from maize	Catalase	CNBr-activated Sepharose	SORENSON and SCANDALIOS (1975).
Deoxyribonuclease inhibitor from crude extract of calf thymus	Deoxyribonuclease	Sepharose 4B	LINDBERG (1974).
Heat-stable inhibitor protein of Ca^{2+} - activated cyclic nucleotide phosphodiesterase from bovine brain	Protein modulator	Sepharose 4B	SHARMA et al. (1978).
Human α1-antitrypsin	Concanavalin A	Con A-Sepharose	IKUTA et al. (1981).
Inhibitor of 3';5'cyclic AMP phosphodiesterase from Dictyostelium discoideum	3';5'cyclic AMP phosphodiesterase	CNBr-activated Sepharose 4B	DICOU and BRACHET (1979).
Inhibitor of cathepsin D from potato juice	Cathepsin D	Sepharose 4B	KEILOVÁ and TOMÁŠEK (1976).
Inhibitor of proteolytic enzyme guanidinobenzoatase from lamb's liver	Guanidinobenzoatase	Activated CH-Sepharose 4B	STEVEN et al. (1986).
Inhibitor of trypsin and acrosin from porcine blood plasma	Antibodies against trypsin-kallikrein inhibitor	Sepharose 4B	VESELSKY et al. (1986).
Inhibitor protein of adenylate cyclase (EC 4.6.1.1) and phosphodiesterase (EC 3.1.4.17) from bovine brain	Cibaron Blue F3GA and activator protein	Affi-Gel Blue and Affi-Gel 10	WALLACE et al. (1979).
Inter-α-trypsin inhibitor (ITI) from human serum	Antibodies against ITI from rabbits	Sepharose 6B	SALIER et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Isoinhibitors of chymotrypsin/elastase from Ascaris lumbricoides	α-Chymotrypsin A	Sepharose CL-4B	PEANANSKY et al. (1984).
Ovomucoid (proteinase inhibitor) from duck egg protein	Trypsin	CNBr-activated Sepharose 4B	SHULGIN et al. (1981).
Pancreas trypsin inhibitor (Trasylol) from fluorescein-labeled conjugates of α-chymotrypsin and Trasylol	Bovine α-chymotrypsin	CNBr-activated Sepharose 4B	KASCHE and BUCHTMANN (1981).
Protease (trypsin; chymotrypsin) inhibitor from human male urine	Bovine α-chymotrypsin (EC 3.4.21.1 - containing β-phenylpropionate)	Sepharose CL-6B with trichloro-s- triazine	KESSNER et al. (1979).
Protease inhibitor α1-antichymotrypsin from human serum	Native calf thymus DNA (type V)	Acid-washed cellulose CF11	ABDULLAH et al. (1983).
Protein inhibitor of cysteine proteinases cystatin from chicken egg white	S-Carboxymethylpapain	Sepharose 4B after CNBr- activation	ANASTASI et al. (1983).
Protein inibitor of adenosine3';5'-monopospate dependent protein kinases (EC 2.7.1.37) from rabbit skeletal muscle	Protein kinase catalytic subunit	CNBr-activated Separose 4B	DEMAILLE et al. (1977).
Protein protease inhibitors I;II;III;IV from bovine spleen	Trypsin	Sepharose 4B	FIORETTI et al. (1983).
Proteinaceous α-amylase inhibitor from Phaseolus vulgaris	Salivary or porcine pancreatic α-amylase and IgG of rabbit antiserum against pure α-amylase inhibitor	Affi-Gel 10 and Sepharose 4B	PICK and WOBER (1979).
Ribonuclease inhibitor from rat liver	Ribonuclease	CM- Cellulose	GRIBNAU et al. (1970).
Serine protease inhibitor antithrombin III and α-thrombin (human radiolabeled proteins)	Heparin	Heparin-Sepharose	DAWES (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Trypsin inhibitor from Ascaris lumbricoides	Porcine trypsin	Sepharose 4B after CNBr-activation	GOODMAN and PEANASKY (1982).
Trypsin inhibitor from corn seeds	Trypsin	CNBr-activated Sepharose 2B	LEI and REECK (1986).
Trypsin inhibitors (two types) from albumin fraction of soybean	Anhydrotrypsin	CNBr-activated Sepharose 4B	PUSZTAI et al. (1988).
Trypsin inhibitors from corn and teosinte seeds	Antibodies against corn trypsin inhibitor	CNBr-activated Sepharose 4B	CORFMAN and REECK (1982).
LECTINS			
Arachis hypogaea agglutinin; soybean agglutinin and Ricinus communis agglutinin	Lactose	Sepharose 6B with epichlorohydrin and hydrazine hydrate	ITO et al. (1986).
Arachis hypogaea; soybean or Ricinus communis agglutinins	Lactose in presence of $NaCNBH_3$	Sepharose 4B with epichlorohyd- rin and ammonia solution	MATSUMOTO et al. (1981).
β-Galactosidase binding soluble lectin from amphibian ovary	Lactose	Lactogel = lactose-aminophenyl-agarose gel	FINK DE CABUTTI et al. (1987).
β-Galactosyl-specific lectin from fruiting bodies of Ischnoderma resinosus	Agarose	Sepharose 4B	KAWAGISHI and MIZUNO (1988).
Cold agglutinin from albumin gland of snail Achatina fulica	Sheep gastric mucin	CNBr-activated Sepharose 4B	MITRA et al. (1987).
Concanavalin A	Trehalose	Epoxy-activated Sepharose 6B	KLYASHCHITSKY (1979).
Concanavalin A	Maltose	Sepharose 6B with epichlorohydrin and hydrazine hydrate	ITO et al. (1986).
Concanavalin A	Maltose in presence of $NaCNBH_3$	Sepharose 4B with epichlorohydrin and ammonia solution	MATSUMOTO et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Concanavalin A (Con A) from tissue culture media	Monoclonal antibody against Con A	Sepharose 4B after CNBr-activation	SPURLL and OWEN (1980).
Concanavalin A (inhibition of biospecific hydrophobic interactions with organic solvents)	Dextran	Sephadex G-100	WASEEM and SALAHUDDIN (1983).
Concanavalin A -α and β from jack bean meal	Dextran and p-aminophenyl β-D-glucopyranoside	Sephadex G-100 and Sepharose 4B after CNBr-activation	ODA et al. (1981).
Concanavalin A from jack bean seeds	Dextran	Sephacryl S-300	NANDEDKAR et al. (1987).
Concanavalin A from jack beans (ConA)	Dextran T40 after periodate oxidation with soy bean trypsin inhibitor(purified by trypsin-Sepharose and CoA-Sepharose)	Sepharose 4B after CNBr- activation with attached trypsin	OLSSON and MATTIASSON (1986).
Cytisus-type hemagglutinin II from Ulex europeus	tri-N-Acetylchitotriose	Sepharose 4B with epichlorohydrin or 1;4-bis(2;3-epoxypropoxy)butane after amination by concentrated ammonia activated with succinic anhydride and N-hydroxysuccinimide (carbodiimide technique)	KONAMI et al. (1981).
D-Galactose/ N-acetyl-D-galactosamine-specific lectin from Erythrina cristagalli	D-Galactose	Sepharose 6B with divinyl sulphone	IGLESIAS et al. (1982).
Developmentally regulated lectin from embryonic chick pectoral muscle	p-Aminophenyl-β-D-lactoside	Sepharose 4B with hexamethylenediamine and succinic anhydride	NOWAK et al. (1977).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Fucose-binding lectin from Ulex europeus	p-Aminophenyl-α-L-fucoside	Copolymer N-[tris(hydroxymethyl)methyl] acrylamide/acrylamide-6-hexanoic acid (A-6-NH-Trisacryl H) and N-[tris(hydroxymethyl)methyl] acrylamide/methacryloyl-glycylglycine (MGG-Trisacryl H) (carbodiimide technique)	RACOIS and BOSCHETTI (1978).
Fucose-specific lectin from Aleuria aurantia	H-Active glycopep- tide of desialyzed porcine submaxillary mucine	CNBr-activated Sepharose 4B	KOCHIBE and FURUKAWA (1980).
Galactose-specific lectin from hemolymph of solitary ascidian	Acid-treated agarose	Sepharose 6B	YOKOSAWA et al. (1982).
Galactoside-binding lectins from rabbit; rat; mouse; pig; lamb; calf; and human spleens	Asialofetuin	Sepharose 4B	ALLEN et al. (1987).
Helix pomatia agglutinin from crude extract	N-Acetyl-D-galactosamine	Sepharose 6B with 1;4-bis(2;3-epoxypropoxy)butane in presence of sodium borohydride	SCHREMPF-DECKER et al. (1980).
Hemagglutinin I from Ulex europeus	6-Amino-1-hexyl-L-fucopyranose	Sepharose 4B after CNBr- activation	FROST et al. (1975).
Isolectins of Phaseolus vulgaris	Ovomucoid	Sepharose 4B	FLEISCHMANN et al. (1985).
Japanese horseshoe crab lectin	N-Acetylglutamic acid	Sepharose 6B with epichlorohydrin and hydrazine hydrate	ITO et al. (1986).
L-Fucose binding lectin from Ulex europeus	L-Fucose	L-Fucose and starch cross-linked by epichlorohydrin	ONOZAKI et al. (1979).
Lactose-specific and L-fucose specific lectins from Laccaria amethystina	Group 0 human red blood cell stromas	Included in polyacrylamide gel	GUILLOT et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Lectin from Amaranthus leucocarpus seeds	Erythrocyte membrane residues (stroma)	Stroma obtained by lysis of human red blood cells group A	ZENTENO and OCHOA (1988).
Lectin from Glycine sojae	D-Galactose	Spheron	FILKA et al. (1978).
Lectin from Phaseolus vulgaris	Concanavalin A	Con A- Sepharose	BASU et al. (1981).
Lectin from Ulex europaeus seeds	Maleylated hog stomach peptone	Maleylated peptone polyacrylamide gel prepared by copolymerization	HOŘEJŠÍ (1979).
Lectin from Vicia faba	Chitin	Chitin	DATTA et al. (1984).
Lectin-ferritin conjugates (iodinated agglutinins coupled to ferritin with glutaraldehyde in presence of hapten sugar)	Sulfated glycopeptides released by papain digestion from hog gastric mucin	Sepharose 4B after CNBr-activation	MAYLIE-PFENNINGER and JAMIESON (1979).
Lectins	Immunoglobulin IgG	CNBr-activated Sepharose 4B	FRANZ and ZISKA (1981).
Lectins (30) from 27 plants	Partially desialylated hog gastric mucin or ovomucoid	Sepharose 4B	FREIER et al. (1985).
Lectins (review)	Polysaccharides (native or modified)	Sephadex; Sepharose; acid-treated Sepharose et al.	LIS and SHARON (1981).
Lectins from aqueous extracts of seeds or other lectin-containing fluids (eluted with appropriate hapten sugar)	Sulfated glycopeptides released by papain digestion from hog gastric mucin	Sepharose 4B after CNBr-activation	MAYLIE-PFENNINGER and JAMIESON (1979).
Lectins from seeds of Ricinus communis	Erythrocyte stromata	Erythrocyte stromata	GENAUD et al. (1982).
Lobster agglutinin 1	Fetuin	Sepharose 4B	HERRON et al. (1983).
Mannan-binding proteins (mammalian lectins) from rat serum (S- MBP-I and II) and rat liver (L-MBP)	Mannan; L-MBP and rabbit immunoglobulin G against L-MBP (purified by Sepharose 4B-L-MBP)	Sepharose 4B after CNBr-activation	OKA et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
N-Acetyl-D-galactosamine specific roat lectin from Dolichos biflorus	Blood group A + H substance	CNBr-activated Sepharose 4B	QUINN and ETZLER (1987).
N-Acetyl-D-galactosamine-specific lectin from hog peanut (Ampicaparpaea bracteata) seeds	Synthetic type A blood group trisaccharide α-N-acetyl-D-galactopyranose-(1;3)-[α-L-fucose-(1;2)]-β-D-galactopyranose	α-D-GalNc-(1;3)-[α-D-Fuc-(1;2)]-β-D-Gal-Synsorb	MALIARIK et al. (1987).
N-Acetyllactosamine-specific lectins from nine Erythrina species	Lactose	Sepharose 4B with divinylsulphone	LIS et al. (1985).
Peanut agglutinin	Asialoglycophorin	Affi-Gel 10	STAROSCIK et al. (1983).
Phytohemagglutinin from Agaricus bisporus	Concanavalin A	Sepharose 4B	AHMAD et al. (1984).
Potato lectin	N;N';N"-Triacetylchitotriose	Sepharose 4B with 1;4-butanediol diglycidyl ether and $NaBH_4$ in NaOH	DESAI and ALLEN (1979).
Rice lectin from Oryza sativa embryos	p-Aminophenyl-β-D-N-acetylglucosamine	Agarose-p-aminophenyl-β-D-N-acetylglucosamine	TABARY et al. (1987).
Ricinus communis agglutinin	Lactose	Toyopearl HW-65 with epichlorohydrin and concentrated ammonia solution	MATSUMOTO et al. (1982).
Roat lectin from soybean	N-Acetylgalactosamine or acid-treated agarose	Sepharose 4B with 1;4-butanediol diglycidyl ether or acid-treated Sepharose	GADE et al. (1981).
Sialic acid-binding lectin (carcinoscorpion) from Indian horseshoe crab (Carcinoscorpius rotunda cauda)	Ovine submaximally mucin and concanavalin A	Sepharose 4B after CNBr- activation	BISHAYEE and DORAI (1980).
Sialic acid-specific lectin from lobster	Colominic acid (homopolymer of N-acetyl-neuraminic acid)	Epoxy-activated Sepharose 6B	VAN DER WALL et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Soybean agglutinin	D-Galactosamine hydrochloride	Toyopearl HW-65 with epichlorohydrin; concentrated ammonia solution and succinic anhydride	MATSUMOTO et al. (1982).
Soybean agglutinin	D-Galactosamine	Sepharose 4B with epichlorohydrin; ammonia solution and succinic anhydride	MATSUMOTO et al. (1981).
Vicia graminea (blood group N) lectin	Concanavalin A	Con A- Sepharose	PRIGENT et al. (1982).
Wheat germ agglutinin	Tetra-N-acetylchitotetraose in presence of $NaCNBH_3$	Sepharose 4B with epichlorohydrin and ammonia solution	MATSUMOTO et al. (1981).
LIPIDS			
Acidic phospholipids from lipid extracts of liver and brain of rats	Antibiotic neomycin sulfate	Porous glass beads Glycophase G/CPG-200	PALMER (1981).
Cerebrosides from bovine brain	Magnesium acetate tetrahydrate	Silicic acid Magnammsil	KANT and HALLEN (1985).
Cholinergic proteolipid from nucleus caudatus of the cow	p-Phenyltrimethylamonium	Sephadex LH-20 with 3,3-iminobispropylamine	SARACENO and DE ROBERTIS (1976).
Glycolipids having terminal α1-3-linked N-acetylgalactosamine from sheep and human type A erythrocyte stroma	Helix pomatia lectin	Affi-Gel 10	TORRES and SMITH (1988).
Lipids (polyphosphoinositides)	Neomycin	Glycophase-CPG	SCHACHT (1978).
Lipids from plasma	Dodecylamine	Sepharose 4B	DEUTSCH et al. (1973).
NUCLEIC ACIDS, NUCLEOTIDES AND NUCLEOSIDES			
5'-Adenosine diphosphoribose	3-Aminophenylboronic acid	Affi-Gel 601	MINAGA et al. (1982).
Acid-soluble nucleotides; NAD and diadenosine 5';5'''-tetraphosphate and acid-insoluble polymer poly(ADP-ribose)	m-Aminophenylboronate hemisulfate	Sepharose 4B after CNBr-activation with 6-aminohexanoic acid or Bio-Rex 70 (carbodiimide technique)	ALVAREZ-GONZALES et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Aminoacyl-tRNA from Escherichia coli	Protein synthesis elongation factor Tu from Escherichia coli	Activated CH- Sepharose 4B	LOUIE et al. (1984).
ATP-Citrate lyase messenger RNA (mRNA) from livers of mice	IgG fraction against murine ATP-citrate lyase from rabbits (purified by Protein A-Sepharose)	Protein A-Sepharose	SUL et al. (1984).
Biological active individual histone messenger RNAs from whole cell or polysomal RNA	DNA (cloned sea urchin histone gene fragments)	m-Aminobenzyloxymethylcellulose after diazotization	CHILDS et al. (1979).
Biologically active globin mRNA from mouse	Globin cDNA	Oligo(dT)-cellulose	WOOD and LINGREL (1977).
Biotinylated rabbitβ-globin precursor mRNA (pre-m RNAB)	Streptavidin	Agarose	LAMOND et al. (1988).
Calf thymus DNA and Nicotiana gossei high-molecular-weight leaf RNA	Ethidium bromide with N;N'-methylenebisacrylamide; N;N;N';N'-tetramethylenediamine and ammonium persulfate	Bio-Gel P-4	VACEK et al. (1982).
Calf thymus DNA and PM-2-viral DNA	Histones from chicken erythrocytes	Bio-Gel A15M cross-linked with epichlorohydrin in presence of $NaBH_4$ after CNBr-activation with glycine methyl ester converted through glycyl hydrazide to glycyl azide	YU et al. (1978).
Capped small nuclear RNAs and mRNA	m-Aminophenylboronic acid	CM-Sepharose CL-6B	WILK et al. (1982).
Chromatin fragments containing bovine satellite DNA	Malachite green; (A-T)-specific	DNA affinity adsorbent (Malachite green gel)	WEBER and COLE (1982).
cis-Diol-containing mononucleotides or short oligonucleotides	m-Aminophenylboronic acid	Affi-Gel 601 (with Bio-Gel P6) or Aminoethyl Bio-Gel P-150	PACE and PACE (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Complementary polynucleotide sequences with mercurated polynucleotides (hybrids)	N-Acetylhomocystein thiolactone	Sepharose CL-4B with ethylenediamine	LONGACRE and MACH (1978).
Complexes of DNA hairpins and a singlestranded oligonucleotide	Oligo (deoxyadenilic acid) and oligo(thymidylic acid)	Oligo (dA)-cellulose and oligo (dT)-cellulose	BAUMANN et al. (1987).
Covalently closed circular plasmid DNA from Escherichia coli	Acridine yellow dye	Acridine yellow ED gel	VINCENT and GOLDSTEIN (1981).
Cyclic GMP	Dihydroxyboronyl derivative	Affi-Gel 601 (polyacrylamide gel)	DAVIS and DALY (1979).
DNA labeled with chemically cleavable biotinylated nucleotide Bio-12-SS-dUTP or Bio-19-SS-dUTP	Avidin or streptavidin	Biotin cellulose	HERMAN et al. (1986).
DNA restriction fragments (fractionation)	Malachite green and Phenyl neutral red gels	DNA affinity adsorbents; (A-T)-specific and (G-C)-specific	SCHWARTZ (1981).
Flavin-adenine dinucleotide and coenzyme A	p-Acetoxymercurianiline	CNBr-activated Sepharose 6B	CHIBATA et al. (1980).
High-specific-radioactivity single-stranded DNA (producing DNA hybridization probes)	Single-stranded DNA (trypsin and kallikrein clone)	Aminobenzyloxymethyl-cellulose (ABM-) after diazotization with $NaNO_2$ in presence of HCl	ASHLEY and MAC DONALD (1984).
Individual species of amino acid transfer nucleic acid (tRNAs) (terminating in 5'-AMP group on amino acid acceptor arm)	Monoclonal antibodies against 5'-adenosine monophosphate (5'-AMP) covalently attached to bovine serum albumin	Affi-Gel 10	ZHU et al. (1987).
Inosine and adenosine in human plasma (determination)	3-Aminophenylboronic acid	Affi-Gel 601	PFADENHAUER and TONG (1979).
Low-abudance messenger RNAs from rat liver	Polysomes after reaction with specific IgG (purified by Protein A-Sepharose)	Protein A-Sepharose	KRAUS and ROSENBERG (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Macromolecular RNA	m-Aminophenylboronic acid hemisulphate	CM-BioGel A or CM-Sepharose 4B	PACE and PACE (1980).
Mercurated polynucleotides	N-Acetylhomocysteine thiolactone	Aminoethylcellulose	FEIST and DANNA (1981).
Methionine-specific tRNAs from Escherichia coli	Elongation factor TU.GTP	CNBr-activated Sepharose 4B	FISCHER et al. (1985).
Mono- and dinucleotides	Bovine pancreatic ribonuclease	Sepharose 4B	ARUS et al. (1982).
mRNA coding for rat liver fructose-1;6-bisphosphatase (FBP) (EC 3.1.3.11)	Antibodies against FBP from rabbits (purified by antigen-coupled Sepharose 4B) and oligothymidylic acid	Protein A-Sepharose and cellulose	EL-DORRY (1986).
mRNA encoding Trypanosoma brucei variable surface antigen	Antibodies from anti-variable antigen-specific serum (purified by Protein A-Sepharose)	Protein A-Sepharose CL-4B	SHAPIRO and YOUNG (1981).
mRNA labeled in vivo with thionucleoside analogs from hamster cells	Organomercurial derivative	Phenylmercury agarose Affi- Gel 501	WOODFORD et al. (1988).
mRNAs from Dictyostelium discoideum	Poly(U)	Poly(U)-Sepharose	PALATNIK et al. (1981).
N-Methylated ribonucleosides (determination in urine)	m-Aminophenylboronic acid	Bio-Gel P2-Hydrazide with succinic anhydride	SCHLIMME et al. (1981).
Nascent mercurated DNA from mixture of cellular nucleic acids	N-Acetyl-D;L-homocysteine	Sulfhydryl agarose or Affi-Gel 401	BANFALVI et al. (1985).
Newly synthesized DNA of HeLa cells containing β-2'-deoxy-6-thioguanylate	p-Chloromercuribenzoate	Affi- Gel 501	YOSHIDA et al. (1980a).
Non-polyadenylated mRNA species	Polyadenylic acid	Poly(A) Sepharose 4B	KATINAKIS and BURDON (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Oligo dG-tailed DNA fragments from linker plasmids of Escherichia coli	Oligo dC	Oligo dC-cellulose	FORSDYKE (1984).
Ovalbumin mRNA from hen oviduct polysomes and by hybridization	Oligo(dT) and DNA complementary to ovalbumin mRNA	Cellulose	RHOADS and HELLMANN (1978).
Plasmids containing Z-DNA (left-handed Z-form of poly(dG-dC))	Monoclonal antibodies against Z-DNA	Sephacryl S-1000 (supe rfine; after CNBr-activation)	THOMAE et al. (1983).
Poly(A)	Poly(U)	Poly(U)-Sepharose 4B	OCHOA et al. (1980).
Poly(A)(polyadenylic acid)-containing RNAs from polyribosomes of 10-day-old chick embryos	Poly(U) (polyuridylic acid)	Sepharose 4B after CNBr-activation or CNBr-activated Sepharose	BERMAN et al. (1978).
Poly(A)$^+$ (polyadenylated) RNA from vitellogenic female-locust fatbody	Poly(U)	Poly(U)-Sepharose 4B	APPLEBAUM et al. (1981).
Poly(A) mRNA from oviduct extract	Oligo(dT)	Ultrogel; Trisacryl or cellulose	SENE et al. (1982).
Poly(A)-containing cellular and viral RNAs	Poly(U)	Poly(U)-agarose type 6 and Poly(U)-Sepharose 4B	PHILLIPS et al. (1980).
Poly(A)-containing mRNA	Poly(U)	Glycogen-hydrazidosuccinyl-Sepharose	KLYASHCHITSKY et al. (1981).
Poly(A)RNA from mammalian cells and subcellular components	Polyuridilic acid	Sepharose 4B after CNBr-activation	BYNUM and VOLKIN (1980).
Poly(adenylic acid)-containing ribonucleoproteins	Oligothymidylic acid	Oligo(dT)-cellulose T2	DE MEYER et al. (1980).
Poly(ADP-ribose)	Aminophenylboronic acid	Bio-Rex 70 (carbodiimide technique)	WIELCKENS et al. (1981).
Poly(ADP-ribosyl)ated domains of chromatin from HeLa nuclei (fractionation)	Anti-poly(ADP-ribose) IgG from rabbits	CNBr- activated Sepharose 4B	MALIK et al. (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Polyadenylated and nonpolyadenylated RNA fractions (mRNA for α-lactalbumin and casein) from lactating rat mammary gland	Polythymidylic acid chains and polyuridylic acid	Oligo(dT)-cellulose; type 2 and Poly(U)-Sepharose	NADIN-DAVIS and MEZL (1983).
Polyadenylated RNA	Oligo(dT)	Oligo(dT)-cellulose	NADIN- DAVIS and MEZL (1985).
Polyoma mRNA (by hybridization to complementary immobilized DNA)	Linear polyoma DNA after denaturation (107° C for 2 min.)	Sephadex G-10 after CNBr-activation	SIDDELL (1978).
Prochymosin-specific mRNA from calf tissue	Antibody against prochymosin from rabbits (purified by immobilized prochymosin)	CNBr-activated Sepharose 4B	NISHIMORI et al. (1984).
Ribo- and 3'-deoxyribo analogues	Trimer of (2'-5')-adenylic acid and its 3'-deoxy analogue with (2-carboxyethyl)-ethylidene group in terminating fragment	AH-Sepharose 4B	KVASYUK et al. (1984).
Ribonucleic acids from Escherichia coli	Spermine	Activated CH-Sepharose 4B	SEIBERT and ZAHN (1978).
Ribonucleosides from tissue extracts	m-Aminophenylboronic acid hemisulfate	Bio-Gel P-2 hydrazide beads diazotized with nitrous acid or with succinic anhydride	OLSSON (1979).
Ribonucleotides	Bovine pancreatic ribonuclease (EC 3.1.27.5)	Sepharose 4B with ethylenediamine and O-bromoacetyl-N-hydroxysuccinimide	NOGUES et al. (1983).
RNA Chains initiated with adenosine-5'-O-(3-thiotriphosphate)	p-Chloromercuribenzoate	Affi-Gel 501	CARROLL and WAGNER (1979).
RNA components of splicing complexes	Biotinylated pre-m RNA	Streptavidin-agarose beads	GRABOWSKI (1990).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Separated strands from double-stranded linearized plasmid DNAs or restriction fragments from restriction fragments terminally labeled at both ends with biotinylated nucleosides	Avidin	Avidin agarose	DELIUS et al. (1985).
Supercoiled PM2 DNA	H1 Histone from calf thymus chromatin	Bio-Gel A-0.5m with glycyl azide derivative	LAU et al. (1979).
Synthetic and naturally occurring double-stranded RNAs	Anti-poly(I).poly(C) antibody from rabbits	Cellulose after CNBr-activation	KITAGAWA and OKUHARA (1981).
Thiol-containing purines and ribonucleic acid from baby hamster kidney cells	Mercury(II)acetate	Cellulose with 1-allyloxy-2;3-epoxypropane	MELVIN et al. (1978).
Thymosin α1 precursor mRNA from total calf thymus poly(A)- mRNA	cDNA (synthesis directed by thymosin α1 precursor mRNA)	Oligo(dT)-cellulose	REY-CAMPOS et al. (1983).
Transcriptionally active DNA with chemically cleavable bionylated nucleotide Bio-12-SS-dUTP from chicken erythrocyte nuclei	Avidin	Avidin-D Agarose	ROSEMAN et al. (1986).
Transfer ribonucleic acids from Bacillus subtilis labeled with ^{35}S	Antibodies against N-[9-(β-D-ribofuranosyl)purin-6-.ylcarbamoyl]-L-threonine conjugated with bovine serum albumin from rabbits	CNBr-activated Sepharose 4B	VOLD et al. (1981).
tRNA from Escherichia coli	Ethidium bromide (3;8-diamino-5-ethyl-6-phenylphenantridinium bromide)	Sepharose 4B after CNBr-activation with 3;3'-diaminodipropylamine and succinic anhydride	THOMAS and SCHECHTER (1978).
tRNA Trp from Escherichia coli	Antibodies against N^6-(Δ^2-isopentenyl)adenosine	CNBr-activated Sepharose 4B	VOLD et al. (1979).
tRNAs containing N^6-(Δ^2-isopentenyl)adenosine derivatives	Anti-N^6-(Δ^2-isopentenyl)adenosine antibody	Protein A- agarose (immobilizing agent iminothiolane)	MC LENNAN and RANEY (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
tRNAs from yellow lupin seeds	Spermine	CNBr-activated Sepharose 4B	JOACHIMIAK et al. (1979).
Tyrosine tRNA from bovine liver	Ricinus communis agglutinin type I	Affi-Gel 10	GARCIA and SINGHAL (1979).
Vaccina complementary RNA (hybridization)	Vaccina single stranded Ehrlich ascites tumor DNA heated at 100 °C with diazobenzoic acid (azoDNA)	Sepharose CL-4B after CNBr-activation with 1;5-diaminopentane (carbodiimide technique)	DICKERMAN et al. (1978).
Viroid RNAs from crude; plant polysaccharides containing nucleic acid fractions	Acridine yellow and phenyl neutral red	DNA affinity adsorbents; structure- and GC-specific (crosslinked bisacrylamide gels)	ROHDE (1982).
PROTEINS; TRANSFER RECEPTORS AND BINDING PROTEINS			
Acetylcholine receptor from Torpedo marmorata	α-Cobratoxin	Nylon tubes	YANG et al. (1981).
Acetylcholine receptors from electric eel	α-Neurotoxin	Nylon tubes activated by partial hydrolysis + coupling through carbodiimide-activated COOH-groups	SUNDARAM (1983).
Acetylcholine receptors from Torpedo californica electroplax	Cobrotoxin	Sepharose 6B after CNBr-activation	FROEHNER and RAFTO (1979).
Activated dexamethasone-receptor from rats	Calf thymus DNA	Cellulose (adsorption with drying)	BODWELL et al. (1984).
Activated progesterone receptor from oviducts of chicks	ATP	Sepharose 4B with adipic acid dihydrazide	MILLER and TOFT (1978).
Active acetylcholine receptor fragment obtained by tryptic digestion of acetylcholine receptor from electric organ of Torpedo californica	Naja naja siamensis toxin	Sepharose 2B after CNBr-activation	BARTFELD and FUCHS (1979).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Active mitochondrial phosphate carrier	Organomercury(II) chloride	Affi-Gel 501	DE PINTO et al. (1982).
Active progesterone receptor	Monoclonal antireceptor antibody	Protein A-Sepharose (cross-linking with dimethyl pimelimidate)	LOGEAT et al. (1985).
Alkaline phosphatase-protein A fusion protein (fusion of protein A gene and Escherichia coli gene encoding alkaline phosphatase)	Immunoglobulin IgG	IgG-Sepharose 4B	NILSSON et al. (1985).
α and β-Adrenergic receptors from hepatic plasma membrane	Alprenolol	Sepharose 6B with 1;4-bis(2;3-epoxypropoxy)butane activated by $Na_2S_2O_3$; reduced with dithiothreitol in presence of EDTA	WOOD et al. (1979).
α2-Adrenergic receptor from porcine brain	Yohimbinic acid monohydrate	Diaminodipropylamine agarose (carbodiimide technique)	REPASKE et al. (1987).
α2-Adrenergic receptors from human platelet membranes	3- Benzazepine with α2-adrenergic antagonist activity (coupling in presence of potassium persulfate $K_2S_2O_8$)	Sepharose CL-4B with 1;4-bis(2;3-epoxypropoxy)butane and sodium thiosulfate reduced with dithiothreitol	REGAN et al. (1982).
α2-Macroglobulin receptor from human fibroblasts	α2-Macroglobulin	Affi-Gel 15	MARYNEN et al. (1984).
Androgen acceptor from rat prostatic chromatin	Deoxyribonucleic acid	Cellulose - coupling of DNA according Alberts and Herrick (1971)	HIREMATH et al. (1980).
Androgen receptor from rat uteri	17α-Epoxypropyl-dihydrotestosterone	Thiopropyl-Sepharose 6B	RAY et al. (1986).
Angiotensin II receptors	[ε-6-(biotinylamido)hexyllysine] angiotensin II	Succinoylavidin Sepharose 4B	FINN and HOFMANN (1985).
Arginine-glycine-aspartic acid adhesion receptors (of fibronectin and vitronectin) from placental tissue	120-kDa Chymotryptic fragment of fibronectin or Gly-Arg-Gly-Asp-Ser-Pro-Lys	CNBr-activated Sepharose	PYTELA et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Auxin receptor proteins	2-Hydroxy-3;5-diiodobenzoic acid	Epoxy-activated Sepharose 6B	TAPPESER et al. (1981).
B Lymphocyte receptor for C3d fragment (CR2) of complement and Epstein-Barr virus	Monoclonal antibody against human CR2	CNBr-activated Sepharose 4B	WEIS et al. (1986).
B12-Binding proteins from rabbit serum	Aminopropyl cob(I)alamin	Sephacryl S-200 after CNBr-activation	JACOBSEN and HUENNEKENS (1986).
Benzodiazepine binding proteins from human serum and rat kidney; lung; skeletal muscle and brain	Chlorazepate and delorazepan	Sepharose with adipic acid hydrazide	MARTINI et al. (1981).
Benzodiazepine receptor from bovine cortical membranes	Benzodiazepine	Adipic acid dihydrazide agarose	SIGEL et al. (1982).
Benzo[a]pyrene binding protein	Benzo[a]pyrene (carcinogen)	Epoxy-activated Sepharose 6B	COLLINS and MARLETTA (1986).
β-Adrenergic receptor from turkey erythrocyte membranes	Alprenol	Sepharose 4B with 1;4-butanediol diglycidylether activated by $Na_2S_2O_3$ reduced with dithiothreitol in presence of EDTA	KELLEHER et al. (1983).
Binding protein for Tityus γ toxin - from venom of scorpion Tityus serrulatus serrulatus	Wheat germ agglutinin	Glutaraldehyde-activated Ultrogel	NORMAN et al. (1983).
Binding protein specific for 3-methylcholanthrene from rat hepatic cytosol	1-Hydroxy-3-methylcholanthrene	Epoxy- activated Sepharose 6B	ARNOLD et al. (1987).
Binding receptor proteins of cytotoxic cells eosinophils from normal human peripheral blood	Human anti-tetanus immunoglobulin G in presence of fetal calf serum	Antigen tetanus toxoid attached to p-aminobenzoyl cellulose	THORNE and FRANKS (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Biologically active macrophage receptor (glycoprotein) for third component of complement (C3) from rabbit alveolar macrophages	Human C3 b	Sepharase after CNBr-activation	SCHNEIDER et al. (1981).
Bovine striatal dopamine D-2 receptor	Haloperidol	Sepharose CL-6B with 1;4-butanediol diglycidyl ether	RAMWANI and MISHRA (1986).
C3b/C4b Receptor (CR1) from membranes of human erythrocytes	Procion Red HE3B and monoclonal antibodies against CR1	Matrex Gel Red A and CNBr-activated Sepharose	WONG et al. (1985).
Ca^{2+} -Binding regulatory protein calmodulin from Dictyostelium discoideum	2-Chloro-10-(3-aminopropyl)phenothiazine	Sepharose 4B	JAMIESON and FRAZIER (1983).
Ca^{2+} -Dependent mannose-binding proteins from human or roe-deer livers or rat fibroadenoma	Mannosylated bovine serum albumins; RNase B; invertase or mannan	Sepharose 4B after CNBr-activation	GABIUS and ENGELHARDT (1987).
Calcium-binding proteins (S-100 and calmodulin)	N-(6-Aminohexyl)-5-chloro-1-napthalenesulfonamide	Epoxy- activated Sepharose 6B or CNBr-activated Sepharose 4B	ENDO et al. (1981).
Calmodulin (displacement by amphiphilic peptides)	Melittin (calcium-dependent affinity)	Sepharose 4B using triethyldiamine for neutral CNBr-activation	COX et al. (1985).
Calmodulin from bovine cerebrum	Perphenazine	Sepharose 4B with 1;4-bis(2;3-epoxypropoxy)butane	ROCHER et al. (1986).
Carbohydrate binding proteins discoidins I and II (with agglutination activity) from Dictyostelium discoideum	Fucosamine	Agarose-ε-aminocaproyl-fucosamine	KILPATRICK et al. (1978).
Cholic acid-binding proteins from rat liver cytosol	Cholic acid	AH-Sepharose 4B (carbodiimide technique)	PATTINSON (1981a).
Cholinergic receptor proteins from torpedo electric organ	Carbachol- or decamethonium-analogues or gallamine amide amine	Sepharose	HOPFF et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cobalamin-binding proteins	Aminopropylcobalamin	Sephacryl S-200 beads	JACOBSEN et al. (1981).
Colony-stimulating factor (CSF) - from human urine	IgG fractions from normal rabbit serum and anti-CSF serum (purified by Protein A-Sepharose)	CNBr-activated Sepharose 4B	WAHEED and SHADDUCK (1989).
Complement component C4-binding protein from human serum	Antibodies against C4bp contaminants	Sepharose 4B	VILLIERS et al. (1981).
Complement-fragment-iC3b-binding proteins from human spleen membranes and leukocytes	Human iC3b	CNBr-activated Sepharose 4B	MICKLEM and SIM (1985).
Complex of solubilized opioid receptor in NG 108-15 cells with [(leucine-enkephalinylhydrazido)succinyl] biocytinamide	Avidin	Avidin-conjugated agarose	NAKAYAMA et al. (1986).
Corticosteroid binding globulin and sex hormone binding globulin from human serum	Cortisol-21-hemisuccinate	AH-Sepharose 4B (carbodiimide technique)	FERNLUND and LAURELL (1981).
Cryptococcus-specific supressor T-cell factors	Cryptococcal culture filtrate antigen	Sepharose 4B with 1;4-butanediol diglycidyl ether	MOSLEY et al. (1986).
Cyclic AMP-adenosine binding protein	9-(p-Aminobenzyl)adenine	Succinylaminopropylaminopropyl-Sepharose	ENDRIZZI et al. (1979).
Cyclic AMP-binding protein from Neurospora	Cyclic AMP (through N^6-amino group with 8-carbon spacer)	Cyclic AMP-agarose	TREVILLYAN and PALL (1982).
Cytokinin-binding protein from tobacco culture cells	N^6-(Δ^2- Isopentenyl)adenosine after periodate oxidation (after coupling with $NaBH_4$)	CNBr-activated Sepharose 4B with adipic acid dihydrazide	CHEN et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cytoplasmic progesterone receptor from human uterus	11-Deoxycorticosterone hemisuccinate coupled to bovine serum albumin	CNBr-activated Sepharose 4B	SMITH et al. (1981).
Cytosolic estrogen receptors from estrogenprimed rabbit uterus	Heparin and estradiol	Heparin-Sepharose and 17β-estradiol-17-hemisuccinyldiaminodipropylaminoagarose 4B	MADHOK and LEUNG (1981).
D-Galactose-binding hemagglutinins from Ascidia malaca	Hydrolyzed-agarose	Sepharose CL-6B treated with acid	PARRINELLO and CANICATTI (1982).
D-Glucose transport protein from human erythrocyte membranes	Wheat germ lectin	Wheat germ Lectin-Sepharose	FROMAN et al. (1981).
D1 Dopamine receptor from rat corpus striatum	(RS)-5-(4-Aminophenyl)-8-chloro-3-methyl-2;3;4;5-tetrahydro-1H-3-benzazepin-7-ol-succinate	Epoxy-activated Sepharose 6B with hexamethylenediamine in presence of ethanolamine and coupling by carbodiimide technique	GINGRICH et al. (1988).
DNA-binding protein (OBF1) from yeast cells	DNA	Cellulose	EISENBERG et al. (1990).
DNA-binding proteins 1 and 2 from human serum	Native calf thymus DNA	Acid-washed cellulose (Whatman CF11) in suspension with DNA dried under blower (cool air) for several hours; after suspension in absolute ethanol irradiation with mercury lamp (15 min.)	HOCH and MC VEY (1977).
Dopamine receptor from canine brain	Haloperidol hemisuccinate	Sepharose 4B with N;N'-iminobispropylamine; succinic anhydride and N;N'-iminobispropylamine	ANTONIAN et al. (1986).
Epidermal growth factor receptor from human epidermoid carcinoma cells	Wheat germ agglutinin	Wheat germ agglutinin-Sepharose CL-4B	NAPIER et al. (1987).
Estradiol receptor from calf uteri	17α-(Carboxymethyl)-17β-estradiol	Sepharose with 3;3'- diaminodipropylamine	MONCHARMONT et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Estrogen receptor from calf uterus	Dichlorotriazinyl dye Reactive Orange 14	Sepharose CL-6B	BOND and NOTIDES (1987).
Estrogen- and glucocorticoid-receptor complexes from MCF-7 cells	Single-stranded calf thymus DNA	Cellulose CF 11	CHONG and LIPPMAN (1982).
Eukaryotic mRNA cap binding protein	2';3'-O-[1-(2-Carboxyethyl)ethylidene]-7-methylguanosine or 7-(5- carboxypentyl) guanosine 5'-diphosphate	AH-Sepharose 4B	RUPPRECHT et al. (1981).
Fatty acid binding protein from rat renal basolateral membrane	1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide (Oleate-Sepharose)	AH-Sepharose 4B	FUJII et al. (1987).
Fc-γ receptors from guinea pig peritoneal macrophages in complexes with rabbit IgG	Protein A	Protein A-Sepharose	JANUSZ et al. (1986).
Fibronectin receptor from Streptococcus pyogenes	Fibronectin	CNBr-activated Sepharose CL-4B	STING et al. (1989).
Foetal steroid-binding protein from human serum	Androstanediol-3-hemisuccinate	Sepharose 4B after CNBr- activation with hexamethylenediamine (carbodiimide technique)	WILKINSON et al. (1986).
Folate-binding protein from cow's milk	Folate	CNBr-activated Sepharose 6B with 1;3-diaminopropan-2-ol (carbodiimide technique)	SALTER et al. (1981).
G-Actin-binding protein (17K protein)	DNase I	DNase I-bound agarose	HOSOYA et al. (1982).
γ-Aminobutyric acid (GABA) receptor from rat brain	Benzodiazepine 1012-S	CNBr-activated Sepharose 4B with adipic acid dihydrazide and sodium iodoacetate	TAGUCHI and KURIYAMA (1984).
Glucocorticoid hormone receptor from rat liver cytosol	17β-Carboxylic acid of dexamethasone (via N-hydroxybenzotriazole ester of dexamethasone)	Affi-Gel 102	HAPGOOD et al. (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glucocorticoid receptor from human spleen tumors	Biotin-dexamethasone conjugates (9-fluoro-16 α-methyl-11 β; 17-dihydroxy-1; 4-androstadiene-3-one-17 β-carboxylic acid coupled to biotin using pentamethylenediamine as spacer)	Avidin-Sepharose	MANZ et al. (1983).
Glucocorticoid-receptor complex from rat liver	ATP after periodate oxidation	Sepharose 4B after CNBr-activation with adipic acid dihydrazide	MOUDGIL and JOHN (1980).
Glycated and non-glycated human serum albumin (separation)	3- aminophenylboronic acid	Glycogel B immobilized to agarose	VIDAL et al. (1992).
Glycine receptor from rat spinal cord	Aminostrychnine and wheat germ agglutinin (WGA)	Affi-Gel 10 and WGA-Sepharose 6MB	PFEIFFER et al. (1982).
Gonadotropin receptor from rat	Human chorionic gonadotropin	Affi-Gel 10	AUBRY et al. (1982).
Group-specific component (vitamin D-binding protein) from human plasma	Cibacron Blue F3GA	Affi-Gel Blue	CHAPUIS-CELLIER et al. (1982).
Growth-hormone receptor from rabbit livers	Human growth hormone	AF-formyl Toyopearl	YAGI et al. (1987).
Guanine nucleotide-binding protein from turkey erythrocytes	Guanosine triphosphate-p-phenylenediamine	Sepharose 4B after CNBr-activation with 6-aminohexanoic acid (carbodiimide technique)	NIELSEN et al. (1980).
Haemagglutinins (galactosyl/lactosyl- and lactosyl-binding proteins) from Botrylloides leachii	Lactose	Acid-treated Sepharose 4B with 1;4-butanediol diglycidyl ether	SCHLUTER and EY (1984).
Haeme-binding protein from rabbit serum	Haemin with 1;1'-carbonyldiimidazole	Aminoethyl-agarose	TSUTSUI and MUELLER (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Haptoglobin from human plasma	Chicken cyanomethemoglobin in presence of cyanoborohydride; concanavalin A and immunoglobulin G against chicken hemoglobin from rabbits	Sepharose CL-4B after periodate oxidation; Affi-Gel Con A and Protein A-Sepharose (coupling of IgG by use of carbodiimide technique)	RADEMACHER and STEELE (1987).
HeLa transcription factors IIIB and IIIC from plasmids linearized by T4 polymerase and labeled with biotin-11-dUTP	Avidin	Biotin-cellulose (prepared by bisoxirane coupling method)	KASHER et al. (1986).
Heme-binding proteins	Hemin acylimidazolide	Sepharose CL-6B after CNBr-activation with diaminoethane	TSUTSUI (1986).
Hepatic glucagon receptor from rat liver membranes	Wheat germ lectin	CNBr-activated Sepharose CL-6B	HERBERG et al. (1984).
Hepatic glucocorticoid receptor from rat	N-Hydroxysuccinimidate-ester of 21-(4-carboxyphenoxy)progesterone (= deoxycorticosterone)	Sepharose 2B with 1;4-butanediol diglycidyl ether	GRANDICS et al. (1984).
High-affinity receptors for activated α2-macroglobulin (α2M)	Human α2M	CNBr-activated Sepharose or Affi-Gel 15 or 10	HANOVER et al. (1983).
Histamine-producing cell stimulating factor from secondary mixed lymphocyte cultures supernatants from spleen cells of mice	Cibacron Blue F3GA dye	Blue Ultrogel	DY et al. (1986).
Human acetylcholine receptor	α-Naja-toxin	Sepharose 4B	KALIES et al. (1979).
Human β-interferon receptor	Wheat germ lectin and anti-interferon immunoglobulin	Wheat germ lectin-Sepharose 4B and IgG crosslinked to Protein A-Sepharse by use of dimethyl pimelimidate	ZHANG et al. (1986).
Human placental insulin receptor	Succinoylavidin	CNBr- activated Sepharose 4B	FINN et al. (1984).
Hyaluronate-binding proteins from bovine nasal cartilage	Hyaluronate with molecular weight of 52000	AH-Sepharose (carbodiimide technique)	TENGBLAD (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
IgE-Binding and IgG-binding factors from human lymphoblastoid cell lines	Human IgE and IgG and lentil-lectin	CNBr-activated Sepharose 4B and Lentil-lectin-Sepharose 4B	JENSEN and SAND (1986).
Insulin binding α1 serum globulin	Insulin antibodies	Affi-Gel 10	GJEDDE and GJEDDE (1980).
Insulin receptor from adipocyte plasma membranes of rats with anti-insulin IgG	Protein A	Protein A-Sepharose CL-4B	HEINRICH et al. (1980).
Insulin receptor from human placental membranes	Insulin	Sepharose 4B with 3;3'-diaminodipropylaminosuccinyl N-hydroxysuccinimide ester	SIEGEL et al. (1981).
Insulin receptor from human placental membranes with insulin-dependent tyrosine protein kinase activity	Wheat germ agglutinin and insulin	Agarose-bound wheat germ agglutinin and Sepharose with 3;3'-diaminodipropylamine and succinic anhydride	PETRUZELLI et al. (1984).
Insulin receptors from human placenta	Wheat germ lectin and insulin	Wheat germ lectin-Sepharose 6MB and Reacti-Gel 6X	NEWMAN and HARRISON (1985).
Insulin-like growth factor I (IGF-I) receptor kinase from human placenta	Wheat germ agglutinin and IGF-I with 8-amino acid leader sequence (Met-Lys-Lys-Tyr-Trp-Ile-Pro-Met) and Thr substitution for Met at position 59	Wheat germ agglutinin- agarose and Affi-Gel 10	YU et al. (1986a).
Insulin-like growth factor I receptor from human placental membranes	Wheat germ agglutinin and insulin	CNBr-activated Sepharose 4B and Affi-Gel 10	TOLLEFSEN et al. (1987).
Intrinsic factor receptors from porcine and human ilea	Intrinsic fator bound to immobilized monocarboxylic acid derivatives of vitamin B 12	Sepharose 4B after CNBr-activation with 3;3'-diaminodipropylamine (carbodiimide technique for coupling of B 12)	KOUVONEN and GRASBECK (1979).
Kainic acid receptor from frog brain membranes	Domoic acid	AH-Sepharose 4B (carbodiimide technique)	HAMPSON and WENTHOLD (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Lactogenic and somatogenic hormone-binding sites from rabbit liver or mammary gland in complexes with biotinylated human growth hormone	Streptavidin	Affi-Gel 10	HAEUPTLE et al. (1983).
Lactogenic and somatogenic receptor from rabbit liver or mammary membranes	Biotinylated growth hormone	Affi-Gel 10 with streptavidin	KRAEHENBUHL and BONNARD (1990).
Lagan binding factor (sequence-specific DNA binding protein) from fetal calf thymus	Double-stranded synthetic oligonucleotides	Teflon fiber (OSS-2)	DUNCAN and CAVALIER (1988).
Laminin receptors from plasma membranes of murine EHS basement membrane tumor	Laminin	Sepharose after CNBr-activation	VON DER MARK and RISSE (1987).)
Ligandin (protein binding organic anions	Sulphobromophtalein	Sepharose 4B	WOLKOFF et al. (1979).
Lipid transfer protein complex (LTC) from human plasma	Antibodies against LTC from goat or rabbit antisera	Affi-Gel 10	BUSCH et al. (1986).
Lipoteichoic acid and glucan-binding proteins from Streptococcus mutans	Antibodies against wall-associated protein antigen A of Streptococcus mutans serotype c from rabbits	CNBr-activated Sepharose	RUSSELL (1981).
Local anesthetic binding protein from mammalian axonal membranes	Quinacrine mustard	Affi-Gel 10	GREENBERG and TSONG (1984).
Lung β2-adrenergic receptors from bovine lung	Adsorbed autoantibodies to β2-receptors	Protein A-Sepharose CL-4B	FRASER and VENTER (1982).
Malignancy-associated DNA-binding protein from human serum	Concanavalin A and DNA from human spleen	Con A-Sepharose and CM-cellulose type CM 23	SCHRODER et al. (1979).
Membrane dicarboxylate binding proteins from Bacillus subtilis	L-Malate or L-glutamate	Sepharose 4B with 1;4-butanediol diglycidyl ether	KAY (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Membrane glycoprotein receptors for Vicia graminea lectin from human red cells	Vicia graminea lectin	CNBr-activated Sepharose 4B	PRIGENT et al. (1983).
Membrane-bound prolactin receptors from mammary gland of rabbits	Ovine prolactin	Affi-Gel 10	NECESSARY et al. (1984).
Messenger RNA Cap-binding protein from rabbit reticulocyte ribosomes	p-Aminophenyl γ-ester of 7-methylguanosine 5'- triphosphate	Sepharose 4B after CNBr-activation	WEBB et al. (1984).
Mineralocorticoid receptors from rabbit kidney cytosol	3-O-Carboxymethyloxime deoxycorticosterone and 17β-carboxylic acid dexamethasone derivative	Sepharose CL-4B after CNBr-activation with nonamethylenediamine (carbodiimide technique)	LOMBES et al. (1987).
Molybdate-stabilized L-cell glucocorticoid receptor	Deoxycorticosterone	Deoxycorticosterone-agarose	HOUSLEY et al. (1985).
Muscarinic acetylcholine receptor from porcine cerebrums	3-(2'- Aminobenzhydryloxy)tropane	Epoxy-activated Sepharose 6B	HAGA and HAGA (1983).
Nerve growth factor receptors from rat pheochromocytoma and human melanoma cells	Wheat germ agglutinin	Affi-Gel 15	GROB et al. (1983).
Neuronal nicotinic receptors from chicken brain	Monoclonal antibody against nicotinic acetylcholine receptors from Electrophorus	Sepharose CL-4B	WHITING and LINDSTROM (1986).
Neurotensin receptor from bovine brain	Neurotensin	Affi-Gel 10	MILLS et al. (1988).
Nicotine receptor from rat brain	(R;S)-6-(2-Hydroxyethyl)nicotine	Epoxy-activated Sepharose 6B	ABOOD et al. (1983).
Nicotinic acetylcholine receptor from chick brain	Monoclonal antibody against receptors from Electrophorus electric organ	Sepharose CL-4B after CNBr-activation	WHITING and LINDSTROM (1986).
Nuclear androgen receptor	Dihydrotestosterone-17β-succinate or 17β-acetate of dihydrotestosterone-7α-undecanoic acid	Affi-Gel 102	DE LARMINAT et al. (1984).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Nuclear estrogen receptor	17β-Estradiol-17-hemisuccinate	Sepharose 4B with ovalbumin	SCHNEIDER and GSCHWENDT (1980).
Nuclear factor III (origin recognition protein C) from Hela nuclear extracts	E.coli DNA and pKB 67-88 DNA	Cellulose	O'NEIL and KELLY (1988).
Oestrogen receptor from calf mammary gland	Heparin	CNBr-activated Sepharose 4B	ROTONDI and AURICCHIO (1979).
Oestrogen-receptor complexes from rat uterine cytosol	Oligodeoxythymidylate	Oligo(dT)-cellulose	MYATT et al. (1982).
Opiate binding components from rat brain membranes	6-Succinylmorphine	Affi-Gel 102	CHO et al. (1983).
Opiate receptor from Triton-solubilized neural membranes of rat brain	14-β-Bromoacetamidomorphine hydrochloride	AH- Sepharose 4B (carbodiimide technique)	BIDLACK et al. (1981).
Organic anion binding protein from rat liver plasma membrane fractions	Bilirubin and sulfobromophthalein	CNBr-activated Sepharose 4B	REICHEN and BERK (1979).
Penicillin-binding proteins 5 and 6 (penicillin-sensitive D- alanine carboxypeptidases) from Escherichia coli membranes	6-Aminopenicillanic acid	Sepharose 4B with epibromohydrin; NH_4OH; succinic anhydride; NaOH; N-hydroxysuccinimide and diisopropyl carbodiimide	AMANUMA and STROMINGER (1980).
Phosphate carrier from pig heart mitochondria	Mersalyl (sodium O-((3-(hydroxymercuri)-2-methoxypropyl)-carbonyl)- phenoxyacetate) coupled using N-ethoxycarbonyl-2-ethoxy-1;2-dihydroquinoline	AM Ultrogel A-4-R with epichlorohydrin and ammonia	TOURAILLE et al. (1981).
Phosphomannosyl-enzyme receptor from bovine liver	Glycoproteins from Dictyostelium discoideum secretions	Affi-Gel 10	FISCHER et al. (1982).
Phytohemagglutinin-receptor glycoproteins from porcine splenic lymphocytes	Phytohemagglutinin	Affi-Gel 10	DUPUIS et al. (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Progesterone receptor components from high-salt-prepared chick oviduct cytosol	3-Oxo-4-androstene-17β-carboxylic acid activated with N-hydroxybenzotriazol	Sepharose CL-4B with diaminododecane	RENOIR et al. (1984).
Progesterone receptor from human uterine tissues	16α-Ethyl-19nor-3-oxo-4-androsterone-17β-carboxylic acid with N-hydroxysuccinimide	Affi-Gel 10 with cystamine and diamine	MANZ et al. (1982).
Progesterone-receptor complex from chick oviduct cytosol	Aurintricarboxylic acid	AH-Sepharose 4B	MOUDGIL et al. (1985).
Prolactin receptor - from bovine mammary gland	Human growth hormone	Affi-Gel 10	ASHKENAZI et al. (1987).
Prolactin receptor from solubilized preparation of mouse microsomal membranes	Hormone prolactin	Activated CH-Sepharose 4B	LISCIA and VONDERHAAR (1982).
Protein binding vitamin D and its metabolites from human plasma	Rabbit skeletal-muscle G-actin	Affi-Gel 15	HADDAD et al. (1984).
Protein binding vitamin D from human plasma	Cibacron Blue F3GA	Affi-Gel Blue	GIANAZZA et al. (1984).
Proteins binding adriamycin from various tissues	Adriamycin	Adipic acid hydrazide-Sepharose	LUCACCHINI et al. (1979).
Proteins with Ca^{2+}-dependent binding to chromaffin granule membrane	Chromaffin granule membranes	CNBr-activated Sepharose 4B	CREUTZ (1981).
Putative androgen and estrogen receptors from mouse brain and kidney	Denaturated calf thymus DNA	Cellulose	FOX et al. (1979).
Putative Na^+/D-glucose cotransporter (protein) from pig kidney brush border membranes	3-Aminophlorizin or 3-aminophloretin	Activated Affi-Gel 15	KITLAR et al. (1988).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Receptor for 1;25-dihydroxyvitamin D3 (1;25-$(OH)_2D_3$) from chicken intestinal mucosa	Monoclonal antibodies against 1;25-$(OH)_2D_3$	CNBr-activated Sepharose	PIKE et al. (1987).
Receptor for human tumor necrosis factor-α (TNF-α) - from a subclone of human histiocytic lympoma cell line U937	Polyclonal antibodies against TNF-α from rabbits (purified by TNF-α-Affi-Gel 10)	CNBr-activated Sepharose CL-4B	STAUBER et al. (1988).
Receptor for nerve growth factor (NGF) from A875 melanoma cells	β-NGF	CNBr-activated Sepharose 4B	PUMA et al. (1983).
Receptor for quinine- and quinidine-dependent antibodies from human Bernard-Soulier platelets of patients	Wheat germ agglutinin	Ultrogel AcA 22	KUNICKI et al. (1981).
Receptors for peanut agglutinin from Lewis lung carcinoma cells	Peanut agglutinin	CNBr-activated Sepharose 4B	ADACHI et al. (1984).
Retinol-binding protein from serum and urine from patient with tubular damage due to multiple myeloma	Retinoic acid and prealbumin	AH-Sepharose 4B and CNBr-activated Sepharose 4B	FEX and HANSSON (1978).
Riboflavin-carrier protein from egg white; yolk and blood from laying hens	Analogs of riboflavin	Aminoalkylated Sepharose 4B or Bio-Gel P-150	MERRILL and MC CORMICK (1978).
Rifamycin-binding protein from Escherichia coli	N-Demethylrifampicin	CNBr-activated Sepharose 4B	SEYDEL et al. (1981).
S-100-Binding proteins and calmodulinbinding proteins (with Ca^{2+} - dependent affinity) from bovine brain	S-100 Protein and calmodulin	Sepharose 4B	GOPALAKRISHNA et al. (1985).
Saliva receptor from Streptococcus mutants	Monoclonal antibody	CNBr-activated Sepharose 4B	ACKERMANS et al. (1985).
Sequence-specific DNA binding proteins from HeLa (human) cells	Sequence-specific synthetic DNA labeled with ^{32}P	Sepharose CL-2B after CNBr-activation	KADONAGA and TJIAN (1986).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Sex hormone binding globulin from human plasma	Androstanediol-3-hemisuccinate and Cibacron Blue F3G-A	Sepharose 4B after CNBr-activation with hexamethylenediamine (carbodiimide technique) and Sepharose 4B heated to 45 $^{\circ}$ C	IQBAL and JOHNSON (1979).
Sex steroid binding protein	Androstanediol hemisuccinate	3;3'-Diaminodipropylamine Sepharose CL-4B	SANTA-COLOMA et al. (1987).
Soluble [^{125}I-Tyr$_{10}$]monoiodoglucagon receptor from rat liver membranes	Wheat germ lectin	CNBr-activated Sepharose CL-4B	IYENGAR and HERBERG (1985).
Specific binding protein for human growth hormone (HGH) from human serum	HGH	Affi-Gel 15	HERINGTON et al. (1986).
Specific growth hormone binding protein from rabbit liver cytosol	Concanavalin A and human growth hormone	Con A- Sepharose and Affi-Gel 15	YMER et al. (1984).
Specific laminin-binding protein from murine fibrosarcoma cells	Laminin	Sepharose 4B	MALINOFF and WICHA (1983).
Steroid holoreceptors from mouse liver and kidney (characteristics of DNA-binding sites)	Oligo(dG); oligo(dT); oligo(dC) and oligo(dA)	Oligo(dG)-;oligo(dT)-;oligo(dC)- and oligo(dA)-celluloses	GROSS et al. (1982).
Target cell receptor for IgE from rat basophilic leukemia cells	Monoclonal IgE	CNBr-activated Sepharose CL-4B	CONRAD and FROESE (1978).
Testicular lutropin receptor	Human choriogonadotropin	Agarose-adipic acid hydrazide	JALLAL et al. (1986).
Tetrodoxin binding component of voltage-sensitive sodium channel from electroplax of Electrophorus electricus in immune complex with monoclonal antibody	Protein A	Protein A-Sepharose	NAKAYAMA et al. (1982).
Thyroid hormone receptors from rat liver nuclei	L-Triiodothyronine	AH-Sepharose with diactivated N-hydroxysuccinimide ester derived from glutaric acid	LATHAM et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Thyroxine-binding globulin (TBG) from pooled whole human serum	Antibodies against TBG from sheep	CNBr-activated Sepharose 4B	KENNEDY et al. (1983).
Tissue factor from human brain and placenta	Monoclonal immunoglobulin IgG1 HTF1-7B8	Affi-Gel 10	CARSON et al. (1987).
Transferrin receptor from various human cell lines in tissue culture (lymphocytes; fibroblasts; carcinoma)	Transferrin (ovalbumin-linked Sepharose 4B as a control for nonspecific binding)	CNBr-activated Sepharose 4B	STEIN and SUSSMAM (1983).
Tumor-derived capillary endothelial cell growth factor from rat	Heparin	Heparin-Sepharose	SHING et al. (1984).
Two soluble forms of human interleukin-2 receptor from Chinese hamster ovary cells	Interleukin-2	Silica-based polyaldehyde NuGel-AF coupled by reductive amination in presence of sodium cyanoborohydride	WEBER et al. (1988).
Type I insulin-like growth factor (IGF) receptor from human placenta	Biosynthetic IGF I	CNBr-activated Sepharose	MALY and LUTHI (1986).
Urate binding protein from human serum	8-Amino-2;6-dihydroxypurine	CNBr-activated Sepharose 4B with adipic acid hydrazide	MAZZONI et al. (1982).
Vitamin D-binding protein from human plasma	25-Hydroxyvitamin D_3-3β-(1;2-epoxypropyl)-ether	Sepharose CL-6B with butanediol diglycidyl ether (in presence of sodium borohydride); sodium thiosulfate and dithiotreitol	LINK et al. (1986).
Z-DNA-binding proteins (containing proteins with strand transferase activity) from human lymphoblastic cell line	Biotinylated Z-DNA (left-handed conformation of the DNA double helix)	Agarose-streptavidin	FISHEL et al. (1990).

PROTEINS AND PEPTIDES CONTAINING -SH GROUP

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
-SH Fragment recieved after CNBr cleavage of S-sulphenyl sulphonate derivative of 3-phosphoglycerate kinase (EC 2.7.2.3)	p-Aminophenylmercuric acid	Sepharose 4B	FATTOUM et al. (1978). p
Amino acids; peptides and proteins containing -SH grou	4-Chlorocarbonyl-2-nitrophenyl-hydrazonopropanedinitrile	Spheron Ara 1000	PODHRADSKY et al. (1985).
β-^{32}P-Labeled[γ-S]GTP	p-Chloromercuribenzoate	Bio-Gel A-15M with ethylenediamine	REEVE and HUANG (1983).
Cysteine-containing fragment from β-chain of bovine hemoglobin	2;2'-dipyridyl disulphide	Silochrom (silica of 680-700 Å pore size diameter)	EGOROV et al. (1977).
Cysteine-containing histones	p-Chloromercuribenzoate	Sepharose 4B with ethylenediamine	RUIZ-CARRILLO (1974).
Cysteinyl peptides from potato and rabbit muscle phosphorylases (EC 2.4.1.1)	2-Pyridyl disulphide hydroxypropylether	Thiopropyl-Sepharose 6B	NAKANO et al. (1980).
Sulfhydryl-containing peptides from parvalbumin; mercaptalbumin and ceruloplasmin	Glutathione-2-pyridyl disulfide	Activated thiol-Sepharose 4B	EGOROV et al. (1975).
PROTEINS AND OTHER BIOLOGICALLY ACTIVE COMPOUNDS			
Actin and actin-associated proteins from cell homogenate	Myosin	Insoluble myosin thick filaments	LEONARDI and RUBIN (1981).
Actin (protein component) from membrane fragments enriched in acetylcholine receptor from Torpedo californica electroplax	Deoxyribonuclease I	Sepharose 2B after CNBr-activation	STRADER et al. (1980).
ADP-Ribosylated nuclear proteins from rat liver nuclei	m-Aminophenyl boronic acid	Hydrazide derivative of Bio-Gel P60	OKAYAMA et al. (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Albumin from human serum	Cholic acid	AH-Sepharose 4B	PATTINSON et al. (1980).
α-Cobratoxin	Acetylcholine receptor	Nylon tubes activated by partial hydrolysis + coupling by cross-linking with glutaraldehyde or through carbodiimide-activated COOH-groups	SUNDARAM (1983).
α-Fetoprotein (AFP) from human amniotic fluid (15th and 18th weeks pregnancy)	Monoclonal antibodies against human AFP	CNBr-activated Sepharose 4B	STENMAN et al. (1981).
α-Fetoprotein from human cord serum	Antibodies against human α-fetoprotein from rabbit	CNBr-activated Sepharose 4B	WATANABE et al. (1982).
α-Fetoprotein in serum of patients with tumor of hepatic parenchyma	Concanavalin A	Con A-Sepharose 4B	BUAMAH et al. (1984).
α-Neurotoxin (α-cobratoxin) from Naja naja siamensis	Acetylcholine receptor	Nylon tubes	YANG et al. (1981).
α2-Macroglobulin from human plasma	Cibacron Blue F3GA	Affi-Gel Blue	ARNAUD and GIANAZZA (1982).
Amnion interferon (human)	Bovine plasma albumin	Sepharose 4B	FERREIRA et al. (1981).
Antithrombin III from defibrinated plasma	N-Acetylated heparin (degradated by heparinase)	AH-Sepharose 4B	TOMONO et al. (1984).
Antithrombin III from human plasma (fraction IV-1)	Heparin (immobilization by six different methods)	Sepharose CL-4B or AH-Sepharose	MITRA et al. (1986).
Apohaemopexin from human blood serum and Cohn fraction IV	Haemin	Sepharose 4B with epichlorohydrin; 2-mercaptoethanol; 1;4-dibromobutane and ammonia solution	ŠTROP et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Apolipoprotein A-I-containing lipoproteins from human blood	Antibodies against human apolipoproteins from rabbits (purified by apolipoproteins coupled to CNBr-activated Sepharose 4B)	Formyl-Cellufine (formylated derivative of cellulose)	OHTA et al. (1989).
Apolipoprotein B containg lipoproteins (LDL) - etracorporeal removal from patients with familial hypercholesterolemia	F(ab')$_2$ fragments of sheep anti-LDL antibodies	Sepharose CL 4B	RIESEN et al. (1988).
Apolipoprotein E enriched very-low-density lipoproteins from human plasma	Heparin	Heparin-Sepharose CL-6B	WEISWEILER (1988).
ATP-hemoglobin	ATP (periodate-oxidized)	Sepharose 4B with adipic acid dihydrazide	HSIA et al. (1986).
Bacteriophage T4 proteins essential for DNA replication and genetic recombination from Escherichia coli	Bacteriophage T4 helix-destabilizing protein (product of gene 32)	Affi-Gel 10	FORMOSA et al. (1983).
β-Galactosidase-tagged replication initiator protein from Escherichia coli	p-Aminophenyl-β-D-thio-galactoside	CNBr- activated Sepharose 4B with hexamethylenediamine and succinic anhydride	GERMINO et al. (1983).
Bile pigments from microsomal preparations	Human serum albumin	Bio-Gel A-5m	OKUDA et al. (1983).
Biologically active pertussis toxin from Bordetella pertussis	Heat-treated bovine fetuin	CNBr-activated Sepharose CL-4B	CHONG and KLEIN (1989).
Blood coagulation Factor V from human plasma	Monoclonal antibody against Factor V	Sepharose	KATZMANN et al. (1981).
Carcinogen binding proteins from mouse liver (= receptor-like binding proteins for aromatic hydrocarbon carcinogens)	1- Aminopyrene	Epoxy-activated Sepharose 6B	COLLINS et al. (1985).
Catecholamines from human urine	m-Aminophenylboronic acid	Boric acid gel	GELIJKENS and DE LEENHEER (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Cholera toxin	NAD^+	Sepharose 4B with 6-hexanoic acid	ANTONI et al. (1978).
Cholesterol-induced high density lipoproteins from hypercholesterolemic rat plasma	Concanavalin A	Con A- Sepharose 4B	MITAMURA (1982).
Cholinergic proteins	(10-Aminodecyl) trimethylammonium	Sepharose with spermine and succinic anhydride	RIGGIO et al. (1980).
Chromatin proteins from Ehrlich ascites tumour cells	Cibacron Blue F3GA	Blue Sepharose CL-6B	KRISTENSEN and HOLTLUND (1980).
Chylomicrons from rat lymph (lipoprotein)	Heparin	Heparin-Sepharose CL-6B	BORENSZTAJN et al. (1985).
Coagulation factor VIII from human plasma	Heparin	CNBr- activated Sepharose 4B	MADARAS et al. (1978).
Collagens and procollagens of different type - from human tissue after limited proteolysis	2;2'-Dipyridyl disulphide	Thiol-activated Sepharose 4B	DEYL and ADAM (1989).
Complement component C3b from human; rabbit and bovine serum	Human factor H	Sepharose 4B	SCOTT and FOTHERGILL (1982).
Complement component proenzymic Clr from human plasma	Anti-Cls immunoglobulins (purified by Cls-Sepharose 4B)	Sepharose 4B after CNBr-activation	VILLIERS et al. (1982).
Conjugate of ovalbumin with biotin-labeled synthetic copolymer of D-glutamic acid and D-lysine	Monomeric avidin	CNBr- activated Sepharose 4B	LIU et al. (1979).
Dermatan sulfate and heparin (fractionation) from their commercial preparations	Heparin cofactor II (noncovalently bound)	Concanavalin A-agarose	GRIFFITH and MARBET (1983).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Digoxin (automated assay)	Ouabain-bovine serum albumin conjugate	Sephadex G-10 after periodate oxidation and borohydride reduction	LEFLAR et al. (1984).
Diphtheria toxin	Cibacron Blue F3GA	Blue Sepharose CL-6B	ANTONI et al. (1983).
Enantiomers (sulphoxide; sulphoxime; benzoylamino acid - separation)	Bovine serum albumin	Activated CH-Sepharose 4B	ALLENMARK and BOMGREN (1982).
Enantiomers of warfarin and tryptophan	Serum albumin	Sepharose 4B	LAGERCRANTZ et al. (1981).
Estrogen-regulated cytoplasmic protein	Monoclonal antibody	Affi-Gel 10	ADAMS et al. (1983).
Exotoxin A of Pseudomonas aeruginosa	NAD	Agarose-N^6-(aminohexyl)carbamoylmethyl-NAD	CUKOR and NOWAK (1982).
Factor VIII from human plasma	Anti-human factor VIII-vWf from rabbit	Sephacryl S-1000 after periodate oxidation	HORNSEY et al. (1986).
Ferredoxin from bovine liver mitochondria	Cytochrome c (from horse heart) with free carboxyl groups modified with histamine	Sepharose 4B with epichlorohydrin; ethylenediamine and bromoacetylic acid	SHKUMATOV et al. (1983).
Fibronectin from human blood	Denatured collagen	Spheron 300 after CNBr-activation	MITINA et al. (1985).
Fibronectin from plasma	Gelatin	CNBr-activated Sepharose 4B	RUOSLAHTI et al. (1982).
Flavoproteins	Flavins	CNBr-activated Sepharose 4B with diaminoalkyl spacers or aminoalkyl derivatives of Bio-Gel P-150	MERRIL and MC CORMICK (1980a).
Fodrin subunits from bovine brain	Calmodulin	Activated Sepharose 4B	GLENNEY and WEBER (1985).
Folates from tissue extract of rat liver or from Lactobacillus casei	Folate-binding protein from dried whey of cow's milk	CNBr-activated Sepharose 4B	SELHUB et al. (1988).
Globin; albumin or hemopexin from bovine blood	Hematoporphyrin or hematin	Sepharose 4B with hexamethylenediamine	OLSEN (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glycated albumin from blood samples of diabetics and non- diabetics	m-Aminophenylboronic acid	GLYCOGEL (boronate agarose)	RENDELL et al. (1985).
Glycated hemoglobin from blood of uremic patients	m-Aminophenylboronic acid	Sepharose CL-6B activated with carbonyl diimidazole	BRUNS et al. (1984).
Glycosylated haemoglobins from diabetic and non-diabetic individuals	m-Aminophenylboronic acid	PBA-Matrex Gel	MIDDLE et al. (1983).
Haemopexin from Cohn Fraction IV-4 of human serum	Haemin with 1;1'-carbonyldiimidazole	Aminoethyl-agarose	TAKAHASHI et al. (1985).
Heavy meromyosin and tropomyosin	Acetamidofluorescein-labeled actin (stabilized with phalloidin)	CNBr-activated Sepharose 4B with anti-fluorescein IgG	LUNA et al. (1982).
Hemoglobin (equilibrium measurements at physiological pH)	Cytoplasmic fragment from human erythrocyte membrane	CNBr-activated Sepharose 4B	CHETRITE and CASSOLY (1985).
Heparan sulfate proteoglycans from human neuroblastoma cells	Human platelet factor-4 (purified by Sepharose with intestinal mucosa heparin)	Sepharose 4B	MARESH et al. (1984).
Heparan sulfate species	Oxyheparan sulfates (after brief periodate oxidation)	CNBr-activated Sepharose 4B with adipic acid dihydrazide	FRANSSON (1982).
Heparin	Human antithrombin III	Bio-Gel A 50m	NADER et al. (1981).
Heparin	Human antithrombin III	CH-Sepharose	NESHEIM et al. (1986).
Heterogenous nuclear ribonucleoprotein (hnRNP) from buffalo and goat liver poly A-hnRNP particles	Oligo-deoxythymidine	Oligo- dT cellulose	KUMAR and ALI (1986).
High density lipoproteins from human serum	Antibodies against apolipoprotein A-I (apo A-I) from goat serum (purified by apo A- I-Sepharose)	CNBr-activated Sepharose 4B	MC VICAR et al. (1984).
High density lipoproteins from plasma of patient with alcoholic hepatitis	Heparin	CNBr-activated Sepharose 4B	MARCEL et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
High mobility group nonhistone chromosomal proteins from chicken erythrocyte	Histone H5 from chromatin	CNBr-activated Sepharose 2B	WEN and REECK (1984).
Human α-1-fetoprotein	Antibodies of intermediate avidity derived from immune complexes	CNBr-activated Sepharose	FOLKERSEN et al. (1985).
Human fibroblast interferon	Concanavalin A	Con A-Sepharose	FRIESEN et al. (1981).
Human high density lipoproteins	Heparin	Sepharose 6B	WEISGRABER and MAHLEY (1980).
Human immune interferon	Triazinyl dyes	MATREX Blue A and B; MATREX Green A; MATREX Orange A; MATREX Red A and CM Affi-Gel Blue; DEAE Affi-Gel Blue	COPPENHAVER (1985).
Human leukocyte interferon from leukocytes infected with Newcastle disease virus	Concanavalin A	Con A-Sepharose	GROB and CHADHA (1979).
Human low density lipoproteins	Concanavalin A	Con A- Sepharose 4B	SEGANTI et al. (1980).
Human lymphoblastoid interferon from Namalwa cells	Monoclonal antibody to human leukocyte interferon	CNBr-activated Sepharo- se	SECHER and BURKE (1980).
Human serum albumin	Cibacron Blue F3G	Epoxy-activated Sepharose 6B	LEATHERBARROW and DEAN (1980).
Hyaluronic acid	Proteoglycan monomers and common tryptic fragment of two link proteins	CH-Sepharose 4B	LE GLEDIC et al. (1982).
^{125}I-succinoylstreptavidin	Iminobiotin hydrobromide	AH- Sepharose 4B	HOFMANN et al. (1980).
Immunotoxins containing abrin A and ricin A-chains	Cibacron Blue F3GA	Blue Sepharose CL-6B	KNOWLES and THORPE (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Initiation factors derived from native 40S ribosomal subunits from embryonic chick muscle	Myosin mRNA and globin mRNA	CNBr-activated Sepharose 4B	HEYWOOD and KENNEDY (1979).
Interferon-γ from human leukocytes (IFN-γ)	Monoclonal antibodies against IFN-γ	CNBr-activated Sepharose 4B	MIYATA et al. (1986).
Interferon-induced human guanylatebinding protein	Guanosine 5'- monophosphate (GMP)	GMP-agarose	CHENG et al. (1985).
Intermediate filament proteins from Ehrlich ascites tumour cells and cells of five main classes of Chordates	Single-stranded calf thymus DNA (heated to 100° C; cooled rapidly and mixed with cellulase)	Cellulase CF11 with DNA lyophilized; mixed in absolute ethanol and ultraviolet light-irradiated for 1 min in ice-bath	NELSON and TRAUB (1982).
Lactoferrin from human whey	Cibacron Blue F3G-A	Blue Sepharose	BEZWODA and MANSOOR (1986).
Lipoprotein complexes from human aorta	Antibodies against lipoprotein LDL from rabbits	CNBr-activated Sepharose 4B	CAMEJO et al. (1985).
Lipoprotein subfractions from human plasma	Heparin	CNBr-activated Sepharose 4B	MARCEL et al. (1981).
Lipoproteins containing apolipoprotein B (LP-B) from human plasma	Affinity-purified antibodies against LP-B	Sepharose CL-4B	MC CONATHY et al. (1985).
Lipoproteins from low density lipoprotein fraction of rat plasma	Heparin	Sepharose 4B	SURI et al. (1984).
Low- and high-density lipoproteins from human plasma	Cholic acid and cholesterol hydrogen succinate	Sepharose 4B after CNBr-activation with hexamethylenediamine; cystamine or cysteamine	WICHMAN (1979).
Lymphocytosis-promoting factor from Bordetella pertussis	Fetuin	Sepharose 4B	ASKELOF and GILLENIUS (1982).
Macrophage migration inhibition factor	Fucosamine	Fucosamine ε– amino caproic acid agarose	FOX et al. (1978).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Major membrane protein from Toxoplasma gondii	Monoclonal antibody	CNBr-activated Sepharose 4B	KASPER et al. (1983).
Maltodextrin-specific pore protein (maltoporin) from outer membrane of Echerichia coli	Soluble starch	Sepharose 6B with 1;4-butanediol diglycidyl ether	CLUNE et al. (1984).
Measles virus proteins from infected hamster brain tissue	IgG from rabbit hyperimmune anti-measles serum	CNBr-activated Sepharose 4B	SWOVELAND (1986).
Membrane proteins from lymphocytes of patients with chronic lymphocytic leukemia	Phenylboronic acid	PBA-50 Matrex Gel	WILLIAMS et al. (1982).
Migration inhibitory factor	Gangliosides from bovine brain	Sepharose 4B with poly-L-lysine	LIU et al. (1982).
Myosin subfragment 1 from rabbit fast or slow twitch	N^6-(6-Aminohexyl)-ADP; G-actin or F-actin	Sepharose 4B after CNBr-activation	WINSTANLEY et al. (1979).
Natural murine γ-interferon (MuIFN-γ)	Monoclonal antibody against MuIFN-γ	DEAE Affi-Gel Blue	GREENFIELD et al. (1986).
Nuclear factor I (NF-I; site-specific DNA binding protein) from crude HeLA nuclear extract	Nonspecific (Escherichia coli) DNA and plasmid (pKB67-88) containing 88 copies of NF-I binding site	Cellulose Cellex 410	ROSENFELD and KELLY (1986).
Oligomeric α-toxin from Staphylococcus aureus Wood 46	Digitonin	Sepharose 4B	SCHAEG et al. (1985).
Osteogenin (bone inductive protein) - from demineralized rat incisors	Heparin	Heparin-Sepharose CL-6B	KATZ and REDDI (1988).
Partialy biotinylated S-1 subfragment (heavy chain) of rabbit skeletal muscle myosin in complex with streptoavidin	Lipoic acid	Affi-Gel 102 (carbodiimide technique)	YAMAMOTO and SEKINE (1987).
Peroxidase activity regulatory protein	Peroxidase	CNBr- activated Sepharose 4B	DUMITRESCU et al. (1982).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Pertussis toxin (protein) from Bordetella pertussis	Cibacron Blue F3GA and fetuin	Affi-Gel Blue and CNBr-activated Sepharose 4B	SEKURA et al. (1983).
Phytochrome from dark-grown rye	Cibacron Blue 3GA	Affi-Gel Blue	SMITH and DANIELS (1981).
Phytochrome from oat seedlings	Cibacron Blue F3GA	Affi-Gel Blue	SONG et al. (1981).
Plant tubulin from azuki bean	Ethyl N-(3-carboxyphenyl)carbamate	Sepharose 4B with ethylenediamine	MIZUNO et al. (1981).
Plasma membrane fractions from calf thymocytes	Ouabanin	CH- Sepharose 4B (carbodiimide technique)	SZAMEL et al. (1987).
Plasma proteins from late pregnancy plasma	Heparin	Heparin-Sepharose	TEISNER et al. (1983).
Plasminogen activator from human blood plasma	Fibrin	Hyflo Super-Cel or Celite	HUSAIN et al. (1981).
Poly(A)-containing ribonucleoproteins from cryptobiotic embryos of Artemia salina	Oligothymidylic acid	Oligo (dT)-cellulose T2	DE MAYER et al. (1980).
Polymerization-competent cytoplasmic actin from rabbit skeletal muscle	DNAse I	Sepharose 4B after CNBr-activation	ZECHEL (1980).
Pregnancy-specific β2/1-glycoprotein from human retroplacental pregnancy serum	Cibacron blue 3G-A; Procion turquoside MX-G and red H-8BN; and anti-normal human serum antibodies	Matrex Gel	MC FARTHING et al. (1982).
Protamine and different histone fractions (study of interaction)	Histone H4	CNBr-activated Sepharose 4B	KHRAPUNOV et al. (1980).
Protein synthesis initiation factor 2 from chicken reticulocytes	β; γ-Methylene guanosine 5'triphosphate treated with periodate	Sepharose with hydrazide	LEE (1984).
Proteins from Drosophila melanogaster 80S ribosomes	5S Ribosomal RNA or poly(A) after periodate oxidation	CNBr- activated Sepharose 4B with adipic acid dihydrazide	STARK and CHOOI (1985).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Proviral coding strand of avian myeloblastosis virus (AMV) from DNA of leukemic chicken myeloblast	AMV 35S viral RNA	CNBr- activated Sepharose	SOM and NAYAK (1978).
Rec A protein and Rec A nucleoprotein complexes - from soluble cell extracts prepared from Escherichia coli	Rec A protein from Escherichia coli	Affi-Gel 10	FREITAG and MC ENTEE (1988).
Recombinant human leukocyte interferon from Escherichia coli	Monoclonal antibodies against leukocyte interferon	Affi-Gel 10	STAEHELIN et al. (1981).
Ribosomal proteins from 30S and 50S ribosomal subunits from Escherichia coli	Chloramphenicol and erythromycin	Epoxy activated Sepharose 6B	SURYANARAYANA (1983).
Ribosomal proteins from Escherichia coli	5S RNA and tRNA after periodate oxidation	CNBr-activated Sepharose 4B with adipic acid dihydrazide	BURRELL and HOROWITZ (1977).
Ribosomal proteins from rat liver	Mixed elongator-tRNAs from Saccharomyces cerevisiae after periodate oxidation	Sepharose 4B after CNBr-activation with adipic acid dihydrazide	ULBRICH et al. (1980).
Ricin toxin (protein) from Ricinus communis	p-Aminophenyl-1-thio-β-D-galactopyranoside	Sepharose 4B	SIMMONS and RUSSELL (1985).
Stabilized band 3 protein from human erythrocyte membrane	p- (Chloromecuri) benzoic acid	Sepharose 4B with ethylenediamine	LUKACOVIC et al. (1981).
Streptavidin from Streptomyces avidinii	Iminobiotin hydroxysuccinimide hydrobromide	CL-Sepharose activated by p- nitrophenyl chloroformate method with hexamethylenediamine	BAYER et al. (1986).
Subcomponent C1q of first component of complement from rabbit serum	Human IgG	Sepharose 6B with ethylenediamine; p-nitrobenzoylamide; reduced by sodium dithionite and diazotized with sodium nitrite	MORI (1980).
Sulphamethizole and salicylic acid	Monomeric human serum albumin	Activated CH-Sepharose 4B	NAKANO et al. (1980).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Thioethers from urine (determination)	p-Chloromercuribenzoate	Affi-Gel 501	BUFFONI et al. (1982).
Thiol compound dihydrolipoic acid	p-Aminophenylarsine oxide	CNBr-activated Sepharose 4B	HANNESTAD et al. (1982).
Thioredoxin fA and fB from leaf extract	Cibacron Blue F3GA and fructose-bisphosphatase (EC 3.1.3.11)	Blue-Sepharose CL-6B and CNBr-activated Sepharose 4B	BUC et al. (1984).
Tissue factor (initiator of blood coagulation) from human brain	Human factor VII	Affi-Gel 15	GUHA et al. (1986).
Tubulin from bovine brain	Deacetylcolchicine	Sepharose 4B with 1;5-diaminepentane and succinic anhydride	KOCHA et al. (1986).
Tubulin from rat brain	Lactoperoxidase	Affi-Gel 10	ROUSSET and WOLFF (1980).
Two iodinated tetanus toxin fractions	Partial hydrolysed fractions of polysialogangliosides	Porous silica beads Spherosil XOC 005 coated with DEAE-dextran (dried at 80 °C) with periodate oxidation and $NaBH_4$ reduction	LAZAROVICI et al. (1984).
Urinary folates	Folate-binding protein (purified by methotrexate-Sepharose 4B)	Sepharose 4B	SELHUB et al. (1980).
Venom component toxins from Chironex fleckeri	Monoclonal antibodies against Chironex venom	Affi-Gel 10	NAGUIB et al. (1988).
Venom from Chironex fleckeri (sea wasp)	Anti-venom antibody	Sepharose 4B	CALTON and BURNETT (1986).
SPECIFIC PEPTIDES			
Actin-binding fragment of fibronectin	Actin	Sepharose 2B	KESKI-OJA and YAMADA (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Arginine-containing peptides after modification with cyclohexane-1;2-dione and digestion by protease	m-Aminophenylboronic acid	Epoxy-activated Sepharose 6B	ROSE et al. (1981).
Biantennary glycopeptides possesing α-L-fucosyl group attached to L-asparagine-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl residue	Vicia faba (FAVA) lectin	Sepharose 4B with hydrazide	KATAGIRI et al. (1984).
C-Terminal peptides from protease digests of proteins	Anhydro-bovine trypsin	Sepharose 4B after CNBr-activation	KUMAZAKI et al. (1987).
DNP-Containing peptides from tryptic digest of DNP-labeled ribonuclease	Goat-anti-DNP antibodies	Sepharose 4B after periodate oxidation with polyacrylhydrazide	MIRON and WILCHEK (1981).
Fibrin-binding peptides from cathepsin D digest of human plasma fibrinogen	Fibrin (thrombin-activated immobilized fibrinogen)	Sepharose 6B after CNBr-activation	HORMANN and SEIDL (1980).
Fibrin-binding peptides from chymotryptic digest of human plasma fibronectin	Fibrin (thrombin-activated immobilized fibrinogen) and gelatin	Sepharose 6B after CNBr-activation	SEIDL and HORMANN (1983).
Fragment peptides from proteolytic digest from reduced and carboxymethylated human urinary trypsin inhibitor	Concanavalin A	Con A-Sepharose	WACHTER and HOCHSTRASSER (1981).
Glycated peptides of ribonuclease A after glycation of amino groups in protein with glucose	3-Aminophenylboronic acid	Affi-Gel 601	WATKINS et al. (1985).
Glycopeptide antibiotics (teicoplanin; vancomycin; ristocetin A)	D-Alanyl-D-alanine	Activated CH-Sepharose 4B	CORTI and CASSANI (1985).
Glycopeptide antibiotics (vancomycin and aridicin) from low titer fermentation broth	D-Alanyl-D-alanine	Affi-Gel 10	FOLENA-WASSERMAN et al. (1987).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Glycopeptide I from subtilisin digest of raw starch-adsorbable glucoamylase I from Aspergillus awamori	Raw starch	Raw starch	HAYASHIDA et al. (1982).
Glycopeptides - from Pronase digestion of bovine lens epithelial cells	Concanavalin A	Con A-Sepharose	MOCZAR et al. (1982).
Glycopeptides from baby hamster kidney cells after digestion with Pronase	Concanavalin A and lentil lectin	Con A-Sepharose and Lentil lectin-Sepharose	HUGHES and MILLS (1983).
Glycopeptides from mouse lymphoma BW5147 cells	Datura stramonium agglutinin	Affi-Gel 10	CUMMINGS and KORNFELD (1984).
Glycopeptides from pepsin digest of ovalbumin	Concanavalin A	Con A-Sepharose	IWASE et al. (1981).
Glycopeptides from tryptic digest of reduced; carboxymethylated and citraconylated human urinary trypsin inhibitor	Concanavalin A	Con A-Sepharose	HOCHSTRASSER et al. (1981).
Glycopeptides of synaptosomal plasma membrane glycoproteins from adult rat forebrain	Concanavalin A	Con A-Sepharose 4B	REEBER et al. (1984).
Glycosylated peptides from tryptic digestion of albumin from blood of diabetic patient	m-Aminophenylboronic acid	Affi-Gel 601	IBERG and FLUCKIGER (1986).
Immunoreactive fibroblast growth factor-related polypeptides from lung; plasma; brain; ovary; corpus luteum; pituitary; chrondrosarcoma of bovine; porcine or rat origin	Heparin	Heparin-Sepharose column	BERTOLINI et al. (1989).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Immunoreactive peptides β- and γ-lipotropin (β- and γ-LPH) and β-endorphin (β-EP) from human plasma	Antibodies against conjugate of bovine serum albumin with β- LPH or β-EP from rabbits (purified by Sepharose with coupled antigens)	Activated CH-Sepharose 4B	YAMAGUCHI et al. (1980).
Interleukin 2 (IL2) (peptid) from human T leukemic cells	Monoclonal antibody against IL2	Affi-Gel 10	MINGARI et al. (1985).
Labelled peptide from total proteolytic digest of nitrotyrosyl 3-phosphoglycerate kinase (EC 2.7.2.3)	Antibodies against nitrotyrosine from goat	Sepharose 4B	DESVAGES et al. (1980).
Macroglycopeptides from trypsin digest of human platelet plasma membranes	Wheat germ agglutinin	CNBr-activated Sepharose 4B	CARNAHAN and CUNNINGHAM (1983).
Mannose containing glycopeptides from human teratocarcinoma- derived cells	Concanavalin A	Con A-Sepharose	RASILO et al. (1980).
Murein precursors (lactyl-pentapeptides of the structure R-D-Ala-D-Ala)	Vancomycin	CH-Sepharose 4B	DE PEDRO and SCHWARZ (1980).
Peptides containing arginine or lysine residues at COOH- terminal position	Anhydrotrypsin	Sepharose 4B after CNBr- activation	ISHII et al. (1983).
Peptides; apolipoproteins; melittin (probing affinity of peptides for amphiphilic surfaces)	Egg yolk L-α-lecithin	Polystyrene-divinylbenzene beads	RETZINGER et al. (1985).
Phycoerythrin chromopeptides from Porphyridium cruentum	D;L- Histidine	Sepharose 4B with epichlorohydrin in presence of $NaBH_4$	RABIER et al. (1984).
Polypeptide from mitochondria (m.W. 32000) of brown adipose tissue from rats	Guanosine-5'-diphosphate	GDP-agarose	RICQUIER et al. (1979).
Radioactively labelled glycopeptides from human myeloma IgG	Lentil and pea lectins	Lentil lectin-Sepharose and Sepharose	KORNFELD et al. (1981).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Seminalplasmin (antimicrobial peptide) from bull spermatozoa	Calmodulin	Sepharose 6B using triethylamine for neutral CNBr- activation	COMTE et al. (1986).
Teicoplanin (glycopeptide antibiotic) from human plasma and urine (determination)	D-Alanyl-D-alanine	D-Alanyl-D-alanine-ε-aminocaproyl Sepharose CL-6B (prepacked column)	RIVA et al. (1987).
Thyroglobulin glycopetides from porcine thyroid glands and follicles in serum-free culture	Concanavalin A; leukophytohemagglutinin; erythrophytohemagglutinin (L- and E-PHA); Bandieraea simplicifolia; ricin; Lotus tetraglonolobus and wheat germ agglutinin	Con A-Sepharose; Lentil-Sepharose; Phaseolus vulgaris E-PHA and L-PHA-agarose; Wheat Germ Agglutinin-agarose; Bandieraea simplicifolia and Ricin-agarose and Lotus tetraglonolobus-agarose	RONIN et al. (1986).
VIRUSES			
Bovine viral diarrhea virus and its surface proteins	Crotalaria juncea lectin	Crotalaria juncea lectin column	KARNAS et al. (1983).
Bovine viral diarrheavirus	Crotolaria juncea lectin	Sepharose 2B	MORENO-LOPEZ et al. (1981).
Carnation mottle virus	Antibody	CNBr-activated Sepharose 4B	DE BORTOLI and ROGGERO (1985).
Encephalomyocarditis virus (interaction)	Glycophorin (sialoglycoprotein in human erythrocyte surface membrane)	Wheat germ agglutinin-Sepharose	PARDOE and BURNESS (1981).
Hepatitis A virus from human faeces	Immunoglobulin G purified from convalescent serum from patient with hepatitis A virus	Sepharose 4B after CNBr-activation	ELKANA et al. (1979).
Influenza virus	α- and β-Ketosides of neuraminic acid derivatives	Sepharose 4B	HOLMQUIST and NILSSON (1979).
Influenza viruses	γ-Globulin of rabbits immunized with influenza virus	Disulfide-linked γ-globulin with N-acetyl homocysteine thiolactone	SWEET et al. (1974).

Table 9.1. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Murine type-C virus p30 precursor protein	Single-stranded DNA	Cellulose	OROSZLAN et al. (1976).
Plant virus	Antibodies	Antibodies cross-linked by glutaraldehyde	LADIPO and DE ZOETEN (1971).
Poliovirus	Antibody	Sepharose 4B	BROWN et al. (1979).
Rabies virus	Concanavalin A and 8 other lectins	Con A- Sepharose and ULTROGEL AcA	DELAGNEAU et al. (1981).
Soybean mosaic virus (SMV)	Monoclonal antibodies specific for SMV	Affi-Gel 10	DIACO et al. (1986).
T4 Phage	Poly DL-lysine	Sepharose 2B	SUNDBERG and HOEGLUND (1973).
T80 Virus in complex with antibodies against porcine enterovirus (detection of antibodies to animal virus)	Rabbit anti-porcine serum antiserum and normal rabbit serum or γ-globulins isolated from them	Sera cross-linked with ethyl chloroformate or CNBr-activated Sepharose 4B	HAZLETT (1977).
Tick-borne encephalitis virus (TBE)	Monoclonal antibodies to TBE virus	CNBr-Sepharose	GRESIKOVA et al. (1984).
Tobacco mosaic virus	Anti-tobacco mosaic virus-antibodies	p- Aminobenzylcellulose	GALVEZ (1966).
Viral protein F2 from Sendai virus	Anti-Sendai virus F2 protein IgG from rabbit	Protein A-Sepharose CL-4B	VAN DER ZEE and WELLING (1985).

compound was isolated from the crude material by a single chromatographic step, combinations of bioaffinity chromatography with additional purification procedures are far more numerous. The general principles that must be observed in order to achieve a successful isolation were discussed in detail in Chapter 8. The conditions should be modified in particular instances on the basis of these principles. In order to provide the reader with information on individual affinity ligands that have actually been used, or commercially available immobilized affinity ligands, commercial names are given in all instances if they have been published. A review of these, with an indication of their chemical nature and the associeted procedures, is given in Table 9.4.

9.2 HIGH-PERFORMANCE LIQUID BIOAFFINITY CHROMATOGRAPHY

In high performance liquid bioaffinity chromatography (HPLBAC) the biospecificity of bioaffinity chromatography is combined with a high performance (pressure) technology based on rigid particles of uniform, small size (high performance liquid chromatography, HPLC). A comparison of bioaffinity chromatography methods using soft gel or porous and nonporous small hard particles has already been shown in Fig. 1.2. The advantages of HPLBAC are discussed in Section 2.2.

The essential characteristics of HPLBAC are summarized by Ohlson et al. (1989) in their review: "High performance affinity chromatography - A new tool in biotechnology". The separation times in HPLBAC are short (minutes) compared with hours for traditional, soft-gel bioaffinity chromatography. Time is saved not only in the application and elution phases but also during washing steps, and this can be of great importance when dealing with crude samples. In preparative applications, speed can also mean improvements in the quality of the compound being isolated, especially for fragile biomolecules that tend to denature during chromatography. The peaks of purified components (which are called ligates by these authors) are much sharper than those found with soft gel chromatography because of the reduced diffusion distances in the smaller HPLBAC beads. Resolution is preserved by running the chromatography under HPLC conditions to minimize dead volumes and other extra-column effects. The

bioaffinity aspect of HPLBAC often makes it much more selective than standard separation modes, such as ion-exchange and hydrophobic interaction. Sharper peaks simplify detection. The signal-to-noise ratio can be raised several fold. Furthermore, peak height (rather than area) can often be used to give quantitative estimates of sample components. HPLBAC users have at their disposal a wide selection of HPLC equipment, including high-speed pumps, sophisticated injection units, detectors of various kinds, auto-sampling devices and data handling capabilities. This enables them to fine-tune the separation process conveniently and to promote higher productivity.

Table 9.2 shows that HPLBAC has been applied in almost all areas of traditional bioaffinity chromatography. The table is organized in the same way as Table 9.1 in preceding section.

HPLBAC appears under many guises, some of them bearing commercial trademarks: fast affinity chromatography (FAC), fast protein liquid affinity chromatography (FPLAC), high speed affinity chromatography (HSAC), high performance affinity chromatography (HPAC) and high performance immunoaffinity chromatography (HPIAC) for antibody-antigen applications.

Pre-activated columns, containing e.g. epoxy, aldehyde, tresyl or other coupling groups, where the ligand is directly coupled during its passage through the column are offered by an ever-increasing number of suppliers. This is convenient and saves time in preparing HPLBAC columns because users do not have to concern themselves with the activation and packing of the support. A list of suppliers of HPLBAC matrices can be found in the review of Ohlson et al. (1989) mentioned above. The addresses of suppliers published in Science Supplement 1990 and by Taylor (1991) are presented in Table 6.5.

The rapid separation obtained with HPLBAC makes this technique especially valuable for analytical assays where time plays an important role. The analysis of drugs and metabolites in serum or plasma can easily be performed within minutes with high recovery rates. Employment of sensitive detection methods permits the analysis of minute amount of samples (Ernst-Cabrera and Wilchek, 1988a). Ohlson et al. (1988 b) analyzed transferrin in clinical serum samples. The analytical procedure lasted only

Table 9.2

High-performance liquid bioaffinity chromatography (HPLBAC)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
ANTIBODIES			
Anti-benzodiazepine antibodies	1-(2-aminoethyl)-7-chloro-1,3-dihydro-5-(2-fluorophenyl)-2H-1,4-benzodiazepine-2-one dihydrochloride	Ultraaffinity-EP column	GOLDMAN et al. (1986).
Antibodies against dexamethasone-21 hemisuccinate-bovine serum albumin conjugates from rabbits	[^{3}H]dexamethasone	Ultraffinity-EP column (with epoxy groups)	FORMSTECHER et al. (1986).
Antibodies against bovine serum albumin	Bovine serum albumin	Partisil with γ-glycidoxypropyltrimethoxysilane with acid hydrolysis diol silicia oxidated with $NaIO_4$ and reduced with $NaBH_4$;activated with chloroformates or 1,1'-carbodiimidazole	ERNST-CABRERA and WILCHEK (1986).
Human IgG antibody	Human IgG	Separon HEMA 1000	TAYLOR (1985).
Immunoglobulin G from rabbit serum	Protein A	Suspension of fluid-impervious silica microspheres in aqueous solution of γ-glycidoxypropyl trimethoxysilane after heating at 95° C oxidized by periodic acid and Protein A attached by reductive amination using cyanoborohydride	VARADY et al. (1988).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Immunoglobulins in blood serum	Protein A from Staphylococcus aureus	LiChrospher Si 4000 diol-bonded silica with (3-glycidoxypropyl) trimethoxysilane activated with 1,1'-carbonyldiimidazole	CROWLEY and WALTERS (1983).
Monoclonal antibodies (IgG 1 or 3) from mouse ascites	Protein A	Selecti Spher-10 Protein A	OHLSON and WIESLANDER (1987).
ANTIGENS AND HAPTENS			
B27 antigen from detergent solubilized membranes of human lymphocytes	Mouse monoclonal antibodies against human B27 antigen after biotinylation of periodate oxidized carbohydrate moieties by biotin hydrazide	Glass beads (diameter 1mm) after derivatization with 3-aminopropyl-triethoxysilane;N-hydroxysuccinimide and N,N'-dicyclohexyl carbodiimide coated with streptavidin	BABASHAK and PHILLIPS (1988).
Blood group A-active oligosaccharides	Monoclonal anti-blood group A antibody of IgM isotype	Silica Selectisper-10 concanavalin A column	DAKOUR et al. (1987).
Cortisol from plasma and urine	Antibodies against cortisol-3-carboxymethyloxime-bovine serum albumin from rabbits	LiChrosorb Si 60 with γ-glycidoxypropyltrimethoxysilane; diethyl ether and sodium metaperiodate	NILSSON (1983).
5,5-Diphenylhydantoin (phenytoin) from human plasma	Phenytoin-specific polyclonal immunoglobulin	LiChrosorb Si 60 with glycidoxypropyltrimethoxy derivate; oxidized to aldehyde form;converted to carboxylic acid derivate and esterified with N-hydroxysuccinimide	JOHANSSON (1986).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Human immunoglobulin G (IgG) and Insulin	Antibodies against human IgG from goat and Monoclonal antibodies against beef insulin	Porous glass spheres (LiChrosphere Si 1000) with glycidoxypropyltrimethoxysilane after periodate oxidation	SPORTSMAN and WILSON (1980).
Human serum albumin (HSA)	Anti-HSA-antibodies from rabbit	Lichrosorb Si 60 with γ-glycidoxypropyltrimethoxysilane (Glycosil) with glycol groups (from oxirane groups converted by heating) after periodate oxidation	OHLSON et al. (1978).
Immunoglobulin E (IgE) - from serum of both adult and pediatric patients	Monoclonal antibodies against human IgE	Controlled-pore glass beads with carbonyl diimidazole-reactive side chains derived on their glycophase surface with attached Protein A	PHILLIPS et al. (1985).
Protein A - monitoring in a fermentation brot	Immunoglobulin G (IgG)	IgG-HPLIC column	OHLSON et al. (1989).
Transferrin in human serum	Rabbit polyclonal antibodies against human transferrin	Selecti-Spher-10 Activated Tresyl HPLAC columns	OHLSON et al. (1988a).
ENZYMES			
Dehydrogenases:			
Alcohol dehydrogenase (EC 1.1.1.1) from horse liver and lactate dehydrogenase (EC 1.1.1.27) from pig heart	Cibacron Blue F3G-A with 1,6-diaminohexane	Silica gel (LiChrosorb Si 60) with γ-glycidoxypropyltrimethoxy silane	LOWE et al. (1981).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
L-Lactate dehydrogenase from crude rabbit muscle extract	Procion Blue MX-R dye	Spherical silica Spherisorb with γ-glycidoxypropyltrimethoxysilane (heated at 90 °C) and hydrolysed to diol-silica	CLONIS et al. (1986).
L-Lactate dehydrogenase from crude extracts of rabbit muscle	Procion Blue MX-R	TSK gel 65000 PW or Dynospheres XP-3507	CLONIS (1987).
Lactate dehydrogenase from pig heart and rabbit muscle and alcohol dehydrogenase from horse liver	N6-(6-Aminohexyl)-AMP	LiChrosorb Si60 with γ-glycidoxypropyltrimethoxysilane (Glycosil) with glycol groups (from oxirane groups converted by heating) after periodate oxidation	OHLSON et al. (1978).
Lactate dehydrogenase isoenzymes (EC 1.1.1.27)	Cibacron Blue F3G-A with 1,6-diaminohexane	Silica gel (LiChrosorb Si 60) with γ-glycidoxypropyltrimethoxy silane	LOWE et al. (1981).
Glucose oxidase (fungal)	Concanavalin A	LiChrosorb Si 100 or 1000 with γ-glycidoxypropyltrimethoxysilane or after hydrolysis with tresyl chloride or after periodate oxidation with $NaBH_4$ stabilization	BORCHERT et al. (1982).
Glutathione S-transferases (EC 2.5.1.18) from rat liver and Walker 256 rat carcinoma cell cytosols	Glutathione (GSH)	GSH covalently bonded through sulfur to 15 Å spacer arm on macroporous (1000 Å) polymethacrylate gel	LA CRETA et al. (1988).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Hexokinase (EC 2.7.1.1) and 3-phosphoglycerate kinase (EC 2.7.2.3) from yeast	Cibacron Blue F3G-A with 1,6-diaminohexane	Silica gel (LiChrosorb Si 60) with γ-glycidoxypropyltrimethoxy silane	LOWE et al. (1981).
Isolipase (study of interaction)	Colipase	LiChrosorb diol with glycidoxypropyltrimethoxysilane after periodate oxidation	MAHE et al. (1986).
β-Lactamase from fermentation media of Aspergillus	Cephalosporing C (best from investigated different ligands)	TSK 400PW after CNBr-activation (best from investigated different matrices)	ROGERS et al. (1985).
Lipoprotein lipase (purification of commercial bovine enzyme)	Heparin	TSK gel Heparin-5PW	NAKAMURA et al. (1988).
Lysozymes and N-bromosuccinimide-oxidized lysozymes	Chitin	Celite 545	YAMADA et al. (1985).
Perioxidase from horse radish	Concanavalin A	LiChrosorb Si 100 or 1000 with γ-glycidoxypropyltrimethoxysilane or after hydrolysis with tresyl chloride or after periodate oxidation with $NaBH_4$ stabilization	BORCHERT et al. (1982).
Horseradish peroxidase	Concanavalin A	Silica (250Å) with 3-isothiocyanatopropyltriethoxysilane	KINKEL et al. (1984).
Proteinases and their zymogenes:			
Bovine trypsin (EC 3.4.4.4)	Soy bean trypsin inhibitor	Aminosilanized LiChrospher 500 NH_2 with glutaraldehyde	KASCHE et al. (1981).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Dipeptidyl-peptidase IV (DPP IV) from plasma membranes of rat liver	Monoclonal antibodies against DPP IV	Succinylimide activated column Microanalyzer	JOSIC et al. (1987).
Human α-thrombin	L-Arginyl methyl ester	Polystyrene cross-linked with 2% divinylbenzene (Bio-Beads SX-2) chlorosulphonated	MULLER et al. (1986).
Pepsin (EC 3.4.23.1)	ε-Aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester	Separon H 1000 with epichlorohydrin	TURKOVA et al. (1981).
Plasminogen and plasmin from plasminogen activated by urokinase	p-Aminobenzamidine	Hydrophilic vinyl-polymer gel Toyopearl HW 65S with chloroacetylglycylglycine (carbodiimide technique)	SHIMURA et al. (1984).
Thrombin (purification of commercial bovine thrombin)	Heparin	TSK gel Heparin-5PW	NAKAMURA et al. (1988).
Trypsin	BPTI trypsin inhibitor	Partisil with γ-glycidoxypropyltrimethoxysilane;with acid hydrolysis diol silica oxidated with $NaIO_4$ and reduced with $NaBH_4$; activated with chloroformates or 1,1'-carbodiimidazole	ERNST-CABRERA and WILCHEK (1986).
Trypsin-family proteases	m-Aminobenzamidine	Toyopearl HW-65	KANAMORI et al. (1986).
Trypsins (resolution of varoius pancreatic trypsins)	Soybean trypsin inhibitor	Epoxy Partisil-10	KHURANA and HO (1989).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Trypsins (bovine and Streptomyces griseus)	p-Aminobenzamidine	Asahipak GS-gel with 6-aminohexanoic acid	ITO et al. (1985).
Ribonuclease A from bovine pancreatic extract	Cibacron Blue F3G-A with 1,6-diaminohexane	Silica gel (LiChrosorb Si 60) with γ-glycidopropyltrimethoxy silane	LOWE et al. (1981).
ENZYME MODIFIED DERIVATIVES			
Anhydrochymotrypsin	L-Tryptophan (L-Trp)	Toyopearl HW65S with N-chloroacetyl-6-aminohexanoic acid (ester moiety after coupling of L-Trp hydrolyzed with dilute alkali)	SHIMURA et al. (1985).
Sialic acid-containing salivary - type amylase from patients' sera	Concanavalin A	Con A column	MORIYAMA et al.. (1991).
GLYCOPROTEINS AND SACCHARIDES			
N-Acetylneuraminic and N-glycolylneuraminic acids - from ganglioside oligosaccharides from human or bovine brains	Wheat germ agglutinin	Nucleosil 300-Å silica gel with γ-glycidoxypropyltrimethoxysilane converted at 95 °C to diol-silica,oxidized by periodate and coupling by reductive amination using cyanoborohydride	RASSI et al. (1989).
Concanavalin A-binding proteins - from human serum	Concanavalin A (Con A)	Con A-immobilized hydrophilic polymer support Gelpack	MANABE et al. (1988).
Detergent-solubilized glycoproteins from human embryonal lung fibroblasts	Concanavalin A and Pisum sativum agglutinin	Silica LiChrospher Si 1000Å with 3-isothiocyanatopropyltriethoxysilane	RENAUER et al. (1985).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Fetuin from fetal calf serum	Wheat germ agglutinin (WGA)	Suspension of fluid-impervious silica microspheres in aqueous solution of γ-glycidoxypropyl trimethoxysilane after heating at 95° C oxidized by periodic acid and WGA attached by reductive amination using cyanoborohydride	VARADY et al. (1988).
Glucosides	Concanavalin A	LiChrosorb Si 100 or 1000 with γ-glycidoxypropyltrimethoxysilane or after hydrolysis diol-silica with tresyl chloride or after periodate oxidation with $NaBH_4$ stabilization	BORCHERT et al. (1982).
N-Glycanase-released and reduced oligosaccharides and products derived from these oligosaccharides	Lectins: leukoagglutinating phytohemagglutinin;concanavalin A; Datura stramonium agglutinin and Vicia villosa agglutinin (in presence of $NaCNBH_3$)	SynChropak GPC-500 after reductive amination and periodate oxidation (after coupling solid $NaBH_4$)	GREEN et al. (1987).
Glycoproteins (α 1-acid human;canine and bovine glycoproteins;asialofetuin; fetuin; horse radish peroxidase and avidin)	Concanavalin A and wheat germ agglutinin	Nucleosil silica gel with γ-glycidoxypropyltrimethoxysilane converted at 95°C to diol-silica;oxidized by periodate and coupling by reductive amination using sodium cyanoborohydride	RASSI et al. (1988).
Glycoproteins from human serum	Phytohaemagglutinin from Phaseolus vulgaris	LiChrospher Si 1000 with γ-glycidoxypropyltrimethoxysilane and after hydrolysis diol silicia with tresyl chloride	BORREBAECK et al. (1984).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Human transferrin after incubation with sialidase and β-galactosidase (detection of glycosidase activity)	Caster bean lectin RCA 120 and concanavalin A	Lectin affinity columns Shimazu LA-RCA 120 column or LA-Con A column	HARADA et al. (1987).
4-Methylumbelliferyl α-D-mannopyranoside	Concanavalin A	Hypersil WP-300 with 3-glycidoxypropyltrimethoxysilane and 1,1'-carbonyldiimidazole	MOORE and WALTERS (1987).
Neutral oligosaccharides (fractionation) from mouse immunoglobulin G after hydrazinolysis;N-acetylation and $NaBH_4$ reduction after sialidase treatment	Lectin RCA 120	Lectin RCA 120 affinity HPLC column	MIZUOCHI et al. (1987).
Nucleotide sugars (separations of UDP-glucose;UDP-galactose)	UDP-N-acetyl-D-glucosamine and UDP-N-acetyl-D-galactosamine); Lectin RCA120	Poly(acrylic ester)gel (WG003) with epoxy residues	TOKUDA et al. (1985).
Nucleotide sugars (resolution)	Isolectins from castor beans	Polyacrylic ester gel (WG003) containing epoxy residue	HARADA et al. (1986).
Oligosaccharide $(Glc)_4$-alditol	Monoclonal antibody against glucose-containing tetrasaccharide hapten $(Glc)_4$ covalently coupled to keyhole limpet hemocyanin	Selecti Spher-10 Activated Tresyl column;microparticulate silicas	OHLSON et al. (1988b).
Plasma membrane glycoproteins - from rat liver and Morris hepatoma	Concanavalin A	Eupergit C1Z ConA (non-porous) or TSK 5PW-non porous and ConA coupled to p-nitrophenyl-activated silica;size pore 27.5 nm or Eupergit C30N ConA (pore size 50 nm)	JOSIC et al. (1988).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
HORMONES			
Arginine vasopressin	Bovine neurophysin II	Controlled-pore glass CPG-2000 or nonporous glass beads (type 1-W)	SWAISGOOD and CHAIKEN (1985).
Arg8-vasopresin	Neurophysin II	ACCELL-C6-OSu; highly cross-linked agarose (emulsified in organic solvent and activated by tresyl chloride method);controlled pore glass or non-porous glass	FASSINA et al. (1986).
INHIBITORS			
Antithrombin III - from human plasma	Heparin	TSK gel Heparin-5PW	NAKAMURA et al. (1988).
α-1-Proteinase inhibitor from human plasma	Procion Red HE-3B	Hydrophilic vinyl-polymer Fractogel TSK HW 65 with sodium sulfate in several portions	GUNZER and HENNRICH (1984).
Trypsin inhibitor from bovine pancreas (BPTI)	Trypsin	Partisil with γ-glycidoxypropyltrimethoxysilane;with acid hydrolysis diol silicia oxidated with $NaIO_4$ and reduced with $NaBH_4$; activated with chloroformates or 1,1'-carbodiimidazole	ERNST-CABRERA and WILCHEK (1986).
LECTINS			
Carbohydrates and lectins	Glycamines	Epoxy-activated methacrylate resin Shodex M-6140G	HONDA et al. (1987).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Concanavalin A	Glucosamine.HCl	Silica LiChrosorb Si 60 and LiChrospher Si 100;500 and 4000 with (3-glycidoxypropyl) - trimethoxysilane and 1,1'-carbonyldiimidazole	WALTERS (1982).
Lectins from chicken and human sera (stepwise elution with various competing sugars)	Oligosaccharides of ovalbumin converted to glycamine derivatives by reductive amination	Shodex M-6140G (Epoxy-activated methacrylate polymer)	HONDA et al (1988).
Ricinus communis agglutinin I and II;soybean agglutinin;Arachis hypogaea agglutinin	Lactose	TSK gels G3000PW with epichlorohydrin and hydrazine hydrate	ITO et al. (1986).
NUCLEIC ACIDS; NUCLEOTIDES AND NUCLEOSIDES			
Adenine nucleosides;adenine nucleotide and triazine dyes	Alcohol dehydrogenase (EC 1.1.1.1) from horse liver	LiChrospher 1000 with γ-glycidoxypropyltrimethoxysilane;by heating obtained diol-silica activated with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride)	NILSSON and LARSSON (1983).
Monomeric ribonucloside compounds from human urine and human serum	m-Aminobenzeneboronic acid	LiChrosorb Si 100 with γ-chloropropyltrimethoxysilane	HAGEMEIER et al. (1983b).
Nucleic acid bases and nucleosides	Thymine	Glycidyl methacrylate polymerized with ethylene glycol dimethacrylate in presence of diluent	KATO et al. (1977).
Nucleosides;nucleotides and carbohydrates	3-Aminobenzene boronic acid	LiChrosorb Si 100 with γ-glycidoxypropyltrimethoxysilane	GLAD et al. (1980).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Ribo- and deoxyribonucleosides (separation)	m-Aminobenzeneboronic acid	LiChrosorb Si 100 with γ-chloropropyltrimethoxysilane	HAGEMEIER et al. (1983a).
Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) - separation	Histones	Porous silica (60 Å) after heating to 150° C under vacuum with 3-glydoxypropyltrimethoxysilane;received epoxy-silica converted to diol by treatment with sulfuric acid at 90°C;diol-silica activated with 1,1,1-trifluoromethanesulfonyl chloride	HOLSTEGE et al. (1988).
PROTEINS: TRANSFER RECEPTORS AND BINDING PROTEINS			
Antigen-specific lymphocyte receptors - from lymphocytes of mice immunized with P2 sequence of bovine myelin basic protein (MBP)	Biotinylated monoclonal antibodies (MAb) against P2 sequence of MBP (coupling of biotin hydrazine to carbohydrate moieties of MAb after periodate oxidation)	Carbonyl diimidazole glycophase-derivatized controlled-pore glass beads with streptavidin	PHILLIPS and FRANTZ (1988).
Calmodulin from crude pea plant extracts	Peptide melittin in presence of Ca^{2+}	300 Å-pore beaded glycidopropyl-silica	FOSTER and JARRETT (1987).
Calmodulin and S 100 β-protein (review)	Phenothiazine or napthalenesulfonamide	Silica particles	MARSHAK et al. (1985).
Calmodulin (Ca^{2+} -dependent protein activator of variety of enzymes) from bovine brain	2-(Trifluoromethyl)-10-(3'-aminopropyl)phenothiazine	SynChropak CM-300 with N-hydroxysuccinimide in presence of carbodiimide with β-alanine and N-hydroxy succinimide in presence of carbodiimide	JARRET (1986).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Growth hormone receptors from rabbit livers	Human growth hormone	AF-formyl Toyopearl	YAGI et al. (1989).
Plasma membrane proteins from human fibroblasts	Procion triazine dyes or mannan	Silica (pore size 250 Å) with 3-aminopropyltriethoxysilane or 3-isothiocyanatopropyltriethoxysilane	KINKEL et al. (1984).
Plasma membrane proteins from rat liver	Concanavalin A	Eupergit 30N (containing epoxy active groups) or CNBr-activated TSK 5PW gel	JOSIC et al. (1987).
Specific T-cell phosphorylcholine receptor from membranes of T cells from mice immunized with phosphorylcholine	Monoclonal anti-idiotypic antibody after biotinylation of periodate oxidized carbohydrate moieties by biotin hydrazine	Streptavidin-coated glass beads	PHILLIPS (1989).
Steroid-binding progesterone receptors - from human breast cancer cells or human uterine tissue	16-α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione (Organon 2058 attached via 21-hydroxyl)	Epoxy-activated silica Ultraffinity-EP column	BOYLE and VAN DER WALT (1988).
OTHER BIOLOGICALLY ACTIVE COMPOUNDS			
Catecholamines and its analogues in human urine (determination)	Boronic acid	Affi-Gel 601	SPEEK et al. (1983).
Enantiomers (D,L-tryptophan;D,L-5-hydroxytryptophan;D,L-kynurenine and D,L-3-hydroxykynurenine)	Bovine serum albumin	Resolvosil	ALLENMARK et al. (1983).
2-Hydroxycarboxylic acids from urine (vanillylmandelic;vanillyllactic etc.)	Aminophenylboronic acid	Boric Acid Gel	HIGA and KISHIMOTO (1986).

Table 9.2. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Individual bile acids in serum and bile (determination)	3α-Hydroxysteroid dehydrogenase	Amino Propyl-CPG 180Å with glutaraldehyde	KAMADA et al. (1982).
Interleukin-2 previously denoted as T cell growth factor (recombinant human) from microbial (E.coli) and mammalian (Chinese hamster ovary cells) sources	Soluble subunit of human interleukin-2 receptor	Silica-based NuGel P-AF polyaldehyde poly-N-hydroxysuccinimide	WEBER and BAILON (1990).
Myosin and actin (cardiac muscle proteins) from cardiac tissue of guinea pigs	Sodium salicylate	Ultraaffinity-EP column	STROHSACKER et al. (1988).
Pharmacologically active racemic sulfoxides	Bovine serum albumin (BSA)	BSA-Silica column	ALLENMARK et al. (1984).
Racemic barbiturates (optical resolution)	Bovine serum albumin (BSA)	Resolvosil-BSA 7 micrometer column	ALLENMARK et al. (1988).
Recombinant leukocyte A interferon from Escherichia coli	Monoclonal antibody	Polyhydroxy silica support NuGel P;GP-500 oxidized with sodium m-periodate and with cyanoborohydride during coupling	ROY et al. (1984).
Recombinant leukocyte A interferon from Escherichia coli	Monoclonal antibody	Polyhydroxy silica support NuGel P;GP-500 oxidized with sodium m-periodate and with cyanoborohydride during coupling	ROY et al. (1985).
Serum bile acids	3α-hydroxysteroid dehydrogenase	Cellulofine GC-200m (beaded cellulose)	HASEGAWA et al. (1983).
Sterols	Tomatine	Silica LiChrosorb Si 100 with γ-glycidoxypropyltrimethoxysilane	CSIKY and HANSSON (1986).

7 min and the system showed high precision. The column was very stable: its activity did not change after more than 500 cycles.

Another application of clinical interest concerns the determination of disease-induced alterations in the concentration of blood serum immunoglobulins. Using HPLBAC, it is possible rapidly to isolate and quantify immunoglobulin in serum. It is an inexpensive method, since the immobilized ligand can be reused many times. Other methods, including such techniques as radial immunodiffusion, immunoelectrophoresis, nephelometry and radioimmunoassays for measuring immunoglobulins are less sensitive, more time-consuming, and expensive.

The future trends of HPLBAC, published by Ohlson et al. (1989), concern analytical and preparative biotechnology. Processes such as the production of monoclonal antibodies or of recombinant DNA specified proteins and peptides have created a need for the rapid determination of specific biomolecules in complex mixtures at several stages during production. HPLBAC could be used for monitoring biomolecules in complex mixtures, possibly providing on-line analysis in bioreactors and down-stream processing. Fig. 9.1 shows the monitoring of Protein A in a fermentation broth of *Staphylococus aureus*. Ohlson et al. (1989) used HPLBAC routinely to optimize the culture time and consumption of media in the fermentation. HPLBAC has an important role in the quantitative and qualitative analysis of biologically active molecules, which leads to an assessment of their purity, potency and safety.

Ohlson et al. (1989) also show that, with a weakly interactive affinity system (K_{diss} of $1\text{-}10^{-4}$M) and many binding sites per unit volume, chromatography can be more dynamic. Fast isocratic separations with high performance (narrow peaks) can be performed without compromising the selectivity introduced by the affinity ligand. Weak affinities between molecular binding sites often occur naturally. However, recombinant DNA and hybridoma methods could provide ligands selected for weak affinities. Ohlson et al. (1988 a) studied weak affinity interactions (K_{diss} of 10^{-2}-10^{-4}M) between oligosaccharide antigens and preselected monoclonal antibodies. They achieved excellent isocratic chromatographic separation of the carbohydrate antigens in a crude extract on HPLBAC column with immobilized monoclonal antibodies. In biotechnology, weak

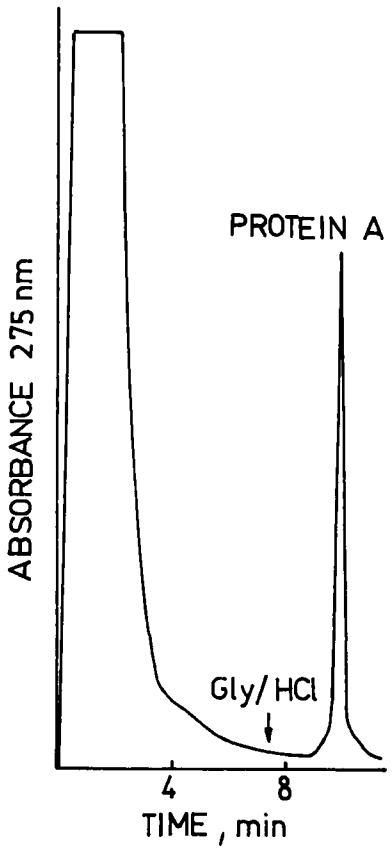


Fig. 9.1. Monitoring of Protein A in a fermentation broth using an IgG-HPLAC column (10μm, 10 x 0.5 cm). Conditions: mobile phase, initially 0.1 M NaH_2PO_4 pH 7.0, 0.15 M NaCl, changed to 0.1 M glycine/HCl, pH 2.2 (at arrow) to elute Protein A. Flow rate, 4 ml min^{-1}, sample, 0.96 ml fermentation broth, temperature, 22 °C. Reproduced with permission from S. Ohlson et. al., Trends Biotechnol., 7 (1989) 179-186.

HPLBAC can be utilized to monitor product quality, e.g. by following the glycosylation patterns of protein drugs.

HPLBAC can also be used in clinical chemistry where complex samples, such as blood and urine, are commonly encountered.

9.3 LARGE-SCALE ISOLATION USING BIOAFFINITY CHROMATOGRAPHY

Large-scale bioaffinity chromatography is usually performed in industrial laboratories and most information is thus proprietary (Ohlson et al., 1989). Table 9.3 (set

up in the same way as the preceding Tables 9.1 and 2) lists papers describing the large-scale isolation of biologically active substances. The commercial names of solid supports and immobilized affinity ligands mentioned in these three tables are collected in Table 9.4, which contains trade names, nature and producers.

Janson (1984) reviewed the state of the art of large-scale bioaffinity purification and discussed its future prospects. Ernst-Cabrera and Wilchek (1987) described the factors which have to be considered in adopting bioaffinity chromatography in industry. Among these factors belongs the availability of a suitable carrier material, which should have good physical properties, and the ability to bind suitable amounts of affinity ligands. Methods for the immobilization of ligands are required which lead to a stable linkage of the ligand to the carrier, thus avoiding leakage of the ligand and hence contamination of the desired product. The ligand to be chosen has to be sufficiently specific and available in large quantities at reasonable prices. Efficient procedures are needed in order to elute the adsorbed compound without loss of biological activity and without affecting the affinity matrix in order to provide a chromatographic system which can be used many times.

The advantages of the application of immunoaffinity chromatography for the large-scale purification of pharmaceutically active proteins have been described by Ernst-Cabrera and Wilchek (1988 b). The realization that proteins can be of therapeutic value, coupled with the feasibility of their large-scale production by the new technologies, has underscored the fact that the purification step is actually the bottleneck in the supply of these compounds. Polyclonal or monoclonal antibodies are suitable affinity ligands, because they can be produced against any compound, even if the latter is only partially purified. Futhermore, monoclonal antibodies can be selected with any desired affinity, thereby making the use of biospecific columns, prepared by their immobilization, very attractive. They show absolute specifity for only one single epitope, the smallest immunologically sub-molecular group on an antigen. Monoclonal antibodies can be produced in large quantities by a hybridoma technology developed by Koehler and Milstein (1975).

Table 9.3

Large-scale biospecific adsorbtion

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
ANTIBODIES			
Anti-DNA antibodies from serum of patients with active Systemic Lupus Erythematotus	Single- and double-stranded DNA	Films of highly polymerized insoluble collagen with hydrazine; $NaNO_2 + HCl$	EL-HABIB et al. (1984).
Immunoglobulin G	Histidine	Sepharose 4B with 1,4-butanediol diglycidyl ether	KANOUN et al. (1986).
Monoclonal antibody against human tumor antigen (250 kDa glycoprotein/proteoglycan from melanoma cells)	Protein A	Protein A-Sepharose CL-4B	LEE et al. (1986).
ANTIGENS AND HAPTENS			
Complement component Clq from human plasma	Antibodies against purified Clq from rabbits	Agarose-polyaldehyde microsphere beads (APMB) (produced by encapsulating polyacrolein microspheres with agarose)	GAZITT et al. (1985).
Epstein-Barr virus membrane antigen (MA) gp 340	Monoclonal antibodies against MA	Sepharose CL-4B after CNBr-activation	RANDLE et al. (1985).
Hepatitis B surface antigen	Concanavalin A	Con A-Sepharose	NEURATH et al. (1978).
ENZYMES			
Adenylosuccinate synthetase (EC 6.3.4.4) from Escherichia coli	Procion Red HE-3B	Sepharose 4B	CLONIS and LOWE (1981).
Alkaline phosphatase (AP) (EC 3.1.3.1) from human placental extract	Antibodies against AP from rabbits	Sepharose 4B	DOELLGAST et al. (1977).

Table 9.3. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Catechol-O-methyl transferase (EC 2.1.1.6) from pig liver	Catechol	Sepharose 4B after CNBr-activation with 4,4'-diaminophenylmethane after diazotisation	GULLIVER and TIPTON (1978).
Dehydrogenases:			
Alcohol dehydrogenase (EC 1.1.1.1) from equine liver	Cibacron Blue F3GA	Blue Sepharose	ROY and NISHIKAWA (1979).
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from baker's yeast	Phosphoryl residues	P-cellulose LG	TAKAGAHARA et al. (1978).
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from yeast	N^6-(6-Aminohexyl)-AMP	CNBr-activated Sepharose 4B	CHAFFOTTE et al. (1977).
3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) from Rhodopseudomonas spheroides	Procion Red H3B and Procion Blue MX-4GD dyes	Sepharose 4B	SCAWEN et al. (1982).
3-Hydroxybutyrate dehydrogenase and malate dehydrogenase from Rhodopseudomonas sphaeroides	Procion Red H-3B	Sepharose 4B	ATKINSON et al. (1981).
IMP dehydrogenase (EC 1.2.1.14) from Escherichia coli	8-(6-Aminohexyl)-AMP	Sepharose 4B	GILBERT et al. (1979).
IMP dehydrogenase (EC 1.2.1.14) from Escherichia coli	Inosine 2',3'-O-[1-(6-aminohexyl)-levulinic acid amide]-acetal 5'-monophosphate	Sepharose 4B	CLONIS and LOWE (1980).
Lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle	Procion Blue MX-R dye	Preparative-grade silica LiChrosorb Si60 with γ-glycidoxypropyltrimethoxysilane converted to glycol form by heating at 70° C at pH 3.5	SMALL et al. (1983).

Table 9.3. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Lactate dehydrogenase from ox heart	NAD	Nonporous quartz fibre silylated with mercaptopropyltrimethoxysilane and to the SH groups covalently coupled tresyl chloride-activated dextran	WIKSTROM and LARSSON (1987).
Malate dehydrogenase (EC 1.1.1.37) from Rhodopseudomonas spheroides	Procion Red H3B and Procion Blue MX-4GD dyes	Sepharose 4B	SCAWEN et al. (1982).
Oestradiol-17-β dehydrogenase (EC 1.1.1.62) of human placenta	Oestrone hemisuccinate	Sepharose 4B with ethylenediamine	NICOLAS et al. (1972).
Pyruvate dehydrogenase from Escherichia coli	Ethanolamine	Sepharose 2B with 1,4-bis(2,3.epoxypropoxy)butane	VISSER et al. (1978).
DNA-Dependent RNA polymerase (EC 2.7.7.6) from Escherichia coli	Deoxyribonucleic acid	Cellulose - coupling of DNA according ALBERTS and HERRICK (1971)	BURGESS and JENDRISAK (1975).
Kinases:			
Glucokinase (EC 2.7.1.2) from rat liver	N-(6-Aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose	Sepharose 4B after CNBr-activation	HOLROYDE et al. (1976).
Glycerokinase from Bacillus stearothermophilus	Procion Blue MX-3G	Sepharose 6B	ATKINSON et al. (1981).
Glycerokinase (EC 2.7.1.30) from Bacillus stearothermophilus	Procion Blue MX-3G	Sepharose CL-4B (stirring at 40° C)	SCAWEN et al. (1983).
Glycerokinase (EC 2.7.1.30) from Bacillus stearothermophilus	Procion Blue MX-3G	Sepharose 4B	HAMMOND et al. (1986).
Hexokinases from rat kidney and liver or crude yeast extract	N-(8-Aminooctanoyl)-2-amino-2-deoxy-D-glucopyranose	CNBr-activated Sepharose 4B	WRIGHT et al. (1978).
Nucleoside diphosphate kinase (EC 2.7.4.6) from pig heart	Cibacron Blue 3G-A	Sepharose 4B	LASCU et al. (1981).

Table 9.3. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Nucleoside diphosphate kinase from brewer's yeast	Cibacron Blue 3GA	Agar cross-linked with epichlorohydrin	PORUMB et al. (1985).
Phosphoglycerate kinase (EC 2.7.2.3) from Saccharomyces cerevisiae	Cibacron blue 3G-A	Sepharose 4B	KULBE and SCHUER (1979).
Lecithin-cholesterol acyltransferase (EC 23.1.43)	Cibacron Blue F3GA	Affi-Gel blue and DEAE-Affi-Gel blue	CHEN and ALBERS (1985).
Penicillinases - from Staphylococcus aureus and Bacillus lichemiformis	D-(-)-Penicillamine after coupling treated with acetic anhydride	Sepharose 4B activated with divinyl sulphone	KISS et al. (1988).
Proteinases:			
Angiotensin I converting enzyme (EC 3.4.15.1) from human plasma	Immunoglobulin G of rabbit anti-baboon lung angiotensin I -converting enzyme serum	CNBr-activated Sepharose 4B	LANZILLO et al. (1980).
Carboxypeptidases A and B from bovine activated pancreatic juice	Soybean trypsin inhibitor (Kunitz) and ε-amino-n-caproyl-D-tryptophan (removal of trypsin and chymotrypsin)	Sepharose 4B after CNBr-activation	REECK et al. (1971).
Carboxypeptidase G from Pseudomonas species	Procion Red H-8BN	Sepharose 6B	ATKINSON et al. (1981).
Carboxypeptidase G2 from Pseudomonas sp.	Procion Red H-8BN	Sepharose 6B	SHERWOOD et al. (1985).
Renin (EC 3.4.99.19) (cleavage of angiotensin I from angiotensinogen) from mouse submaxillary gland	Pepstatin with N-hydroxysuccinimide	Sepharose with hexamethylenediamine	SUZUKI et al. (1981).
Rhizopuspepsin isozymes (EC 3.4.23.9) from Rhizopus chinensis	Pepstatin	AH-Sepharose 4B	OHTSURU et al. (1982).

Table 9.3. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Pseudocholinesterase from equine serum	Procion Red HE-7B	Sepharose 4B	ATKINSON et al. (1981).
GLYCOPROTEINS AND SACCHARIDES			
Fibronectins from bovine and human plasma	Gelatin	Gelatin-Ultrogel	ROULLEAU et al. (1982).
Polymethyl polysaccharides	p-Nitrophenyl palmitate	Aminoalkylsilyl silicate	HINDSGAUL and BALLOU (1984).
INHIBITOR			
Inhibitor of 3',5' cyclic AMP phosphodiesterase from Dictyostelium discoideum	Cibacron Blue F3GA	Affi-Gel Blue	DICOU and BRACHET (1981).
LECTIN			
Peanut agglutinin	Arabinogalactan	Arabinogalactan cross-linked with epichlorohydrin	MAJUMDAR and SUROLIA (1978).
PROTEINS: TRANSFER RECEPTORS AND BINDING PROTEINS			
Calmodulin and S-100 protein from brain	Melittin	CNBr-activated Sepharose 4B	KINCAID and COULSON (1985).
Oestrogen receptor from calf mammary gland	Heparin	CNBr-activated Sepharose 4B	ROTONDI and AURICCHIO (1979).
Soluble forms of human interleukin-2 receptor produced by gene-linked co-amplication technology in Chinese hamster ovary cells	Recombinant human interleukin-2	Silica-based polyaldehyde Nu Gel-AF	WEBER et al. (1988).
OTHER BIOLOGICALLY ACTIVE COMPOUNDS			

Table 9.3. (continued)

Substances isolated	Affinity ligands	Solid supports or immobilized affinity ligands	References
Albumin from human plasma	Cibacron Blue F3GA	Blue-Trisacryl	SAINT-BLANCAD et al. (1982).
Cholera toxin from Vibrio cholerae	Receptor ganglioside for cholera toxin GM1 from bovine brain hydrolyzed to lysoGM1	Porous silica beads Spherosil X0C 005 with DEAE-dextran after periodate oxidation with $NaBH_4$ after coupling	TAYOT et al. (1981).
Human lymphoblastoid interferon from cell line Namalva induced with Newcastle disease virus	Antibodies against human leukocyte interferon from sheep	CNBr-activated Sepharose 4B	BRIDGEN et al. (1977).
Interferon from human leukocytes	Monoclonal antibody against human leukocyte interferon from mice bearing transplantable tumours	CNBr-activated Sepharose	SECHER and BURKE (1980).
Interferon α-2a synthesized in Escherichia coli	Monoclonal antibodies against human leukocyte interferon	N-Hydroxysuccinimide ester-activated Sepharose FF	HOCHULI et al. (1987).
Intermediate filament protein vimentin from Ehrlich ascites tumor cells	Single-stranded calf thymus DNA	CF-11 Cellulose	NELSON and TRAUB (1982).
Recombinat leukocyte A interferon	Monoclonal antibodies	Affi-Gel 10	TARNOWSKI and LIPTAK (1983).
SPECIFIC PEPTIDE			
Glycopeptide antibiotic aridicin from clarified fermentation broth	D-Ala-D-Ala	Affi-Gel 10	FOLENA-WASSERMAN et al. (1987).

Table 9.4.

Comercial names of solid supports mentioned in Tables 9.1,2 and 3

*Tradename**	*Nature*	*Producer***
ACCELL-C6-0Su	Highly cross-linked agarose	(1)
Actigel	Cross-linked agarose	(2)
Affi-Gel 10-501	Cross-linked agarose	(3)
Affi-Gel 601,731	Polyacrymide gels	(3)
Affi-Prep matrix	Pressure-stable polymer	(3)
AGATP,	Agarose with N^6-(aminohexyl) ATP	(4)
AGNAD	Agarose with nicotinamide adenine dinucleotide (NAD)	(4)
Asahipak GS	Hydrophilic vinyl polymers	(5)
Avicel PH-102	Microcrystaline cellulose	(6)
Bio-Gel A	Agarose	(3)
Bio-Gel P	Polyacrylamide gels	(3)
Bio-Glass	Porous glass	(3)
Bio-Rex 70	Acrylic polymer	(3)
Celite 545	Silica	(7)
Cellex	cellulose	(3)
Cellulofine GC	Beaded cellulose	(8)
Cellulose CF 111	Cellulose	(9)
CPG	Controled-pore glass	(1) (10) (11) (12)
Davisil	Octadecyl-bonded silica	(14)
Dynabeads	Magnetic parcticles	(15)
Eupergit	Glycidyl-methacrylate copolymer	(16)
Eupergit C1Z	Non-porous Eupergit	(16)
Fractogel TSK HW	Hydrophilic vinyl polymers	(17)
Glycerol- CPG	Glycerolpropyl-glass	(11)
Glycogel	m-Aminophenylboronic acid agarose	(13)
Glycophase	Glycerol-coated glass	(13)
Glycophase G/CPG	Triethoxypropyl glycidosilane-coated glass	(13)
Hi Trap Columns	Highly cross-linked agarose	(18)
Hypersil WP-300	Silica	(14)
Indubiose	Agarose	(19)
LiChrospher or LiChrosorb Si; Diol or non-porous Monospher	Silica	(17)

Table 9.4. (continued)

*Tradename**	*Nature*	*Producer***
Matrex Cellufine gels	Cellulose	(20)
Matrex dyes	Agarose with different dyes	(20)
Nucleosil	Silica	(21)
NuGel	Polyhydroxy silica	(22)
Partisil	Silica	(2) (13) (23)
Porasil	Silica	(1)
Reacti-Gel 6X	Cross-linked agarose	(13)
Resolvosil	Silica with albumin	(21)
Selecti Spher 10	Silica	(21)
Selecti Spher 10 Activated Tresyl	Activated tresyl silica	(24)
Separon HEMA	Hydroxyethyl methacrylate copolymer	(25)
Sephacryl	Allyl-dextran after cross-linking with N,N'-methylene-bis-acrylamide	(18)
Sephadex	Dextran gels	(18)
Sepharose	Agarose	(18)
Sepharose CL	Agarose after cross-linking with 2,3-dibromopropanol and desulphurization	(18)
Spheron	Hydroxylethyl methacrylate copolymer	(26)
Spherosil XOB-015	Silica	(27)
Shodex M-6140G	Epoxy-activated methacrylate polymer	(28)
Silochrom	Organo-silica support	(29)
Sorbsil	Silica	(30)
SynChropak	Glycerolpropyl silica	(31)
Toyopearl HW 65S	Hydrophilic vinyl polymer	(32)
Trisacryl GF	Hydrophilic acrylate polymer	(33)
TSK	Silica	(32)
TSK 5PW	Polystyrene divinylbenzene	(34)
Ultraaffinity-EP column	Epoxy-activated silica	(35)
Ultrogel A	Agarose	(33)
Ultrogel AcA	Polyacrylamide-agarose gel	(33)

*See Chapter 5 and Table 6.4, where most derivatives of cited solid supports are shown

** Addresses of producers (page 578)

Addresses of producers

(1) Waters Associates, Milford, MA 01757, USA

(2) Sterogene Biochemicals, San Gabriel, CA 91775, USA

(3) Bio-Rad Laboratories, Richmond, CA 94804, USA

(4) P.L. Biochemicals, Milwaukee, Wisc., USA

(5) Asahi Chemical Industry Co., Tokyo, Japan

(6) FMC Corporation, Philadelphia, PA, USA

(7) Manville Filtration and Minerals, Denver, CO 80217, USA

(8) Seikagaku Kogyo, Tokyo, Japan

(9) Whatman, Maidstone, U.K.

(11)Electro-Nucleonics, Inc., Fairfield, NJ 07006, USA

(12)Corning Glass Works, Corning, NY 14831, USA

(13)Pierce Chemical Company, Rockford, IL 61105, USA

(14)Alltech Associates, Inc., Deerfield, IL 60015, USA

(15)Dynal A.S., Oslo, Norway

(16)Röhm Pharma GMBH, Darmstadt, FRG

(17)E.Merck, D-6100, Darmstadt, FRG

(18)Pharmacia-LKB Biotechnology, Inc., S-75104, Uppsala, Sweden

(19)Industrie Francaise Biologique, Gennevillers, France

(20)Amicon Co., Danvers, MA 01923, USA

(21)Machery-Nagel, D-5160, Dürren, FRG

(22)Separation Industries, Metuchen, NJ 08840, USA

(23)Whatman, Inc., Clifton, NJ 07014, USA

(24)Perstorp Biolytica AB, Lund, Sweden

(25)TESSEK Ltd., 11001 Praque, CR

(26)Lachema, 62133 Brno, CR

27)Rhöne-Poulenc, Usine de Salindres, France

(28)Showa Denko, Shiba-daimon, Tokyo, Japan

(29)VNIIL, Stavropol, Russia

(30)Crosfield Chemicals, Warrington, U.K.

(31)Syn Chrom, Inc., Linden, IN 47955, USA

(32)Toyo Soda Man. Co., Tokyo, Japan

(33)Reactifs IBF, Villeneuve la Garenne, France

(34)Toso Haas, Philadelphia, PA 19105, USA

(35)Beckman Instruments, Inc., San Ramon, CA 94583, USA

However, the practical success of immunoaffinity chromatography in the large-scale preparation of biologically active molecules depends on many critical factors. The factors involved in the preparation of specific antibodies were successfully solved by Eveleigh (1982). According to him, some form of automated system becomes essential for routine immunoaffinity separation. A schematic diagram of this system is shown in Fig. 2.5. For immunoaffinity separation, conventional chromatography columns with a high cross-sectional area to lengthen the ratio, and preferably adaptors to reduce dead space, are quite suitable. All fluid lines, connectors and valve bodies should be of an inert plastic, since thiocyanate and acid are corrosive. A normally closed, two-way sample valve is important, in order to prevent a disastrous loss of the sample by syphoning under these circumstances. A mechanical, programmable, multi-way device designed for the purpose forms the basis of an automated instrument built by Eveleigh (1978).

The peristaltic pump is most suitable, as chaotropes having a corrosive nature are used. A conventional chromatography UV monitor with its associated recorder is important in optimising the operating conditions and, in retrospect, monitoring of unattended repetitive separations. For the same reason it is useful to incorporate active fluid sensors at critical points within the fluid circuitry to detect line breakages and unanticipated loss of buffers, etc. Other protective devices desirable for long-term unattended operation are pressure sensors to indicate impending column blockage or valve malfunctions, and power failure interruptions to avoid a potential loss of synchronization. For a versatile and reliable control system the microprocessor-based programmer has been introduced. An automatic immunospecific affinity chromatographic system for continuous operation has also been described by Folkersen et al. (1978), and repetitive semi-automatic immunoaffinity chromatography has been described by Römer and Rauterberg (1980).

The development of high performance bioaffinity chromatography offers high flow rates and more rigid bioaffinity matrices suitable for large-scale purification (Ernst-Cabrera and Wilchek, 1987). Fig. 9.2 shows the chromatography of D,L -tryptophan on de-fatted bovine serum albumin attached to succinylaminoethyl agarose published by

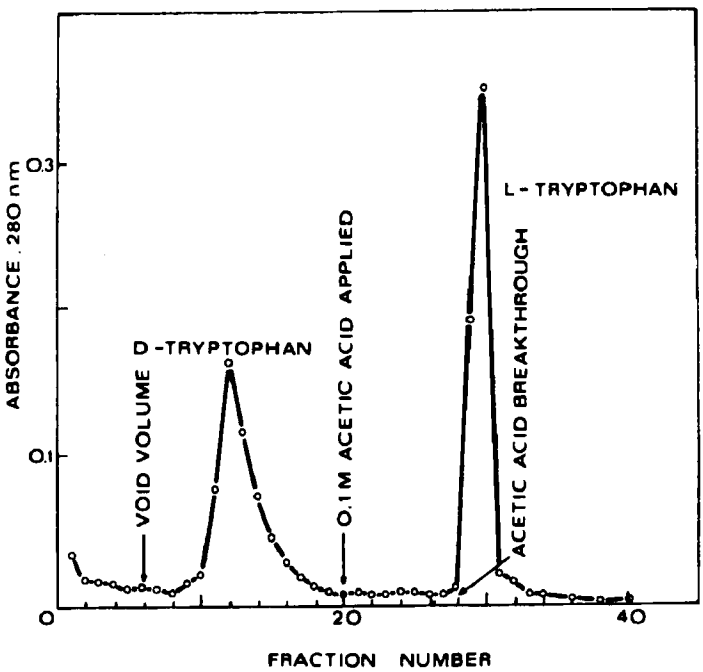


Fig. 9.2. Chromatography of D, L-tryptophan on de-fatted bovine serum albumin-succinylaminoethyl-agarose (250 x 9 mm). D,L-Tryptophan (500 nmole) dissolved in 0.1 ml of 0.1 M borate buffer (pH 9.2) containing 1% (v/v) of dimethyl sulphoxide, was applied to the column (containing a total of 630 nmole of bovine serum albumin). The column was eluted at 30 ml/h with borate buffer (no dimethyl sulphoxide) for 20 tubes, and then with 0.1 N acetic acid. The void volume was determined from the elution volume of dimethyl sulphoxide. Reproduced with permission from K. K. Stewart and R. F. Doherty, Proc. Nat. Acad. Sci. U. S., 70 (1973) 2850 - 2852.

Stewart and Doherty (1973). It was shown that the material eluted with a borate buffer contained only D-tryptophan, whereas that eluted with acetic acid was L-tryptophan only. Evidently, bioaffinity chromatography could be used in a suitable manner for the large-scale resolution of enantiomeric pairs. The different types of bonds of some optical isomers on serum proteins have been more discussed in the first edition of this book (Turková, 1978).

By adopting a systematic, practical approach, separations and purifications of biologically active molecules can become routine procedures rather than an art.

REFERENCES

Aarsman, A. J., Neys, F. and Van den Bosch, H., Biochim.Biophys.Acta, 792 (1984) 363-366.

Abdel-Aal, Y. A. I. and Hammock, B. D., Arch.Biochem.Biophys., 243 (1985) 206-219.

Abdullah, M., Jeremy, R., Davies, H. and Hill, J. A., J.Chromatogr., 347 (1985) 129-136.

Abdullah, M., Siddiqui, A. A., Hill, J. A. and Davies, R. J. H., Arch.Biochem.Biophys., 225 (1983) 306-312.

Abe, Y. and Okazaki, T., Arch.Biochem.Biophys., 253 (1987) 241-248. Abood, L. G., Latham, W. and Grassi, S., Proc.Nat.Acad.Sci. U.S., 80 (1983) 3536-3539.

Abouakil, N., Rogalska, E., Bonicel, J. and Lombardo, D., Biochim.Biophys.Acta, 961 (1988) 299-308.

Abraham, E. C., Perry, R. E. and Stallings, M., J.Lab.Clin.Med., 102 (1983) 187-197.

Absolom, D. and van Regenmortel, M. H. V., FEBS Lett., 81 (1977) 419-444.

Ackermans, F., Klein, J. P., Ogier, J., Bazin, H., Cormont, F. and Frank, R. M., Biochem.J., 228 (1985) 211-217.

Adachi, M., Mizuta, T., Yokota, M., Shibusawa, H., Yamamura, M., Hamada, Y., Uetsuji, S., Yamamoto, M. and Miwa, H., Anticancer Res., 4 (1984) 1-4.

Adams, D. J., Hajj, H., Bitar, K. G., Edwards, D. P. and Mc Guire, W. L., Endocrinology, 113 (1983) 415-417.

Adams, J. B. and McDonald, D., Biochim.Biophys.Acta, 567 (1979) 144-153.

Adamson, E. D., J.Neurochem., 28 (1977) 605-615.

Adinolfi, A. and Hopkinson, D. A., Ann.Hum.Genet., 41 (1978) 399-407.

Aerts, J. M. F. G., Donker-Koopman, W. E., Murray, G. J., Barranger, J. A., Tager, J. M. and Schram, A. W., Anal.Biochem., 154 (1986) 655-663.

Afting, E. G. and Becker, M. L., Biochem.J., 197 (1981) 519-522. Agrawal, A. K. and Rao, V. K. M., Indian J.Exp.Biol., 21 (1983) 553-556.

Agrawal, V. P. and Kolattukudy, P. E., Arch.Biochem.Biophys., 191 (1978) 452-465.

Ahmad, A., Surolia, A. and Bachhawat, B. K., Biochim.Biophys.Acta, 481 (1977) 542-548.

Ahmad, N., Bansal, R., Ahmad, A., Rastogi, A. K. and Kidwai, J. R., Indian J.Biochem.Biophys., 21 (1984) 237-240.

Akasaka, K., Sasaki, H., Shimada, H. and Shiroya, T., Develop Growth Differ., 28 (1986) 85-94.

Aksnes, A., Skotland, T., Flatmark, T. and Jones, T. L., Neurochem.Res., 4 (1979) 385-398.

Alberts, B. and Herrick, G., Methods Enzymol., 21 (1971) 198-217.

Al-Azawie, D. M. A., Read, G. F. and Riad-Fahmi, D., J.Immunol.Methods, 77 (1985) 259-265.

Alessandri, J. -., Christon, R., Arfi, T. S., Riazi, A. and Leger, C. L., J.Chromatogr., 432 (1988) 75-91.

Alessandri, J. M., Leger, C. and Mahe, N., Biochimie, 66 (1984) 663-672.

Alevy, Y. G. and Fleischman, J. B., Mol.Immunol., 17 (1980) 275-280.

Alhadeff, J. A. and Holzinger, R. T., J.Biochem.Biophys.Methods, 6 (1982) 229-233.

Alhadeff, J. A. and Andrews-Smith, G. L., Biochem.J., 187 (1980) 45-51.

Allen, H. J., Cywinski, M., Palmberg, R. and Di Cioccio, R. A., Arch.Biochem.Biophys., 256 (1987) 523-533.

Allenmark, S. Andersson, S. and Bojarski, J., J.Chromatogr., 436 (1988) 479-483.

Allenmark, S., Bomgren, B., Boren, H. and Lagerstrom, P. O., Anal.Biochem., 136 (1984) 293-297.

Allenmark, S., Bomgren, B. and Boren, H., J.Chromatogr., 264 (1983) 63-68.

Allenmark, S. and Bomgren, B., J.Chromatogr., 252 (1982) 297-300.

Alvarez-Gonzales, R., J.Chromatogr., 444 (1988) 89-95.

Alvarez-Gonzales, R., Juarez-Salinas, H., Jacobson, E. L. and Jacobson, M. K., Anal.Biochem., 135 (1983) 69-77.

Alving, C. R. and Richards, R. L., Immunochemistry, 14 (1977) 373-381.

Amanuma, H. and Strominger, J. L., J.Biol.Chem., 255 (1980) 11173-11180.

Amneus, H., Naslund, L., Gabel, D. and Kasche, V., Biochem.Biophys.Res.Commun., 90 (1979) 1313-1320.

Anastasi, A., Brown, M. A., Kembhavi, A. A., Nicklin, M. J. H., Sayers, C. A., Sunter, D. C. and Barrett, A. J., Biochem.J., 211 (1983) 129-138.

Anderson, A., Hoglund, L., Pontis, E. and Reichard, P., Biochemistry, 25 (1986) 867-872.

Anderson, J. K. and Mole, J. E. B. H. J., Biochemistry, 17 (1978) 467-473.

Anderson, C. D. and Hall, P. L., Anal.Biochem., 60 (1974) 417-423.

Andersson, L. and Mosbach, K., Methods Enzymol., 89 (1982) 435-445.

Andersson, K. K., Balny, C., Douzou, P. and Bieth, J. G., J.Chromatogr., 192 (1980) 236-239.

Andersson, L., Jornvall, H. and Mosbach, K., Anal.Biochem., 69 (1975) 401-409.

Andorn, N. and Aronovitch, J., J.Immunol.Methods, 73 (1984) 49-55.

Andrawis, A., Pinsky, A. and Grossman, S., Phytochemistry, 21 (1982) 1523-1525.

Andres, C. M. and Fox, I. H., J.Biol.Chem., 254 (1979) 11388-11393.

Andres, R. Y., Eur.J.Biochem., 62 (1976) 591-600.

Andrews, D. W., Bono, M. R., Kaufman, J. F., Knudsen, P. and Strominger, J. L., Methods Enzymol., 108 (1984) 600-606.

Andrews, P., FEBS Lett., 9 (1970) 297-300.

Ankilova, V. N., Reshetnikova, L. S., Chernaya, M. M. and Lavrik, O. I., FEBS Lett., 227 (1988) 9-13.

Ansari, A. A., Baig, M. A. and Malling, H. V., J.Immunol.Methods, 42 (1981) 45-51.

Ansari, A. A., Biochem.J., 199 (1981) 75-79.

Antoine, J. C., Ternynck, T., Rodrigot, M. and Avrameas, S., Immunochemistry, 15 (1978) 443-452.

Antoni, G., Bigio, M., Borri, G., Casagli, M. C. and Neri, P., Experientia, 39 (1983) 885-886.

Antoni, G., Brizzi, A., Casagli, M. C. and Neri, P., Ital.J.Biochem., 27 (1978) 373-377.

Antoniades, D. and Antonoglou, O., Biochim.Biophys.Acta, 519 (1978) 447-460.

Antonian, L., Antonian, E., Murphy, R. B. and Schuster, D. I., Life Sci., 38 (1986) 1847-1858.

Anwar, R. A. and Vlaovic, M., Can.J.Biochem., 57 (1979) 188-196.

Applebaum, S. W., James, T. C., Wreschner, D. H. and Tata, J. R., Biochem.J., 193 (1981) 209-216.

Arai, K., Iizuka, S., Makita, A., Oikawa, K. and Taniguchi, N., J.Immunol.Methods, 91 (1986) 139-143.

Arends, J., Methods Enzymol., 73 (1981) 166-175.

Arends, J.Immunol.Methods, 25 (1979) 171-175.

Areson, P. D. W., Charm, S. E. and Wong, B. L., Biotechnol.Bioeng., 22 (1980) 2207-2217.

Arnaud, P., Emerson, D. L. and Gianazza, E., Biochim.Biophys.Acta, 749 (1983) 270-275.

Arnaud, P. and Gianazza, E., FEBS Lett., 137 (1982) 157-161.

Arnold, P. S., Garner, R. C. and Tierney, B., Biochem.J., 242 (1987) 375-381.

Artzatbanov, V., Muller, M. and Azzi, A., Arch.Biochem.Biophys., 257 (1987) 476-480.

Arus, C., Nogues, M. V. and Cuchillo, C. M., J.Chromatogr., 237 (1982) 500-505.

Asaoka, K., J.Biochem. Tokyo, 95 (1984) 685-696.

Ashkenazi, A., Madar, Z. and Gertler, A., Moll.Cell.Endocrinol., 50 (1987) 79-87.

Ashley, P. L. and MacDonald, R. J., Anal.Biochem., 140 (1984) 95-103.

Ashman, L. K. and De Young, N. J., Immunochemistry, 14 (1977) 329-336.

Askelof, P. and Gillenius, P., Infection and Immunity, 36 (1982) 958-961.

Atkinson, A., Hammond, P. M., Hartwell, D. R., Hughes, P., Scawen, M. D., Sherwood, R. F., Small, D. A. P., Bruton, C. J., Harvey, M. J. and Lowe, C. R., Biochem.Soc.Trans., 9 (1981) 290-293.

Atrat, P., Deppmeyer, V. and Horhold, C., J.Chromatogr., 189 (1980) 279-283.

Aubert, J. P., Chiroutre, M., Kerckaert, J. P., Helbecque, N. and Loucheux-Lefebvre, M. H., Biochem. Biophys. Res.Commun., 104 (1982) 1550-1559.

Aubry, M., Collu, R., Ducharme, J. R. and Crine, P., Endocrinology, 111 (1982) 2129-2131.

Azaryan, A., Akopyan, T. and Buniatian, H., Biomed.Biochim.Acta, 42 (1983) 1237-1246.

Babashak, V. and Phillips, T. M., J. Chromatogr., 444 (1988) 21-28.

Bachas, L. G., Ashcom, G. S. and Meyerhoff, M. E., Anal.Lett, 19 (1986) 1653-1678.

Bachman, B. K. and Lee, C. Y., Anal.Biochem., 72 (1976) 153-160.

Badia, M. and Querol, E., J.Virol.Meth., 22 (1988) 23-29.

Baecker, P. A. and Wedding, R. T., Anal.Biochem., 102 (1980) 16-21.

Baer, A. and Schiebel, W., Eur.J.Biochem., 86 (1978) 77-84.

Bailyes, E. M., Newby, A. C., Siddle, K. and Luzio, J. P., Biochem.J., 203 (1982) 245-251.

Baines, B. S., Brocklehurst, K., Carey, P. R., Jarvis, M., Salih, E. and Storer, A. C., Biochem.J., 233 (1986) 119-129.

Baksi, K., Rogerson, D. L. and Rushizky, G. W., Biochemistry, 17 (1978) 4136-4139.

Baldwin, T. O., Holzman, T. F., Holzman, R. B. and Riddle, V. A., Methods Enzymol., 133 (1986) 98-108.

Ball, D. J. and Nishimura, J. S., J.Biol.Chem., 255 (1980) 10805-10812.

Ball, E. H. and Singer, S. J., Proc.Nat.Acad.Sci.U.S., 78 (1981) 6986-6990.

Ballard, D. W., Kranz, D. M. and Voss, E. W. J., Proc.Nat.Acad.Sci. U.S., 80 (1983) 5071-5074.

Balow, R. M. and Eriksson, I., Biochem.J., 241 (1987) 75-80.

Bamberg, E., Dorner, F. and Stockl, W., Experientia, 31 (1975) 516-517.

Banfalvi, G., Bhattacharya, S. and Sarkar, N., Anal.Biochem., 146 (1985) 64-70.

Bannikova, G. E., Varlamov, V. P. and Rogozhin, S. V., Bioorg.Khim., 9 (1983) 1505-1510.

Bannon, P., Lessard, F., Lepage, R., Joly, J. G. and Dufresne, L., Clin.Chem., 30 (1984) 485-486.

Bansal, M. R., Kanwar, K. C. and Gupta, G. S., Indian J.Biochem.Biophys., 17 (1980) 484-486.

Baran, M. M., Allen, D. M., Russell, S. R., Scheetz, M. E. and Monthony, J. F., J.Immunol.Methods, 53 (1982) 321-334.

Baranov, V. I., Bioorg.Khim., 9 (1983) 1650-1657.

Barns, R. J. and Elmslie, R. G., Biochim.Biophys.Acta, 445 (1976) 815-818.

Bartfeld, D., FEBS Lett., 105 (1979) 303-306.

Bartfeld, D. and Fuchs, S., Biochem.Biophys.Res.Commun., 89 (1979) 512-519.

Bartlett, D. L., King, C. H. R. and Poulter, C. D., Anal.Biochem., 149 (1985) 507-515.

Bartos, F., Bartos, D., Campbell, R. A., Grettie, D. P., Mc Daniel, R. and Davis, M., Res. Commun. Chem. Pathol.Pharmacol., 23 (1979) 547-559.

Bascomb, N. F., Turner, K. J. and Schmidt, R. R., Plant Physiol., 81 (1986) 527-532.

Basha, S. M. M., Horst, M. N., Bazer, F. W. and Roberts, P. M., Arch.Biochem.Biophys., 185 (1978) 174-184.

Basu, P. S., Ray, M. K. and Datta, T. K., Ind.J.Biochem.Biophys., 18 (1981) 101-104.

Batista-Viera, F., Axen, R. and Carlsson, J., Prep.Biochem., 7 (1977) 103-110.

Bauer, P. I. and Buki, K. G., Acta.Biochim.Biophys., 16 (1981) 135-144.

Baumann, U., Lehmann, U., Schwellnus, K., Van Boom, J. H. and Kuhn, H., Eur.J.Biochem., 170 (1987) 267-272.

Bayer, E. A. and Wilchek, M., J. Chromatogr., 510 (1990) 3-11.

Bayer, E. A., Ben-Hur, H., Gitlin, G. and Wilchek, M., J.Biochem.Biophys.Methods, 13 (1986) 103-112.

Baylin, S. B., Wieman, K. C., O'Neil, J. A. and Roos, B. A., J.Clin.Endocrinol.Metab., 53 (1981) 489-497.

Bazzone, T. J., Sokolovsky, M., Cueni, L. B. and Vallee, B. L., Biochemistry, 18 (1979) 4362-4366.

Beaty, N. B. and Lane, M. D., J. Biol. Chem., 257 (1982) 924-929.

Befort, J. J., Befort, N., Petrissant, G., Remy, P. and Ebel, J. P., Biochimie, 56 (1974) 625-630.

Beikirch, H., von der Haar, F. and Cramer, F., Methods Enzymol., 34 (1974) 503-506.

Bellon, G., Ooyama, T., Hornebeck, W. and Robert, L., Artery, 7 (1980) 290-302.

Benbouzid, A., Guinet, R. and Cloppet, H., J.Chromatogr., 306 (1984) 117-124.

Bendiak, B. and Schachter, H., J.Biol.Chem., 262 (1987) 5775-5783.

Berenson, R. J., Bensinger, W. I. and Kalamasz, D., J.Immunol.Methods, 91 (1986a) 11-19.

Berenson, R. J., Bensinger, W. I., Kalamasz, D. and Martin, P., Blood, 67 (1986b) 509-515.

Berger, E. G., Weiser, M. M. and Isselbacher, K. J., Experientia, 32 (1976) 690-691.

Berghman, L. R., van Beeumen, J., Decuypere, E., Kuhn, E. R. and Vandesande, F., J.Endocrinol., 118 (1988) 381-387.

Berglund, O. and Eckstein, F., Eur.J.Biochem., 28 (1972) 492-496.

Bergstrom, J. D. and Bieber, A. L., Prep.Biochem., 8 (1978) 275-293.

Berliner, L. J., Sugawara, Y. and Fenton II., J. W., Biochemistry, 24 (1985) 7005-7009.

Berman, A. E., Gornaeva, N. P. and Mazurov, V. I., Biokhimiya, 43 (1978) 1830-1837.

Bernard, D., Maurizis, J. C., Chollet, P., Chassagne, J. and Plagne, R., J.Chromatogr., 308 (1984) 322-328.

Berti, A., Liguri, G., Stefani, M., Nassi, P. and Ramponi, G., Physiol.Chem.Phys., 14 (1982) 307-311.

Bertolini, J., Guthridge, M. and Hearn, M. T. W., J.Chromatogr., 491 (1989) 49-60.

Bessler, W. and Schindler, P., Experientia, 35 (1979) 1292-1293.

Bezwoda, W. R. and Mansoor, N., Clin.Chim.Acta, 157 (1986) 89-94.

Bhadra, R. and Datta, P., Biochemistry, 17 (1978) 1691-1699.

Bhakdi, S. and Kayser, W., J.Immunol.Methods, 41 (1981) 365-376.

Bhalla, R. C., Sharma, R. V. and Gupta, R. C., Biochem.J., 203 (1982) 583-592.

Bibor, V. and Verly, W. G., J.Biol.Chem., 253 (1978) 850-855.

Bicsak, T. A. and Harper, E., Anal.Biochem., 145 (1985) 286-291.

Bidlack, J. M., Abood, L. G., Osei-Gyimah, P. and Archer, S., Proc.Nat.Acad.Sci. U.S., 78 (1981) 636-639.

Bihari, V. and Buchholz, K., Biotech.Lett., 6 (1984) 571-576.

Bill, K., Broger, C. and Azzi, A., Biochim.Biophys.Acta, 679 (1982) 28-34.

Billings, P. C., Stclair, W., Owen, A. J. and Kennedy, A. R., Cancer Res., 48 (1988) 1798-1802.

Birkenmeier, G. and Kopperschlager, G., J.Chromatogr., 235 (1982) 237-248.

Bischoff, E., Tran-thi, T. A. and Decker, K. F. A., Eur.J.Biochem., 51 (1975) 353-361.

Bishayee, S. and Dorai, D. T., Biochim.Biophys.Acta, 623 (1980) 89-97.

Bishop, D. F. and Desnick, R. J., J.Biol.Chem., 256 (1981) 1307-1316.

Bizzozero, S. A. and Dutler, H., Arch.Biochem.Biophys., 256 (1987) 662-676.

Blake, D. A. and Goldstein, I. J., Methods Enzymol., 83 (1982) 127-132.

Blake, D. A. and Goldstein, I. J., Anal.Biochem., 102 (1980) 103-109.

Blake, J., Hines, K. K. and Li, C. H., Int.J.Peptide Prot.Res., 20 (1982) 429-433.

Blanchard, K. L. and Richardson, J. P., J.Biol.Chem., 258 (1983) 14091-14097.

Bloom, J. W., Wong, M. F. and Mitra, G., J. Immunol. Methods, 117 (1989) 83-89.

Blumberg, P. M. and Strominger, J. L., Methods Enzymol., 34 (1974) 401-405.

Bober, M. A. and Ownby, C. L., Toxicon, 26 (1988) 301-308.

Boctor, F. N. and Shaheen, H. I., Immunology, 57 (1986) 587-593.

Boctor, F. N., Cheever, A. W. and Higashi, G. I., Immunology, 45 (1982) 237-245.

Bodanszky, A. and Bodanszky, M., Experientia, 26 (1970) 327.

Bodwell, J. E., Holbrook, N. J. and Munck, A., Biochemistry, 23 (1984) 1392-1398.

Bogli, C., Hofer, A. and Furlan, M., Thromb.Haemost., 60 (1988) 308-310.

Bohach, G. A. and Snyder, I. S., Infec.Immunity, 53 (1986) 435-437.

Bok, R. A. and Mangel, W. F., Biochemistry, 24 (1985) 3279-3286.

Bolen, P. L., Roth, K. A. and Freer, S. N., Appl.Environ.Microbiol., 52 (1986) 660-664.

Bond, J. P. and Notides, A. C., Anal.Biochem., 163 (1987) 385-390.

Bond, M. D. and Van Wart, H. E., Biochemistry, 23 (1984) 3077-3085.

Borchardt, R. T. and Schasteen, C. S., Biochim.Biophys.Acta, 708 (1982) 272-279.

Borchardt, R. T. and Cheng, C. F., Biochim.Biophys.Acta, 522 (1978) 49-62.

Borchert, A., Larsson, P. O. and Mosbach, K., J.Chromatogr., 244 (1982) 49-56.

Borensztajn, J., Kotlar, T. J. and Meredith, S. C., J.Biol.Chem., 260 (1985) 13047-13052.

Borrebaeck, C. A. K., Soares, J. and Mattiasson, B., J.Chromatogr., 284 (1984) 187-192.

Boschetti, E., Girot, P., Secheresse, J. P. and Saint-Blancard, J. S., J.Chromatogr., 210 (1981) 469-475.

Bosron, W. F., Li, T. K., Lange, L. G., Dafeldecker, W. P. and Vallee, B. L., Biochem.Biophys.Res.Commun., 74 (1977) 85-91.

Bouriotis, V., Galpin, I. J. and Dean, P. D. G., J.Chromatogr., 210 (1981) 267-278.

Bowers-Komro, D. M., Hagen, T. M. and McCormick, D. B., Methods Enzymol., 122 (1986) 116-120.

Boyle, D. M. and van der Walt, L. A., J.Chromatogr., 455 (1988) 434-438.

Brakstad, O. G. and Maeland, J. A., Acta Pathol.Microbiol.Immun.B, 93 (1985) 323-325.

Brandt, H., Capulong, Z. L. and Lee, E. Y. C., J.Biol.Chem., 250 (1975) 8038-8044.

Brantner, J. H., Medicus, R. G. and McRorie, R. A., J.Chromatogr., 129 (1976) 97-105.

Brattain, M. G., Kimball, P. M., Pretlow, T. G. and Marks, M. E., Biochem.J., 163 (1977) 247-251.

Breddam, K., Sorensen, S. B. and Ottesen, M., Carlsberg Res.Commun., 50 (1985) 199-209.

Breithaupt, T. B. and Light, R. J., J.Biol.Chem., 257 (1982) 9622-9628.

Bridgen, P. J., Anfinsen, C. B., Corley, L., Bose, S., Zoon, K. C. and Ruegg, U. T., J.Biol.Chem., 252 (1977) 6585-6587.

Briske-Anderson, M. and Duerre, J. A., Can.J.Biochem., 60 (1982) 118-123.

Brocklehurst, K., Baines, B. S. and Malthouse, J. P. G., Biochem.J., 197 (1981) 739-746.

Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. and Crook, E. M., Methods Enzymol., 34 (1974) 531-544.

Brodelius, P. E. and Kaplan, N. O., Arch.Biochem.Biophys., 194 (1979) 449-456.

Brodelius, P. E., Lannom, R. A. and Kaplan, N. O., Arch.Biochem.Biophys., 188 (1978) 228-231.

Brodelius, P., Larsson, P. O. and Mosbach, K., Eur.J.Biochem., 47 (1974) 81-89.

Brody, R. S. and Westheimer, F. H., J.Biol.Chem., 254 (1979) 4238-4244.

Broger, C., Bill, K. and Azzi, A., Methods Enzymol., 126 (1986) 64-72.

Brooks, S. P. J. and Storey, K. B., J.Chromatogr., 455 (1988) 291-296.

Brophy, P. J. and Vance, D. E., FEBS Lett., 62 (1976) 123-125.

Brot, F. E., Bell, C. E. and Sly, W. S., Biochemistry, 17 (1978) 385-391.

Brown, A. J. L. and Lee, C. Y., Biochem.J., 179 (1979) 479-482.

Brown, E. and Joyeau, R., Biochimie, 61 (1979) 437-442.

Brown, F., Underwood, B. O. and Fantes, K. H., J.Med.Virol., 4 (1979) 315-319.

Broxmeyer, H. E., Bicknell, D. C., Gillis, S., Harris, E. L., Pelus, L. M. and Sledge, G. W. J., Blood Cells, 11 (1986) 429-446.

Bruck, C., Portetelle, D., Glineur, C. and Bollen, A., J.Immunol.Methods, 53 (1982) 313-319.

Bruns, D. E., Lobo, P. I., Savory, J. and Wills, M. R., Clin.Chem., 30 (1984) 569-571.

Brusdeilins, M., Kuhner, R. and Schumacher, K., Biochim.Biophys.Acta, 840 (1985) 79-90.

Buamah, P. K., Cornell, C. and Skillen, A. W., Clin.Chem., 30 (1984) 1257-1258.

Buc, J., Riviere, M., Gontero, B., Sauve, P., Meunier, J. C. and Ricard, J., Eur.J.Biochem., 140 (1984) 199-202.

Budzik, G. P., Powell, S. M., Kamagata, S. and Donahoe, P. K., Cell, 34 (1983) 307-314.

Buffoni, F., Coppi, C. and Santoni, G., Clin.Chem., 28 (1982) 248-250.

Buki, K. B., Kirsten, E. and Kun, E., Anal.Biochem., 167 (1987) 160-166.

Burchell, B., Biochem.J., 173 (1978) 749-757.

Bureau, D. and Daussant, J., J.Immunol.Methods, 57 (1983) 205-213.

Burger, D., Schleuning, W. D. and Schapira, M., J.Biol.Chem., 261 (1986) 324-327.

Burger, D., Spiranganathan, N., Mc Donald, T. L. and Gorham, J. R., Amer.J.Vet.Res., 44 (1983) 86-90.

Burgess, R. R. and Jendrisak, J. J., Biochemistry, 14 (1975) 4634-4638.

Burrell, H. R. and Horowitz, J., Eur.J.Biochem., 75 (1977) 533-544.

Burrill, P. H., Brannon, P. M. and Kretchmer, N., Anal.Biochem., 117 (1981) 402-405.

Burtin, P. and Gendron, M. C., Immunochemistry, 15 (1978) 245-249.

Burtscher, H. J., Auer, B., Klocker, H., Schweiger, M. and Hirsch-Kauffmann, M., Anal.Biochem., 152 (1986) 285-290.

Busch, S. J., Duvic, C. R., Ellsworth, J. L., Ihm, J. and Harmony, J. A. K., Anal.Biochem., 153 (1986) 178-188.

Busen, W., J.Biol.Chem., 257 (1982) 7106-7108.

Buswell, J. A., Eriksson, K. E. and Pettersson, B., J.Chromatogr., 215 (1981) 99-108.

Bynum, J. W. and Volkin, E., Anal.Biochem., 107 (1980) 406-416.

Callahan, J. W., Shankaran, P., Khalil, M. and Gerrie, J., Can.J.Biochem., 56 (1978) 885-891.

Callaway, J. E., Garcia, J. A. J., Hersh, C. L., Yeh, R. K. and Gilmore-Hebert, M., Biochemistry, 25 (1986) 4757-4762.

Calton, G. J. and Burnett, J. W., Toxicon, 24 (1986) 416-420.

Cambier, J. C. and Neale, M. J., J.Immunol.Methods, 51 (1982) 209-221.

Camejo, G., Hurt, E. and Romano, M., Biomed.Biochim.Acta, 44 (1985) 389-401.

Caperelli, C. A., Chettur, C., Lin, L. Y. and Benkovic, S. J., Biochem.Biophys.Res.Commun., 82 (1978) 403-410.

Carlberg, I. and Mannervik, B., Anal.Biochem., 116 (1981) 531-536.

Carlenor, E., Tang, H. L. and Rydstrom, J., Anal.Biochem., 148 (1985) 518-523.

Carlsen, J., Christiansen, K. and Bro, B., Biochim.Biophys.Acta, 689 (1982) 12-20.

Carnahan, G. E. and Cunningham, L. W., Biochemistry, 22 (1983) 5384-5389.

Carne, A., Anal.Biochem., 121 (1982) 227-229.

Carrea, G., Lugaro, G., Niada, R., Vecchini, P. and Antonini, E., FEBS Lett., 104 (1979) 393-395.

Carroll, A. R. and Wagner, R. R., J.Biol.Chem., 254 (1979) 9339-9341.

Carson, S. D., Ross, S. E., Bach, R. and Guha, A., Blood, 70 (1987) 490-493.

Carter-Su, C., Pillion, D. J. and Czech, M. P., Biochemistry, 19 (1980) 2374-2385.

Cartwright, S. J. and Waley, G., Biochem.J., 221 (1984) 505-512.

Cash, C. D., Maitre, M., Rumigny, J. F. and Mandel, P., Biochem. Biophys.Res. Commun., 96 (1980) 1755-1760.

Cash, C. D., Maitre, M., Ossola, L. and Mandel, P., Biochim.Biophys.Acta, 524 (1978) 26-36.

Caughey, G. H., Viro, N. F., Ramachandran, J., Lazarus, S. C., Borson, D. B. and Nadel, J. A., Arch. Biochem. Biophys., 258 (1987) 555-563.

Chaffotte, A. F., Roucous, C. and Seydoux, F., Eur.J.Biochem., 78 (1977) 309-316.

Chandrasekaran, S. and Dhar, S. C., Arch.Biochem.Biophys., 257 (1987) 395-401.

Chandrika, S. R. and Padmanaban, G., Biochem.J., 191 (1980) 29-36.

Chang, C. S. and Chang, K. P., Proc.Nat.Acad.Sci. U.S., 83 (1986) 100-104.

Chang, J. T. and Chang, G. G., Anal.Biochem., 121 (1982) 366-369.

Chao, J., Chao, L. and Margolius, H. S., Biochim.Biophys.Acta, 80 (1984) 244-249.

Chapuis-Cellier, C., Gianazza, E. and Arnaud, P., Biochim.Biophys.Acta, 709 (1982) 353-357.

Charlton, J. P., J.Chromatogr., 346 (1985) 247-254.

Chaudhuri, S. and Thomas, E. W., Biochem.J., 177 (1979) 115-119.

Chauvin, M. M., Korri, K. K., Tirpak, A., Simpson, R. C. and Scrimgeour, K. G., Can.J.Biochem., 57 (1979) 178-187.

Chen, C. H. and Albers, J. J., Biochem.Biophys.Acta, 834 (1985) 188-195.

Chen, C., Melitz, D. K., Petschow, B. and Eckert, R. L., Eur.J.Biochem., 108 (1980) 379-387.

Chen, C. C. and Somberg, E. W., Biochim.Biophys.Acta, 613 (1980) 514-528.

Chen-Marotel, J., Blouquit, Y., Rosa, R. and Calvin, M. C., J.Chromatogr., 258 (1983) 213-222.

Cheng, C. M. and Hawiger, J., J.Biol.Chem., 254 (1979) 2165-2167.

Cheng, K. C., Krutzsch, H. C., Park, S. S., Grantham, P. H., Gelboin, H. V. and Friedman, F. K., Biochem. Biophys. Res.Commun., 123 (1984) 1201-1208.

Cheng, Y. S. E., Becker-Manley, M. F., Chow, T. P. and Horan, D. C., J.Biol.Chem., 260 (1985) 15834-15839.

Chernikevich, I. P., Voskoboyev, A. I. and Klyashchitsky, B. A., Bioorg.Khim., 7 (1981) 209-216.

Chetrite, G. and Cassoly, R., J.Mol.Biol., 185 (1985) 639-644.

Chiancone, E. and Gattoni, M., J.Chromatogr., 376 (1986) 343-348.

Chiatante, D., Balconi, C., Newton, R. P. and Brown, E. G., Phytochemistry, 27 (1988) 2477-2483.

Chibata, I., Tosa, T. and Matuo, Y., Methods Enzymol., 66 (1980) 221-226.

Chibata, I., Tosa, T. and Matuo, Y., Methods Enzymol., 34 (1974a) 267-271.

Chibata, I., Tosa, T., Sato, T., Sano, R., Yamamoto, K. and Matuo, Y., Methods Enzymol., 34 (1974b) 405-411.

Chibbar, R. N. and van Huystee, R. B., J.Plant Physiol., 116 (1984) 365-373.

Chien, S. F. and Dawson, G., Biochim.Biophys.Acta, 614 (1980) 476-488.

Childs, G., Levy, S. and Kedes, L. H., Biochemistry, 18 (1979) 208-213.

Cho, T. M., Ge, B. L., Yamamoto, C., Smith, A. P. and Loh, H. H., Proc.Nat.Acad.Sci. U.S., 80 (1983) 5176-5180.

Chong, M. T. and Lippman, M. E., J.Biol.Chem., 257 (1982) 2996-3002.

Chong, P. and Klein, M., Biochem.Cell Biol., 67 (1989) 387-391.

Choy, P. C. and Vance, D. E., Biochem.Biophys.Res.Commun., 72 (1976) 714-719.

Choy, Y. M., Lau, K. M. and Lee, C. Y., J.Biol.Chem., 254 (1979) 1159-1163.

Christner, P., Damato, D., Reinhart, M. and Abrams, W., Biochemistry, 21 (1982) 6005-6011.

Chua, M. M., Fan, S. T. and Karush, F., Biochim.Biophys.Acta, 800 (1984) 291-300.

Chung, H. K. and Rhee, S. G., Proc.Nat.Acad.Sci. U.S., 81 (1984) 4677-4681.

Ciechanover, A., Elias, S., Heller, H. and Hershko, A., J.Biol.Chem., 257 (1982) 2537-2542.

Cirakoglu, B. and Waller, J. P., Eur.J.Biochem., 149 (1985a) 353-361.

Cirakoglu, B. and Waller, J. P., Eur.J.Biochem., 151 (1985b) 101-110.

Clemetson, K. J., Naim, H. Y. and Luscher, E. F., Proc.Nat.Acad.Sci. U.S., 78 (1981) 2712-2716.

Clonis, Y. D., J.Chromatogr., 407 (1987) 179-187.

Clonis, Y. D., Jones, K. and Lowe, C. R., J.Chromatogr., 363 (1986) 31-36.

Clonis, Y. D. and Lowe, C. R., Biochem.Biophys.Acta, 659 (1981) 86-98.

Clonis, Y. D. and Lowe, C. R., Eur.J.Biochem., 110 (1980) 279-288.

Clune, A., Lee, K. S. and Ferenci, T., Biochem.Biophys.Res.Commun., 121 (1984) 34-40.

Cohen, C. M., Kramer, R. M. and Branton, D., Biochim.Biophys.Acta, 597 (1980) 29-40.

Coll, R. J. and Murphy, A. J., J.Biol.Chem., 259 (1984) 14249-14254.

Collins, S. and Marletta, M. A., Biochemistry, 25 (1986) 4322-4329.

Collins, S., Altman, J. D. and Marletta, M. A., Biochem.Biophys.Res.Commun., 129 (1985) 155-162.

Colonno, R. J., Callahan, P. L. and Long, W. J., J.Virol., 57 (1986) 7-12.

Colson, P. and Verly, W. G., Eur.J.Biochem., 134 (1983) 415-420.

Comte, M., Malnoe, A. and Cox, J. A., Biochem.J., 240 (1986) 567-573.

Conrad, D. H. and Froese, A., J.Immunol., 120 (1978) 429-437.

Cook, G. M. W. and Buckie, J. W., Methods Enzymol., 184 (1990) 304-314.

Cook, N. D. and Peters, T. J., Biochim.Biophys.Acta, 828 (1985) 205-212.

Coombe, R. G. and George, A. M., Aust.J.Biol.Sci., 29 (1976) 305-316.

Coppenhaver, D. H., IRCS Med.Sci.-Biochem., 13 (1985) 809-810.

Corfman, R. S. and Reeck, G. R., Biochim.Biophys.Acta, 715 (1982) 170-174.

Corti, A. and Cassani, G., Appl.Biochem.Biotech., 11 (1985) 101-109.

Cotton, R. G. H. and Jennings, I. G., Eur.J.Biochem., 85 (1978) 357-363.

Cox, J. A., Comte, M., Fitton, J. E. and De Grado, W. F., J.Biol.Chem., 260 (1985) 2527-2534.

Cozzari, C. and Hartman, B. K., Anal.Biochem., 133 (1983) 120-125.

Craney, C. L. and Goffredo, M. E., Anal.Biochem., 128 (1983) 312-316.

Cranston, J. W., Perini.F., Crisp, E. R. and Hixson, C. V., Biochim.Biophys.Acta, 616 (1980) 239-258.

Cremel, G., Filliol, D. and Waksman, A., Anal.Biochem., 150 (1985) 332-336.

Cresswell, P., J.Biol.Chem., 254 (1979) 414-419.

Creutz, C. E., Biochem.Biophys.Res.Commun., 103 (1981) 1395-1400.

Crewther, P. E., Bianco, A. E., Brown, G. V., Coppel, R. L., Stahl, H. D., Kemp, D. J. and Anders, R. F., J.Immunol.Methods, 86 (1986) 257-264.

Crowley, S. C. and Walters, R. R., J.Chromatogr., 266 (1983) 157-162.

Csiky, I. and Hansson, L., J.Liq.Chromatogr., 9 (1986) 875-886.

Cueni, L. B., Bazzone, T. J., Riordan, J. F. and Vallee, B. L., Anal.Biochem., 107 (1980) 341-349.

Cukor, G. and Nowak, N. A., J.Bacteriol., 149 (1982) 1162-1165.

Cummings, R. D. and Kornfeld, S., J.Biol.Chem., 259 (1984) 6253-6260.

D'Souza, S. and Ananthakrishnan, R., Austr.J.Exp.Biol.Med.Sci., 57 (1979) 245-250.

Dahl, D. and Bignami, A., Biochim.Biophys.Acta, 578 (1979) 305-316.

Dailey, H. A., Methods Enzymol., 123 (1986) 408-415.

Dailey, H. A., Fleming, J. E. and Harbin, B. M., Methods Enzymol., 123 (1986) 401-408.

Dakour, J., Lundblad, A. and Zopf, D., Anal.Biochem., 161 (1987) 140-143.

Dakour, J., Hansson, G. C., Lundblad, A. and Zopf, D., Arch.Biochem.Biophys., 248 (1986) 677-683.

Danielsen, E. M., Noren, O., Sjostrom, H., Ingram, J. and Kenny, A. J., Biochem.J., 189 (1980) 591-603.

Danielsson, B., Buelow, L., Lowe, C. R., Satoh, I. and Mosbach, K., Anal.Biochem., 117 (1981) 84-93.

Dano, K., Moller, V., Ossowski, L. and Nielsen, L. S., Biochim.Biophys.Acta, 613 (1980) 542-555.

Danton, M. J. and Coleman, M. S., Anal.Biochem., 159 (1986) 233-239.

Das, K., Methods Enzymol., 66 (1980) 39-50.

Das, K., Dunnill, P. and Lilly, M. D., Biochim.Biophys.Acta, 397 (1975) 277-287.

Dasch, J. R., Pace, D. R., Waegell, W., Inenaga, D. and Ellingsworth, L., J.Immunol., 142 (1989) 1536-1541.

Datta, P. K., Basu, P. S. and Datta, T. K., Prep.Biochem., 14 (1984) 373-387.

Davidson, M. W., Gray, G. O. and Knaff, D. B., FEBS Lett., 187 (1985) 155-159.

Davis, C. W. and Daly, J. W., J.Cyclic Nucleotide Res., 5 (1979) 65-74.

Davis, G. C., Hein, M. B. and Chapman, D. A., J.Chromatogr., 366 (1986) 171-189.

Dawes, J., Anal.Biochem., 174 (1988) 177-186.

Day, E. D. and Hashim, G. A., Neurochem.Res., 9 (1984) 1453-1465.

Day, E. D., Hashim, G. A., Varitek, V. A. J., Lazarus, K. J. and Paterson, P. Y., J.Neuroimmunol., 1 (1981) 311-324.

De Bari, V. A., Nicotra, J., Blaney, J. F., Schultz, E. F. and Needle, M. A., Clin.Chem., 30 (1984) 1187-1192.

De Bell, K. E., Taplits, M. S., Hoffman, T. and Bonvini, E., Cell. Immunol., 127 (1990) 159-171.

De Bortoli, M. and Roggero, P., Microbiologica, 8 (1985) 113-121.

De Cristofaro, R., Landolfi, R., Bizzi, B. and Castaglo, M., J.Chromatogr., 426 (1988) 376-380.

De Graaf-Hess, A. C. and de Kok, A., FEBS Lett., 143 (1982) 261-264.

De la Rosa, M. A., Diez, J. and Vega, J. M., Rev.Espan.Fisiol., 36 (1980) 177-182.

De Larminat, M. A., Bruchovsky, N., Rennie, P. S., Lee, S. P. and Tertzakian, G., Prostate, 5 (1984) 123-140.

De Martinis, M. L., Mc Intyre, P. and Hoogenraad, N., Biochem.Int., 3 (1981) 371-378.

De Meyer, P., De Herdt, E., Kondo, M. and Slegers, H., J.Biochem.Biophys.Methods, 2 (1980) 311-314.

De Pedro, M. A. and Schwarz, U., FEMS Microbiol.Lett., 9 (1980) 215-217.

De Pinto, V., Tommasino, M., Palmieri, F. and Kadenbach, B., FEBS Lett., 148 (1982) 103-106.

De Schutter, G., Wuytack, F., Verbist, J. and Casteels, R., Biochim.Biophys.Acta, 773 (1984) 1-10.

De Witt, D. L. and Smith, W. L., J.Biol.Chem., 258 (1983) 3285-3293.

Dean, R. T., J.Chromatogr., 150 (1978) 279-283.

Deguchi, T., Sakamoto, Y., Sasaki, Y. and Uyemura, K., J.Biol.Chem., 263 (1988) 7528-7533.

Del Rosso, M., Cappelletti, R., Viti, M., Vannucchi, S. and Chiarugi, V., Biochem.J., 199 (1981) 699-704.

Delagneau, J. F., Perrin, P. and Atanasiu, P., Ann.Virol., 132 (1981) 461-471.

Delius, H., Van Heerikhuizen, H., Clarke, J. and Koller, B., Nucl.Acid.Res., 13 (1985) 5457-5469.

Delori, P. and Tessier, M., Biochimie, 62 (1980) 287-288.

Demaille, J. G., Peters, K. A. and Fischer, E. H., Biochemistry, 16 (1977) 3080-3086.

Den Tandt, W. R., Clin.Chim.Acta, 102 (1980) 199-205.

Denney, R. M., Fritz, R. R., Patel, N. T. and Abell, C. W., Science, 215 (1982) 1400-1403.

Desai, N. N. and Allen, A. K., Anal.Biochem., 93 (1979) 88-90.

Desvages, G., Roustan, C., Fattoum, A. and Pradel, L. A., Eur.J.Biochem., 105 (1980) 259-266.

Deutsch, D. G., Fogelman, D. J. and von Kaulla, K. N., Biochem.Biophys.Res.Commun., 50 (1973) 758-764.

Deutsch, D. G. and Mertz, E. T., Science, 170 (1970) 1095-1096.

Devaney, E. and Howell, K. E., EMBO J., 4 (1985) 3123-3130.

Dey, P. M., Eur.J.Biochem., 140 (1984) 385-390.

Deyl, Z. and Adam, M., J.Chromatogr., 488 (1989) 161-197.

Di Cioccio, R. A., Barlow, J. J. and Matta, K. L., Carbohyd.Res., 127 (1984) 109-120.

Diaco, R., Hill, J. H., Hill, E. K., Tachibana, H. and Durand, D. P., J.Gen.Virol., 66 (1986) 2089-2094.

Dickerman, H. W., Ryan, T. J., Bass, A. I. and Chatterjee, N. K., Arch.Biochem.Biophys., 186 (1978) 218-234.

Dicou, E. and Brachet, P., Biochem.Biophys.Res.Commun., 102 (1981) 1172-1179.

Dicou, E. and Brachet, P., Biochem.Biophys.Res.Commun., 90 (1979) 1321-1327.

Dieckhoff, J., Knebel, H., Heidemann, M. and Mannherz, H. G., Eur.J.Biochem., 151 (1985) 377-383.

Dierickx, P. J., IRCS Med.Sci.-Biochem., 14 (1986) 1007-1008.

Digman, J. D. and Strobel, H. W., Biochemistry, 16 (1977) 1116-1123.

Dimroth, P., FEBS Lett., 141 (1982) 59-62.

Dobrowolska, G., Ber, E. and Muszynska, G., J.Chromatogr., 376 (1986) 421-427.

Dodd, R. Y. and Kobita, J. A., J.Immunol.Methods, 20 (1978) 117-129.

Dodin, G., FEBS Lett., 134 (1981) 20-24.

Doellgast, G. J., Spiegel, J., Guenther, R. A. and Fishman, W. H., Biochem.Biophys.Acta, 484 (1977) 59-78.

Degerman, E., Belfrage, P., Newman, A. H., Rice, K. C. and Manganiello, V. C., J.Biol.Chem., 262 (1987) 5797-5807.

Donner, P., Wagner, H. and Kroger, K., Biochem.Biophys.Res.Commun., 80 (1978) 766-772.

Dousset, J. C., Dousset, N., Foglietti, M. J. and Douste-Blazy, L., Biochim.Biophys.Acta, 664 (1981) 273-277.

Draetta, G. and Klee, C. B., Methods Enzymol., 159 (1988) 573-581.

Drechsel, D., Karic, L. and Glaser, C. B., Anal.Biochem., 143 (1984) 141-145.

Drocourt, J. L., Gangloff, J., Dirheimer, G. and Thang, M. N., Biochem.Biophys.Res.Commun., 97 (1980) 787-793.

Drocourt, J. L., Thang, D. C., Buckingham, R. H. and Thang, M. N., Nucl.Acids Res., 6 (1979) 2919-2928.

Droux, M., Jacquot, J. P., Miginac-Maslow, M., Gadal, P., Huet, J. C., Crawford, N. A., Yee, B. C. and Buchanan, B. B., Arch.Biochem.Biophys., 252 (1987) 426-439.

Drummond, R. J., J.Neurochem., 33 (1979) 1143-1150.

Ducrocq, R., Le Bonniec, B., Carlier, O., Assan, R. and Elion, J., J.Chromatogr., 419 (1987) 75-83.

Duez, C., Frere, J. M., Klein, D., Noel, M. and Ghuysen, J. M., Biochem.J., 193 (1981) 75-82.

Duffey, P. S., Drouillard, D. L. and Barbe, C. P., J.Immunol.Methods, 45 (1981) 137-151.

Dugas, B., Vazquez, A., Gerard, J. P., Richard, Y., Auffredou, M. T., Delfraissy, J. F., Fradelizi, D. and Galanaud, P., J.Immunol., 135 (1985) 333-338.

Duhamel, R. C., Meezan, E. and Brendel, K., Mol.Immunol., 17 (1980) 29-36.

Dumas, B., Legrand, M. G. P. and Fritig, B., Planta, 176 (1988) 36-41.

Dumitrescu, M., Schell, H. D., Mateescu, M. A. and Bentia, T., Rev.Roum.Biochim., 19 (1982) 121-126.

Duncan, C. H. and Cavalier, S. L., Anal.Biochem., 169 (1988) 104-108.

Dunn, S. D. and Tozer, R. G., Arch.Biochem.Biophys., 253 (1987) 73-80.

Dunn, S. D., Anal.Biochem., 159 (1986) 35-42.

Dupuis, G., Doucet, J. P., Bastin, B. and Cardin, J., Can.J.Biochem.Cell Biol., 63 (1985) 932-940.

Durley, R. C., Sharp, C. R., Maki, S. L., Brenner, M. L. and Carnes, M. G., Plant Physiol., 90 (1989) 445-451.

Dvorak, D. J., Gipps, E. and Kidson, C., Nature, 271 (1978) 564-566.

Dy, M., Lebel, B. and Schneider, E., J.Immunol., 136 (1986) 208-212.

Egorov, T. A., Shakhparonov, M. I., Davidovith, Y. A., Losinsky, V. I., Zaslavsky, B. Y. and Rogozhin, S. V., Bioorg.Khim., 3 (1977) 1111-1116.

Egorov, T. A., Svenson, A., Ryden, L. and Carlsson, J., Proc.Nat.Acad.Sci. U.S., 72 (1975) 3029-3033.

Ehlers, M. R. W., Maeder, D. L. and Kirsch, R. E., Biochim.Biophys.Acta, 883 (1986) 361-372.

Eichler, D. C. and Glitz, D. G., Biochim.Biophys.Acta, 335 (1974) 303-317.

Eisenberg, S., Francesconi, S. C., Civalier, C. and Walker, S. S., Methods Enzymol., 182 (1990) 521-529.

Ekanger, R. and Doskeland, S. O., Methods Enzymol., 159 (1988) 97-104.

Ekanger, R., Sand, T. E., Ogreid, D., Christoffersen, T. and Doskeland, S. O., J.Biol.Chem., 260 (1985) 3393-3401.

Ekramoddoullah, A. K. M., Kisil, F. T. and Sehon, A. H., Int.Arch.Allergy Appl.Immunol., 80 (1986) 100-106.

El-Dorry, H. A., Biochim.Biophys.Acta, 867 (1986) 252-255.

El-Dorry, H. A., Bull, H. G., Iwata, K., Thornberry, N. A., Cordes, E. H. and Soffer, R. L., J.Biol.Chem., 257 (1982) 14128-14133.

El-Habib, R., Coulet, P. R., Sanhadji, K., Gautheron, D. G., Laville, M. and Traeger, J., Biotechnol.Bioeng., 26 (1984) 665-669.

Elango, N., Srinivasan, A. and Rao, A. R., J.Biochem.Biophys.Methods, 4 (1981) 233-240.

Elbein, A. D. and Mitchell, M., Arch.Biochem.Biophys., 168 (1975) 369-377.

Elices, M. J. and Goldstein, I. J., Arch.Biochem.Biophys., 254 (1987) 329-341.

Elkana, Y., Thornton, A. and Zuckerman, A. J., J.Immunol.Methods, 25 (1979) 185-187.

Elseweidy, M. M., Stallings, M. and Abraham, E. C., J.Lab.Clin.Med., 102 (1983) 628-636.

Elwell, M. and Hersh, L. B., J.Biol.Chem., 254 (1979) 2434-2438.

Emlen, W. and Burdick, G., J.Immunol.Methods, 62 (1983) 205-215.

Emod, I., Tong, N. T. and Keil, B., Biochim.Biophys.Acta, 659 (1981) 283-291.

Emod, I. and Keil, B., FEBS Lett., 77 (1977) 51-56.

Emoto, Y., Kawamura, T. and Tawada, K., J.Biochem.Tokyo, 103 (1988) 172-176.

Endo, F., Tanoue, A., Ogata, T., Motohara, K. and Matsuda, I., Clin.Chim.Acta, 176 (1988) 143-150.

Endo, M. and Hiyama, N., J.Biochem.Tokyo, 86 (1979) 1559-1565.

Endo, T., Tanaka, T., Isobe, T., Kasai, H., Okuyama, T. and Hidaka, H., J.Biol.Chem., 256 (1981) 12485-12489.

Endrizzi, A., Hrelia, S., Lucacchini, A. and Rossi, C. A., FEBS Lett., 108 (1979) 395-398.

Enevoldsen, B. S., Reimann, L. and Hansen, N. L., FEBS Lett., 79 (1977) 121-124.

Englebienne, P. and Doyen, G., J.Immunol.Methods, 62 (1983) 197-204.

Erdodi, F., Csortos, C., Bot, G. and Gergely, P., Biochem.Biophys.Res.Commun., 128 (1985) 705-712.

Erickson, R. H. and Kim, Y. S., Biochim.Biophys.Acta, 743 (1983) 37-42.

Erickson, R. P. and Sandman, R., Experientia, 33 (1977) 14-15.

Ernst-Cabrera, K. and Wilchek, M., Trends Anal. Chem., 7 (1988a) 58-63.

Ernst-Cabrera, K. and Wilchek, M., Med. Sci. Res., 16 (1988b) 305-310.

Ernst-Cabrera, K. and Wilchek, M., in R. Burgess (Editor), Protein Purification: Micro to Macro, Alan R. Liss, Inc., New York, 1987, pp. 163-175.

Ernst-Cabrera, K. and Wilchek, M., Anal.Biochem., 159 (1986) 267-272.

Eruslanov, B. V., Kramarov, V. M., Smolyaninov, V. V. and Borovik, R. V., Bioorg.Khim., 6 (1980) 1361-1369.

Escribano, M. J., Haddada, H. and de Vaux Saint Cyr, C., J.Immunol.Methods, 52 (1982) 63-72.

Esmann, M., Anal.Biochem., 108 (1980) 83-85.

Esumi, H., Sato, S., Sugimura, T. and Furihata, C., FEBS Lett., 86 (1978) 33-36.

Evans, C. H., Biochem.J., 225 (1985) 553-556.

Eveleight, J.V., J.Chromatogr., 159 (1978) 129-145.

Eveleight, J.V., in T.C.J. Gribnau, J. Visser and R.F.J. Nivard (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982, pp. 293-303.

Evstratova, N. G., Aianyan, A. E., Miroshnikov, A. I., Serebrennikova, G. A. and Evstigneeva, R. P., Biokhimiya, 47 (1982) 1547-1550.

Fähnrich, P. and Irrgang, K., Biotechnol.Lett., 6 (1984) 251-256.

Farmer, E. E. and Easterby, J. S., Anal.Biochem., 141 (1984) 79-82.

Farmer, E. E. and Easterby, J. S., Anal.Biochem., 123 (1982) 373-377.

Farooqui, A. A. and Hanson, W. L., Biochem.J., 242 (1987) 97-102.

Farooqui, A. A., Taylor, W. A. and Horrocks, L. A., Biochem.Biophys.Res.Commun., 122 (1984) 1241-1246.

Farooqui, A. A. and Srivastava, P. N., Int.J.Biochem., 10 (1979) 745-748.

Fassina, G., Swaisgood, H. E. and Chaiken, I. M., J.Chromatogr., 376 (1986) 87-93.

Fattoum, A., Roustan, C., Feinberg, J., Desvages, G. and Pradel, L. A., Eur.J.Biochem., 82 (1978) 161-167.

Fauvel-Lafeve, F., Picard, P., Godeau, G. and Legrand, Y. J., Biochem.J., 255 (1988) 251-258.

Faye, L., Salier, J. P. and Ghorbel, A., J.Chromatogr., 235 (1982) 427-433.

Federico, R., di Lisi, F. and Angelini, R., Plant Sci., 38 (1985) 9-12.

Fedoroff, N. V. and Zinder, N. D., Proc.Nat.Acad.Sci. U.S., 68 (1971) 1838-1843.

Feist, P. L. and Danna, K. J., Biochemistry, 20 (1981) 4243-4246.

Feracci, H. and Maroux, S., Biochim.Biophys.Acta, 599 (1980) 448-463.

Ferenci, T. and Lee, K. S., J.Mol.Biol., 160 (1982) 431-444.

Fernlund, P. and Laurell, C. B., J.Steroid Biochem., 14 (1981) 545-552.

Ferre, J., Yim, J. J. and Jacobson, K. B., J.Chromatogr., 357 (1986) 283-292.

Ferreira, P. C. P., Paucker, M., Golgher, R. R. and Paucker, K., Arch.Virol., 68 (1981) 27-33.

Fex, G. and Hansson, B., Biochim.Biophys.Acta, 537 (1978) 358-365.

Fick Jr., R. B., Naegel, G. P. and Reynolds, H. Y., J.Immunol.Methods, 38 (1980) 103-116.

Fiddler, M. B., Ben-Yoseph, Y. and Nadler, H. L., Biochem.J., 177 (1979) 175-180.

Figueiredo, A. F. S., Takii, Y., Tsuji, H., Kato, K. and Inagami, T., Biochemistry, 22 (1983) 5476-5481.

Filka, K., Čoupek, J. and Kocourek, J., Biochim.Biophys.Acta, 539 (1978) 518-528.

Fink de Cabutti, N. E., Caron, M., Joubert, R., Elola, M. T., Bladier, D. and Herkovitz, J., FEBS Lett., 223 (1987) 330-334.

Finlay, T. H., Troll, V. and Hodgins, L. T., Anal.Biochem., 108 (1980) 354-359.

Finn, F. M. and Hofmann, K., Yale J.Biol.Med., 58 (1985) 597-604.

Finn, F. M., Titus, G., Horstman, D. and Hofmann, K., Proc.Nat.Acad.Sci. U.S., 81 (1984) 7328-7332.

Fioretti, E., Binotti, I., Barra, D., Citro, G., Ascoli, F. and Antonini, E., Eur.J.Biochem., 130 (1983) 13-18.

Fischer, A. M., Yu, X. J., Tapon-Bretau-Diere, J., Muller, D., Bros, A. and Jozefonvicz, J., J.Chromatogr., 363 (1986) 95-100.

Fischer, W., Doi, T., Ikehara, M., Ohtsuka, E. and Sprinzl, M., FEBS Lett., 192 (1985) 151-154.

Fischer, H. D., Creek, K. E. and Sly, W. S., J.Biol.Chem., 257 (1982) 9938-9943.

Fishel, R., Anziano, P. and Rich, A., Methods Enzymol., 184 (1990) 328-340.

Fishel, R. A. and Detmer, K. R. A., Proc.Nat.Acad.Sci. U.S., 85 (1988) 36-40.

Fleischmann, G., Mauder, I., Illert, W. and Rudiger, H., Biol.Chem.Hoppe-Seyler, 366 (1985) 1029-1032.

Foglietti, M. J., J.Chromatogr., 128 (1976) 309-312.

Folena-Wasserman, G., Sitrin, R. D., Chapin, F. and Snader, K. M., J.Chromatogr., 392 (1987) 225-238.

Folkersen, J., Teisner, B., Westergaard, J. G. and Grudzinskas, J. G., J.Immunol.Methods, 77 (1985) 45-54.

Folkersen, J., Teisner, B., Ahrons, S. and Svehag, S. E., J.Immunol.Methods, 23 (1978a) 117-125.

Folkersen, J., Teisner, B., Westergaard, J. and Svehag, S.E., J.Immunol.Methods, 23 (1978b) 137-147.

Fong, S., Fox, R. I., Rose, J. E., Liu, J., Tsoukas, C. D., Carson, D. A. and Vaughan, J. H., J.Immunol.Methods, 46 (1981) 153-163.

Formosa, T., Burke, R. L. and Alberts, B. M., Proc.Nat.Acad.Sci. U.S., 80 (1983) 2442-2446.

Formstecher, P., Hammadi, H., Bouzerna, N. and Dautrevaux, M., J.Chromatogr., 369 (1986) 379-390.

Fornstedt, N., FEBS Lett., 177 (1984) 195-199.

Forsdyke, D. R., Anal.Biochem., 137 (1984) 143-145.

Fořtová, H., Dyr, J. E., Vodrážka, Z. and Kornalík, F., J.Chromatogr., 260 (1983) 522-526.

Foster, W. S. and Jarrett, H. W., J.Chromatogr., 403 (1987) 99-107.

Fougier, S., Nemoz, G., Prigent, A. F., Marivet, M., Bourguignon, J. J., Wermuth, C. and Pacheco, H., Biochem.Biophys.Res.Commun., 138 (1986) 205-214.

Fox, R. A., Macsween, J. M. and Rajaraman, R., Develop.Comp.Immunol., 2 (1978) 147-160.

Fox, T. O., Bates, S. E., Vito, C. C. and Wieland, S. J., J.Biol.Chem., 254 (1979) 4963-4966.

Franěk, F., Saber, M. A., Doskočil, J., Novotný, J. and Fust. G., Mol.Immunol., 16 (1979) 389-394.

Fransson, L. A., Carbohyd.Res., 110 (1982) 127-144.

Fransson, L. A. and Havsmark, B., Int.J.Biol.Macromol., 4 (1982) 73-78.

Frantz, S. C., Henriques, H. F. and Phillips, T. M., J.Chromatogr., 444 (1988) 41-46.

Franz, H. and Ziska, P., Lectins-Biol.,Biochem.,Clin.Biochem., 1 (1981) 179-184.

Fraser, C. M. and Venter, J. C., Biochem.Biophys.Res.Commun., 109 (1982) 21-29.

Freier, T., Fleischmann, G. and Rudiger, H., Biol.Chem.Hoppe-Seyler, 366 (1985) 1023-1028.

Freitag, N. and Mc Entee, K., J.Biol.Chem., 263 (1988) 19525-19534.

French, J. S. and Coon, M. J., Arch.Biochem.Biophys., 195 (1979) 565-577.

Freytag, J. W., Dickinson, J. C. and Tseng, S. Y., Clin.Chem., 30 (1984) 417-420.

Friedman, F. K., Robinson, R. C., Park, S. S. and Gelboin, H. V., Biochem.Pharmacol., 34 (1985) 2051-2054.

Friedman, F. K., Robinson, R. C., Krutzsch, H. C., Grantham, P. H., Park, S. S. and Gelboin, H. V., Biochem. Biophys.Res.Commun., 129 (1985) 926-933.

Friedrichs, W. E. and Grose, C., J.Virol., 49 (1984) 992-996.

Friesen, H. J., Stein, S., Evinger, M., Familletti, P. C., Moschera, J., Meienhofer, J., Shively, J. and Pestka, S., Arch.Biochem.Biophys., 206 (1981) 432-450.

Frigon, R. P. and Stone, R. A., J.Biol.Chem., 253 (1978) 6780-6786.

Frith, G. J. T., Swinden, L. B. and Dalling, M. J., Plant Cell Physiol., 19 (1978) 1029-1041.

Froehner, S. C. and Rafto, S., Biochemistry, 18 (1979) 301-307.

Froman, G., Lundahl, P. and Acevedo, F., FEBS Lett., 129 (1981) 100-104.

Fromant, M., Fayat, G., Laufer, P. and Blanquet, S., Biochimie, 63 (1981) 541-553.

Frost, R. G., Holmes, E. W., Norden, A. G. W. and O'Brien, J. S., Biochem.J., 175 (1978) 181-188.

Frost, R. G., Reitherman, R. W., Miller, A. L. and O'Brien, J. S., Anal.Biochem., 69 (1975) 170-179.

Fujii, S., Kawaguchi, H. and Yasuda, H., J.Biochem.Tokyo, 101 (1987) 679-684.

Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y. and Hakura, K., J.Biol.Chem., 258 (1983) 5045-5049.

Fukami, Y. and Lipmann, F., Proc.Nat.Acad.Sci. U.S., 82 (1985) 321-324.

Fukushima, Y., Hayashi, K. and Motai, H., Agr.Biol.Chem. Tokyo, 49 (1985) 1643-1648.

Fuller, S. A., Philips, A. and Coleman, M. S., Biochem.J., 231 (1985) 105-113.

Funk, M. O., Nakagawa, Y., Skochdopole, J. and Kaiser, E. T., Int.J.Peptide Protein Res., 13 (1979) 296-303.

Furukawa, K. and Nakamura, H., J.Biochem. Tokyo, 96 (1984) 1343-1350.

Gabius, H. J. and Engelhardt, R., J.Chromatogr., 391 (1987) 452-456.

Gade, W., Jack, M. A., Dahl, J. B., Schmidt, E. L. and Wold, F., J.Biol.Chem., 256 (1981) 12905-12910.

Gaida, A. V., Monastyrskii, V. A., Magerovskii, Y. V., Staroverov, S. M. and Lisichkin, G. V., J.Chromatogr., 424 (1988) 385-391.

Galvez, G. E., Virology, 28 (1966) 171-187.

Gambaryan, A. S., Venkstern, T. V., Nedospasov, A. A. and Khomutov, R. M., Bioorg.Khim., 6 (1980) 901-907.

Gamble, H. R., Exp.Parasitol., 59 (1985) 398-404.

Garcia, C. M. and Singhal, R. P., Biochem.Biophys.Res.Commun., 86 (1979) 697-703.

Garcia-Olalla, C. and Garrido-Pertierra, A., Biochem.J., 241 (1987) 573-581.

Gardlik, S., Barber, M. J. and Rajagopalan, K. V., Arch.Biochem.Biophys., 259 (1987) 363-371.

Gaskell, S. J. and Brownsey, B. G., Clin.Chem., 29 (1983) 677-680.

Gazitt, Y., Margel, S., Lerner, A., Wands, J. R. and Shouval, D., Immunol.Lett., 11 (1985) 1-8.

Geiger, R., Clausnitzer, B., Fink, E. and Fritz, H., Hoppe-Seyler's Z.Physiol.Chem., 361 (1980) 1795-1803.

Gelijkens, C. F. and De Leenheer, A. P., J.Chromatogr., 183 (1980) 78-82.

Gemeiner, P. and Viskupic, E., J.Biochem.Biophys.Methods, 4 (1981) 309-319.

Genaud, L., Guillot, J., Betail, G. and Coulet, M., J.Immunol.Methods, 49 (1982) 323-332.

George, J. and Chirikjian, J. G., Nucl.Acid.Res., 5 (1978) 2223-2232.

Geren, C. R., Olomon, C. M., Primrose, D. C. and Ebner, K. E., Prep.Biochem., 7 (1977) 19-31.

Gergely, P., Castle, A. G. and Crawford, N., Int.J.Biochem, 10 (1979) 807-814.

Germino, J., Gray, J. G., Charbonneau, H., Vanaman, T. and Bastia, D., Proc.Nat.Acad.Sci. U.S., 80 (1983) 6848-6852.

Gershenwald, J. E., Bensadoun, A. and Saluja, A., Biochim.Biophys.Acta, 836 (1985) 286-295.

Ghalayini, A. and Eichberg, J., J.Neurochem., 44 (1985) 175-182.

Ghangas, G. S. and Wilson, D. B., Appl.Environ.Microbiol., 54 (1988) 2521-2526.

Ghetie, V. and Sjoquist, J., Methods Enzymol., 108 (1984) 132-138.

Ghetie, V., Mota, G. and Sjoequist, J., J.Immunol.Methods, 21 (1978) 133-141.

Gianazza, E., Emerson, D. L., Dykes, D. and Arnaud, P., Biochem.J., 218 (1984) 969-973.

Gibbons, J. J. J., Pohl, D. A., Tsai, C. C. and Roodman, S. T., Biochim.Biophys.Acta, 670 (1981) 146-149.

Gibson, D. M. and Ullah, A. H., Arch.Biochem.Biophys., 260 (1988) 503-513.

Gibson, G. G. and Schenkman, J. B., J.Biol.Chem., 253 (1978) 5957-5963.

Gietzen, K., Tejcka, M. and Wolf, H. U., Biochem.J., 189 (1980) 81-88.

Gilbert, H. J., Lowe, C. R. and Drabble, W. T., Biochem.J., 183 (1979) 481-494.

Gilead, Z., Gazitt, Y., Klein, G. and Sulitzeanu, D., Methods Enzymol., 74 (1981) 664-675.

Gilliam, A. C., Lang, D. and Lo Spalluto, J. J., J.Immunol., 125 (1980) 874-885.

Gingrich, J. A., Amlaiky, N., Senogles, S. E., Chang, W. K., Mc, Quade, R. D., Berger, J. G. and Caron, M. G., Biochemistry, 27 (1988) 3907-3912.

Girard, R. and Goichot, J., Ann.Immunol.-Inst.Pasteur, C132 (1981) 211-217.

Gjedde, F. and Gjedde, B., Acta Endocrinol., 95 (1980) 139-144.

Glad, M., Ohlson, S., Hansson, L., Mansson, M. O. and Mosbach, K., J.Chromatogr., 200 (1980) 254-260.

Gleeson, P. A., Jermyn, M. A. and Clarke, A. E., Anal.Biochem., 92 (1979) 41-45.

Glencross, R. G., Abeywardene, S. A., Corney, S. J. and Morris, H. S., J.Chromatogr., 223 (1981) 193-197.

Glenney, J. R. J. and Weber, K., Anal.Biochem., 150 (1985) 364-368.

Glick, J. and Oppenheim, J. D., Vox Sang., 49 (1985) 49-57.

Gluck, S. and Caldwell, J., J.Biol.Chem., 262 (1987) 15780-15789.

Godinot, C. and Gautheron, D. C., Methods Enzymol., 55 (1979) 112-114.

Goldman, M. E., Weber, R. J., Newman, A. H., Rice, K. C., Skolnick, P. and Paul, S. M., J. Chromatogr., 382 (1986) 264-269.

Gonchoroff, D. G., Branum, E. L. and O'Brien, J. F., Clin.Chem., 35 (1989) 29-32.

Gong, C. S., Chen, L. F. and Tsao, G. T., Biotechnol.Bioeng., 21 (1979) 167-171.

Goodman, R. B. and Peanasky, R. J., Anal.Biochem., 120 (1982) 387-393.

Gopalakrishna, R., Barsky, S. H. and Anderson, W. B., Biochem.Biophys.Res.Commun., 128 (1985) 1118-1124.

Gordon, G. L. and Doelle, H. W., Eur.J.Biochem., 67 (1976) 543-555.

Gorecki, M., Bar-Eli, A., Burstein, Y., Patchornik, A. and Chain, E. B., Biochem.J., 147 (1975) 131-137.

Gottvaldová, M., Hrabová, H., Sillinger, V. and Kučera, J., J.Chromatogr., 427 (1988) 331-334.

Gould, B. J., Hall, P. M. and Cook, J. G. H., Clin.Chim.Acta, 125 (1982) 41-48.

Gounaris, A. D., Brown, M. A. and Barrett, A. J., Biochem.J., 221 (1984) 445-452.

Grabowski, P. J., Methods Enzymol., 184 (1990) 319-327.

Grabowski, G. A. and Dagan, A., Anal.Biochem., 141 (1984) 267-279.

Grandics, P., Miller, A., Schmidt, T. J. and Litwack, G., Biochem.Biophys.Res.Commun., 120 (1984) 59-65.

Grant, D. W., Magee, A. I. and Hermon-Taylor, J., Eur. J. Biochem., 88 (1978) 183-189.

Grasser-Regallet, F., Scheftel, J. M. and Monteil, H., FEMS Microbiol.Lett., 35 (1986) 239-243.

Gravel, R. A., Lam, K. F., Mahuran, D. and Kronis, A., Arch.Biochem.Biophys., 201 (1980) 669-673.

Green, E. D., Brodbeck, R. M. and Baenziger, J. U., Anal.Biochem., 167 (1987) 62-75.

Greenberg, M. and Tsong, T. Y., J.Biol.Chem., 259 (1984) 13241-13245.

Greenfield, R. S., Kiener, P. A., Chin, D. P., Curry, R. C. and Spitalny, G. L., J.Leukocyte Biol., 40 (1986) 665-676.

Gresikova, M., Russ, G., Novak, M. and Sekeyova, M., Acta Virol., 28 (1984) 141-143.

Grez, M. and Niessing, J., FEBS Lett., 77 (1977) 57-60.

Gribnau, A. A. M., Schoenmakers, J. G. G., van Kraaikamp, M. and Bloemendal, H., Biochem. Biophys. Res.Commun., 38 (1970) 1064-1068.

Griffith, M. J. and Marbet, G. A., Biochem.Biophys.Res.Commun., 112 (1983) 663-670.

Griffiths, B. W. and Godard, A., Can.J.Biochem.Cell Biol., 61 (1983) 130-136.

Griffiths, P., Houlton, A., Adams, M. J., Harvey, M. J. and Dean, P. D. G., Biochem.J., 161 (1977) 561-568.

Grivennikov, I. A., Petukhov, S. P., Bulargina, T. V., Gulyaev, N. N. and Severin, E. S., Biokhimiya, 49 (1984) 1395-1406.

Grob, P. M., Berlot, C. H. and Bothwell, M. A., Proc.Nat.Acad.Sci. U.S., 80 (1983) 6819-6823.

Grob, P. M. and Chadha, K. C., Biochemistry, 18 (1979) 5782-5786.

Groopman, J. D., Donahue, P. R., Zhu, J., Chen, J. and Wogan, G. N., Proc.Nat.Acad.Sci. U.S., 82 (1985) 6492-6496.

Gross, S. C., Kumar, S. A. and Dickerman, H. W., J.Biol.Chem., 257 (1982) 4738-4745.

Grossmann, K., Friedrich, H. and Seitz, H. U., Z.Naturforsch.Sect.C, 39 (1984) 652-655.

Gschwind, H. P., Gschwind, U., Paul, C. H. and Kirschner, K., Eur.J.Biochem., 96 (1979) 403-416.

Guesdon, J. L., Courcon, J. and Avrameas, S., J.Immunol.Methods, 21 (1978) 59-63.

Guha, A., Bach, R., Konigsberg, W. and Nemerson, Y., Proc.Nat.Acad.Sci. U.S., 83 (1986) 299-302.

Guillot, J., Genaud, L., Gueugnot, J. and Damez, M., Biochemistry, 22 (1983) 5365-5369.

Gulliver, P. A. and Tipton, K. F., Eur.J.Biochem., 88 (1978) 439-444.

Gundelfinger, E., Saumweber, H., Dallendorfer, A. and Stein, H., Eur.J.Biochem., 111 (1980) 395-401.

Gunzer, G. and Hennrich, N., J.Chromatogr., 296 (1984) 221-229.

Gurne, D., Chen, J. and Shemin, D., Proc.Nat.Acad.Sci. U.S., 74 (1977) 1383-1387.

Hack, N., Wilkinson, J. M. and Crawford, N., Biochem.J., 250 (1988) 355-361.

Hackenthal, E., Hackenthal, R. and Hilgengeldt, U., Biochim.Biophys.Acta, 522 (1978) 561-573.

Haddad, J. G., Kowalski, M. A. and Sanger, J. W., Biochem.J., 218 (1984) 805-810.

Haeuptle, M. T., Aubert, M. L., Djiane, J. and Kraehenbuhl, J. P., J.Biol.Chem., 258 (1983) 305-314.

Haga, K. and Haga, T., J.Biol.Chem., 258 (1983) 13575-13579.

Hagemeier, E., Boos, K. S., Schlimme, E., Lechtenborger, K. and Kettrup, A., J.Chromatogr., 268 (1983a) 291-295.

Hagemeier, E., Kemper, K., Boos, K. S. and Schlimme, E., J.Chromatogr., 282 (1983b) 663-669.

Hagerman, D. D., J.Biol.Chem., 262 (1987) 2398-2400.

Hahn, L. H. E. and Yamada, K. M., Proc.Nat.Acad.Sci. U.S., 76 (1979) 1160-1163.

Hahn, T., Toker, L., Budilovsky, S., Aderka, D., Eshhar, Z. and Wallach, D., Proc.Nat.Acad.Sci. U.S., 82 (1985) 3814-3818.

Haiech, J., Predeleanu, R., Watterson, D. M., Ladant, D., Bellalou, J., Ullmann, A. and Barzu, O., J.Biol. Chem., 263 (1988) 4259-4262.

Hall, N., Addis, P. and De Luca, M., Biochemistry, 18 (1979) 1745-1751.

Hama, T., Okada, M., Kojima, K., Kato, T., Matsuyama, M. and Nagatsu, T., Mol.Cell.Biochem., 43 (1982) 35-42.

Hammock, B. D., Prestwich, G. D., Loury, D. N., Cheung, P. Y. K., Eng, W. S., Park, S. K., Moody, D. E., Silva, M. H. and Wixtrom, R. N., Arch.Biochem.Biophys., 244 (1986) 292-309.

Hammond, P. M., Atkinson, T. and Scawen, M. D., J.Chromatogr., 366 (1986) 79-90.

Hammond, C. I. and Holland, M. J., J.Biol.Chem., 258 (1983) 3230-3241.

Hampson, D. R. and Wenthold, R. J., J.Biol.Chem., 263 (1988) 2500-2505.

Hannestad, U., Lundqvist, P. and Sorbo, B., Anal.Biochem., 126 (1982) 200-204.

Hanover, J. A., Rudick, J. E., Willingham, M. C. and Pastan, I., Arch.Biochem.Biophys., 227 (1983) 570-579.

Hansen, R. S., Charbonneau, H. and Beavo, J. A., Methods Enzymol., 159 (1988) 543-557.

Hansen, R. S. and Beavo, J. A., Proc.Nat.Acad.Sci. U.S., 79 (1982) 2788-2792.

Hanson, J. W. and Dailey, H. A., Biochem.J., 222 (1984) 695-700.

Hansson, G. C. and Zopf, D., J.Biol.Chem., 260 (1985) 9388-9392.

Hapgood, J. P., Sabbatini, G. P. and von Holt, C., Biochemistry, 25 (1986) 7529-7534.

Harada, H., Kamei, M., Tokumoto, Y., Yui, S., Koyama, F., Kochibe, N., Endo, T. and Kobata, A., Anal.Biochem., 164 (1987a) 374-381.

Harada, H., Kamei, M., Sunada, K., Yui, S. and Koyama, F., J.Chromatogr., 407 (1987b) 299-304.

Harada, H., Kamei, M., Yui, S. and Koyama, F., J.Chromatogr., 355 (1986) 291-295.

Harb, J., Meflah, K., Duflos, Y. and Bernard, S., Eur.J.Biochem., 137 (1983) 131-138.

Harmon, F. R., Anal.Biochem., 103 (1980) 58-63.

Harpaz, N., Flowers, H. M. and Sharon, N., Eur.J.Biochem., 77 (1977) 419-426.

Harpel, P. C., Sullivan, R. and Chang, T., J.Biol.Chem., 264 (1989) 616-624.

Harper, L., Scott, G. K. and Seow, H. F., Comp.Biochem.Physiol.Pt.B, 78 (1984) 231-235.

Harper, J. R., Mahmoudi, N. and Orengo, A., Clin.Chem., 28 (1982) 2378-2382.

Harris, R. B., Ohlsson, J. T. and Wilson, I. B., Arch.Biochem.Biophys., 206 (1981) 105-112.

Harris, J. I., Hocking, J. D., Runswick, M. J., Suzuki, K. and Walker, J. E., Eur.J.Biochem., 108 (1980) 535-547.

Hasegawa, S., Uenoyama, R., Takeda, F., Chuma, J., Baba, S., Kamiyama, F., Iwakawa, M. and Fushimi, M., J.Chromatogr., 278 (1983) 25-34.

Hashida, S., Imagawa, M., Ishikawa, E. and Freytag, J. W., J.Immunoassay, 6 (1985) 111-123.

Hatcher, V. B., Oberman, M. S., Lazarus, G. S. and Grayzel, A. I., J.Immunol., 120 (1978) 665-670.

Hatton, M. W. C., Kaur, H. and Regoeczi, E., Biochem.Soc.Trans., 5 (1977) 1443-1446.

Hauschka, P. V., Mavrakos, A. E., Iafrati, M. D., Doleman, S. E. and Klagsbrun, M., J.Biol.Chem., 261 (1986) 12665-12674.

Hayashida, S., Kunisaki, S., Nakao, M. and Flor, P. Q., Agr.Biol.Chem.Tokyo, 46 (1982) 83-89.

Hayes, J. D., Biochem.J., 233 (1986) 789-798.

Hayunga, E. G. and Sumner, M. P., J.Parasitol., 72 (1986) 283-291.

Hazlett, D. T. G., Immunochemistry, 14 (1977) 473-476.

Hearn, M. T. W. and Davies, J. R., J.Chromatogr., 512 (1990) 23-39.

Hearn, M. T. W., J.Chromatogr., 376 (1986) 245-257.

Hearn, M. T. W., Harris, E. L., Bethel, G. S., Hancock, W. S. and Ayers, J. A., J.Chromatogr., 218 (1981) 509-518.

Hearn, M. T. W., Bethell, G. S., Ayers, J. S. and Hancock, W. S., J.Chromatogr., 185 (1979) 463-470.

Hegazy, M. G., Thysseril, T. J., Schlender, K. K. and Reimann, E. M., Arch.Biochem.Biophys., 258 (1987) 470-481.

Heikinheimo, M., Stenman, U. H., Bang, B., Hurme, M., Makela, O. and Bohn, H., J.Immunol.Methods, 60 (1983) 25-31.

Heinrich, J., Pilch, P. F. and Czech, M. P., J.Biol.Chem., 255 (1980) 1732-1737.

Hellstrom, U., Hammarstrom, M. L., Hammarstrom, S. and Perlmann, P., Methods Enzymol., 108 (1984) 153-168.

Hemler, M., Lands, W. E. M. and Smith, W. L., J.Biol.Chem., 251 (1976) 5575-5579.

Heney, G. and Orr, G. A., Anal.Biochem., 114 (1981) 92-96.

Henriksen, A. Z. and Maeland, J. A., Acta Path.Microbiol.Immunol.Scand.Sect.B, 94 (1986) 257-263.

Herberg, J. T., Codina, J., Rich, K. A., Rojas, F. J. and Iyengar, R., J.Biol.Chem., 259 (1984) 9285-9294.

Herington, A. C., Ymer, S. I. and Stevenson, J. L., Biochem.Biophys.Res.Commun., 139 (1986) 150-155.

Herion, P. and Bollen, A., Bioscience Rep., 3 (1983) 373-379.

Herman, T. M., Lefever, E. and Shimkus, M., Anal.Biochem., 156 (1986) 48-55.

Hermann, K., Kimura, B. and Phillips, M. I., Biochem.Biophys.Res.Commun., 136 (1986) 685-700.

Herron, L. R., Abel, C. A., Vander Wall, J. and Campbell, P. A., Eur.J.Immunol., 13 (1983) 73-78.

Hershfield, M. S., Aiyar, V. N., Premakur, R. and Small, W. C., Biochem.J., 230 (1985) 43-52.

Hershko, A., Heller, H., Elias, S. and Ciechanover, A., J.Biol.Chem., 258 (1983) 8206-8214.

Hertz, C. M., Graves, D. J., Lauffenburger, D. A. and Serota, F. T., Biotechnol.Bioeng., 27 (1985) 603-612.

Hettkamp, H., Legler, G. and Bause, E., Eur.J.Biochem., 142 (1984) 85-90.

Hey, Y. and Dean, P. D. G., Biochem.J., 209 (1983) 363-371.

Heywood, S. M. and Kennedy, D. S., Arch.Biochem.Biophys., 192 (1979) 270-281.

Hibino, T., Anal.Biochem., 147 (1985) 342-352.

Higa, S. and Kishimoto, S., Anal.Biochem., 154 (1986) 71-74.

Hillman, J. D., Biochem J., 179 (1979) 99-107.

Hillyer, G. V. and De Ateca, L. S., Infect.Immun., 26 (1979) 802-807.

Hilpert, W. and Dimroth, P., Eur.J.Biochem., 132 (1983) 579-587.

Hindsgaul, O. and Ballou, C. E., Biochemistry, 23 (1984) 577-584.

Hinman, C. L., Green, C. L., Denno, D., Rauch, H. C. and Hudson, R. A., Mol.Immunol., 22 (1985) 681-688.

Hino, Y. and Minakami, S., J.Biol.Chem., 257 (1982) 2563-2568.

Hirai, K., Matsuda, Y. and Nakagawa, H., J.Biochem.Tokyo, 102 (1987) 893-902.

Hiramatsu, A., Agr.Biol.Chem. Tokyo, 46 (1982) 371-374.

Hiremath, S. T., Loor, R. M. and Wang, T. Y., Biochem.Biophys.Res.Commun., 97 (1980) 981-986.

Hirota, K. and Shimamura, M., J.Chromatogr., 319 (1985) 173-185.

Hissey, P. H., Thompson, K. J. and Bawden, L., J.Immunol.Methods, 78 (1985) 211-216.

Hitchcock, M. J. M., Farrell, C. A., Huybensz, S., Luh, B. Y. and Phelps, D. J., Anal.Biochem., 163 (1987) 219-223.

Hitzeman, R. A. and Price, A. R., J.Biol.Chem., 253 (1978) 8518-8525.

Hizi, A. and Joklik, W. K., J.Biol.Chem., 252 (1977) 2281-2289.

Hoch, S. O. and McVey, E., J.Biol.Chem., 252 (1977) 1881-1887.

Hochstrasser, K., Schonberger, O. L., Rossmanith, I. and Wachter, E., Hoppe-Seyler's Z.Physiol.Chem., 362 (1981) 1357-1362.

Hochuli, E., Gillessen, D. and Kocher, H. P., J.Chromatogr., 411 (1987) 371-378.

Hodgkinson, S. C. and Lowry, P. J., Biochem.J., 205 (1982) 535-541.

Hoffmann, L. G. and Kuo, C. Y., J.Immunol.Methods, 15 (1977) 101-119.

Hofmann, K., Wood, S. W., Brinton, C. C., Montibeller, J. A. and Finn, F. M., Proc.Nat.Acad.Sci. U.S., 77 (1980) 4666-4668.

Hojeberg, B., Blomberg, K., Stenberg, S. and Lind, C., Arch.Biochem.Biophys., 207 (1981) 205-224.

Hokari, S. and Sakagishi, Y., Arch.Biochem.Biophys., 253 (1987) 350-356.

Holden, J. A., Meredith, G. S. and Kelley, W. N., J.Biol.Chem., 254 (1979) 6951-6955.

Holden, J. A. and Kelley, W. N., J.Biol.Chem., 253 (1978) 4459-4463.

Holland, R., Hardie, D. G., Clegg, R. A. and Zammit, V. A., Biochem.J., 226 (1985) 139-145.

Holme, K. R., Hall, L. D., Armstrong, C. R. and Withers, S. G., Carbohyd.Res., 173 (1988) 285-291.

Holmes, E. H. and Hakomori, S. I., J.Biochem.Tokyo, 101 (1987) 1095-1105.

Holmquist, L. and Nilsson, G., Acta Path.Microbiol.Scand.Sect.B, 87 (1979) 129-135.

Holroyde, M. J., Chesher, J. M. E., Trayer, I. P. and Walker, D. G., Biochem.J., 153 (1976) 351-361.

Holstege, C. P., Pickaart, M. J. and Louters, L. L., J.Chromatogr., 455 (1988) 401-405.

Honda, S., Suzuki, K., Suzuki, S. and Kakehi, K., Anal.Biochem., 169 (1988) 239-245.

Honda, S., Suzuki, S. and Kakehi, K., J.Chromatogr., 396 (1987) 93-100.

Honda, T., Fujita, A., Tsubakihara, Y. and Morihara, K., J.Chromatogr., 376 (1986) 385-393.

Hongo, S. and Sato, T., Anal.Biochem., 114 (1981) 163-166.

Hooper, N. M. and Turner, A. J., Biochem.J., 241 (1987) 625-633.

Hopff, W. H., Bodmer, D. M. and Waser, P. G., J.Recept.Res., 4 (1984) 219-229.

Hořejší, V., Biochim.Biophys.Acta, 577 (1979) 389-393.

Hori, K., Kurotsu, T., Kanda, M., Miura, S., Nozoe, A. and Saito, Y., J.Biochem., 84 (1978) 425-434.

Hormann, H. and Seidl, M., Hoppe-Seyler's Z.Physiol.Chem., 361 (1980) 1449-1452.

Hornsey, V. S., Prowse, C. V. and Pepper, D. S., J.Immunol.Methods, 93 (1986) 83-88.

Hosoya, H., Mabuchi, I. and Sakai, H., J.Biochem. Tokyo, 92 (1982) 1853-1862.

Hotta, K. and Goso, K., Anal.Biochem., 110 (1981) 338-341.

Housley, P. R., Sanchez, E. R., Westphal, H. M., Beato, M. and Pratt, W. B., J.Biol.Chem., 260 (1985) 13810-13817.

Hradec, J. and Kříž, O., Biochem.J., 173 (1978) 349-352.

Hsia, C. J. C., Er, S. S., Hronowski, L. J., Persaud, K. and Ansari, M. R., J.Chromatogr., 381 (1986) 153-157.

Hsu, H. H. T., Munoz, P. A., Barr, J., Oppliger, I., Morris, D. C., Vaananen, H. K., Tarkenton, N. and Anderson, H. C., J.Biol.Chem., 260 (1985) 1826-1831.

Huang, K. P., Akatsuka, A., Singh, T. J. and Blake, K. R., J.Biol.Chem., 258 (1983) 7094-7101.

Hubbard, R. A., Schluter, S. F. and Marchalonis, J. J., Methods Enzymol., 108 (1984) 139-148.

Hubert, J. J., Schenk, D. B., Skelly, H. and Leffert, H. L., Biochemistry, 25 (1986) 4156-4163.

Hubscher, U., Pedrali-Noy, G., Knust-Kron, B., Doerfler, W. and Spadari, S., Anal.Biochem., 150 (1985) 442-448.

Hughes, R. C. and Mills, G., Biochem.J., 211 (1983) 575-587.

Humayun, M. Z. and Jacob, T. M., Biochim.Biophys.Acta, 349 (1974) 84-95.

Hunt, J. S., McGiven, A. R., Groufsky, A., Lynn, K. L. and Taylor, M. C., Biochem.J., 227 (1985) 957-963.

Hunt, J. S., MacDonald, P. R. and McGiven, R., Biochem.Biophys.Res.Commun., 104 (1982) 1025-1032.

Hunt, T., Herbert, P., Campbell, E. A., Delidakis, C. and Jackson, R. J., Eur.J.Biochem., 131 (1983) 303-311.

Husain, S. S., Lipinski, B. and Gurewich, V., Proc.Nat.Acad.Sci. U.S., 78 (1981) 4265-4269.

Husic, D. W. and Tolbert, N. E., Arch.Biochem.Biophys., 252 (1987) 396-408.

Iberg, N. and Fluckiger, R., J.Biol.Chem., 261 (1986) 13542-13545.

Ida, S., J.Biochem. Tokyo, 82 (1977) 915-918.

Igarashi, H., Morita, T. and Iwanaga, S., J.Biochem. Tokyo, 86 (1979) 1615-1618.

Iglesias, J. L., Lis, H. and Sharon, N., Eur.J.Biochem., 123 (1982) 247-252.

Ikeda, M., Gutkowska, J., Thibault, G., Boucher, R. and Genest, J., Hypertension, 3 (1981) 81-86.

Ikeda, S. and Ives, D. H., J.Biol.Chem., 260 (1985) 12659-12664.

Ikeda, S., Park, I., Gardner, P. and Ives, D. H., Biochemistry, 23 (1984) 1914-1921.

Ikemoto, F., Dzau, V. J., Haber, E., Takaori, K. and Yamamoto, K., Clin.Sci., 65 (1983) 117-120.

Ikura, K., Sakurai, H., Okumura, K., Sasaki, R. and Chiba, H., Agr.Biol.Chem. Tokyo, 49 (1985) 3527-3531.

Ikuta, T., Okubo, H., Ishibashi, H., Shibata, K., Nagano, M. and Kudo, J., Biochem.Int., 2 (1981) 619-628.

Illum, L., Jones, P. D. E., Kreuter, J., Baldwin, R. W. and Davis, S. S., Int.J.Pharm., 17 (1983) 65-76.

Imagawa, M., Yoshitake, S., Ishikawa, E., Niitsu, Y., Urushizaki, I., Kanazawa, R., Tachibana, S., Nakazawa, N. and Ogawa, H., Clin.Chim.Acta, 121 (1982) 277-289.

Imai, T. and Anderson, B. M., Arch.Biochem.Biophys., 254 (1987) 253-262.

Imam, A., Laurence, D. J. R. and Neville, A. M., Biochem.J., 193 (1981) 47-54.

Imber, M. J., Glasgow, L. R. and Pizzo, S. V., J.Biol.Chem., 257 (1982) 8205-8210.

Inagaki, M., Yokokura, H., Itoh, T., Kanmura, Y., Kuriyama, H. and Hidaka, H., Arch.Biochem.Biophys., 254 (1987) 136-141.

Inagaki, M., Watanabe, M. and Hidaka, H., J.Biol.Chem., 260 (1985) 2922-2925.

Inagaki, K., Miwa, I. and Okuda, J., Arch.Biochem.Biophys., 216 (1982) 337-344.

Inoue, K., Nishimukai, H. and Yamasawa, K., Biochim.Biophys.Acta, 569 (1979) 117-123.

Inoue, M., Hara, M., Nagashima, F., Matsui, S., Mitsuyasu, N. and Morino, Y., Biochim.Biophys.Acta, 659 (1981) 362-369.

Inoue, S., Hashida, S., Kohno, T., Tanaka, K. and Ishikawa, E., J.Biochem. Tokyo, 98 (1985a) 1387-1394.

Inoue, S., Hashida, S., Tanaka, K., Imagawa, M. and Isikawa, E., Anal.Lett.Pt.B-Clin.Biochem., 18 (1985b) 1331-1344.

Inoue, S., Nagamatsu, Y. and Hatanaka, C., Agr.Biol.Chem. Tokyo, 48 (1984) 633-640.

Inouye, Y., Kawaguchi, Y. and Nakamura, S., Chem.Pharm.Bull. Tokyo, 33 (1985) 1544-1551.

Inouye, Y., Kawaguchi, Y. and Nakamura, S., Chem.Pharm.Bull. Tokyo, 32 (1984) 2333-2339.

Iqbal, M. J. and Johnson, M. W., J.Steroid Biochem., 10 (1979) 535-540.

Ishibashi, M., Ito, A., Sakyo, K. and Mori, Y., Biochem.J., 241 (1987) 527-534.

Ishibashi, T. and Imai, Y., J.Lipid Res., 26 (1985) 393-395.

Ishibashi, T., Maru, A., Imai, Y., Makita, A. and Tsuji, I., Biochim.Biophys.Acta, 616 (1980) 218-227.

Ishii, K., Futaki, S., Uchiyama, H., Nagasawa, K. and Andoh, T., Biochem.J., 241 (1987) 111-119.

Ishii, S. I., Yokosawa, H., Kumazaki, T. and Nakamura, I., Methods Enzymol., 91 (1983) 378-383.

Ishii, S. and Kasai, K., Methods Enzymol., 80 (1981) 842-848.

Isobe, M. and Sugiura, M., Chem.Pharm.Bull. Tokyo, 25 (1977) 1987-1991.

Isogai, Y., Yamamoto, Y., Yamamoto, Y. and Nishimura, M., FEBS Lett., 224 (1987) 71-74.

Isojima, S., Koyama, K. and Fujiwara, N., Clin.Exp.Immunol., 49 (1982) 449-456.

Isotova, L. S., Gorodetsky, D. I., Yanonis, V. V., Baratova, L. A., Belyanova, L. P., Timokhina, E. A., Strongin, A. Y. and Stepanov, V. M., Bioorg.Khim., 4 (1978) 397-403.

Ito, N., Noguchi, K., Shimura, K. and Kasai, K. I., J.Chromatogr., 333 (1985) 107-114.

Ito, Y., Yamasaki, Y., Seno, N. and Matsumoto, I., J.Biochem. Tokyo, 99 (1986) 1267-1272.

Ivanov, A. E., Zhigis, L. S., Chekhovskykh, E. A., Reshetov, P. D. and Zubov, V. P., Bioorg.Khim., 11 (1985) 1527-1532.

Iverson, G. M., Eardley, D. D., Janeway, C. A. and Gershon, R. K., Proc.Nat.Acad.Sci. U.S., 80 (1983) 1435-1439.

Iwase, H., Kato, Y. and Hotta, K., J.Biol.Chem., 256 (1981) 5638-5642.

Iyengar, R. and Herberg, J. T., Methods Enzymol., 109 (1985) 13-20.

Iynedjian, P. B., Enzyme, 24 (1979) 366-373.

Izuta, S. and Saneyoshi, M., Anal.Biochem., 174 (1988) 318-324.

Jacob, R. T. and Pattabiraman, T. N., J.Biosciences, 6 (1984) 289-295.

Jacobsen, D. W. and Huennekens, F. M., Methods Enzymol., 123 (1986) 28-36.

Jacobsen, D. W., Montejano, Y. D. and Huennekens, F. M., Anal.Biochem., 113 (1981) 164-171.

Jain, S. K., Pluskal, M. G. and Sarkar, S., FEBS Lett., 97 (1979) 84-90.

Jallal, B., Salesse, R. and Garnier, J., C.R.Acad.Sci.Ser.III.-Vie, 303 (1986) 73-76.

Jambon, B., Montagne, P., Bene, M. C., Brayer, M. P., Faure, G. and Duheille, J., J.Immunol., 127 (1981) 2055-2059.

Jamieson, G. A. J. and Frazier, W. A., Arch.Biochem.Biophys., 227 (1983) 609-617.

Jansen, E. H. J. M., Steenbergen, J., Dejong, F. H. and Vandermolen, H. J., Mol.Cell.Endocrinol., 21 (1981) 109-117.

Janusz, M., Niezgodka, M., Wieczorek, Z. and Lisowski, J., J.Immunol.Methods, 86 (1986) 119-124.

Jarrett, H. W., J.Chromatogr., 363 (1986) 456-461.

Jenny, R., Church, W., Odegaard, B., Litwiller, R. and Mann, K., Prep.Biochem., 16 (1986) 227-245.

Jensen, J. R. and Sand, T. T., Int.Arch.Allergy Appl.Immunol., 81 (1986) 104-111.

Jensen, P. J., Amos, D. B. and Koren, H. S., J.Immunol., 123 (1979) 1127-1132.

Jepsen, S. and Andersen, B. J., Acta Pathol.Microbiol.Scand.Sect.C, 89 (1981) 99-103.

Jergil, B. and Mosbach, K., Methods Enzymol., 34 (1974) 261-264.

Jervis, L., Biochem.J., 197 (1981) 755-758.

Jimbow, K., Jimbow, M. and Chiba, M., J.Invest.Dermatol., 77 (1981) 213-218.

Joachimiak, A., Zwierzynski, T., Radocki, D., Barciszewska, M. and Barciszewski, J., J.Chromatogr., 206 (1981) 600-605.

Joachimiak, A., Barciszewska, M., Barciszewski, J. and Wiewiorowski, M., J.Chromatogr., 180 (1979) 157-162.

Job, D., Durand, R. and Teissere, M., Eur.J.Biochem., 128 (1982) 35-39.

Johansen, J. T., Carlsberg Res.Commun., 41 (1976) 73-80.

Johansson, B., J.Chromatogr., 381 (1986) 107-113.

Johnson, S. J., Stevenson, K. J. and Gupta, V. S., Can.J.Biochem., 58 (1980) 1252-1257.

Josic, D., Hofmann, W., Habermann, R. and Reutter, W., J.Chromatogr., 444 (1988) 29-39.

Josic, D., Hofman, W., Habermann, R., Becker, A. and Reutter, W., J.Chromatogr., 397 (1987) 39-46.

Jouanneau, Y. and Tabita, F. R., Arch.Biochem.Biophys., 254 (1987) 290-303.

Jouve, M. H., Pelmont, J. and Gaillard, J., Arch.Biochem.Biophys., 248 (1986) 71-79.

Juckett, D. A. and Hultquist, D. E., Proc.Soc.Exp.Biol.Med., 172 (1983) 79-83.

Juranka, P. and Chan, V. L., J.Biol.Chem., 260 (1985) 7738-7743.

Kadonaga, J. T. and Tjian, R., Proc.Nat.Acad.Sci. U.S., 83 (1986) 5889-5893.

Kadusevicius, V. A., Sudzhiuvene, O. F. and Pesliakas, J. J., Bioorg.Khim., 9 (1983) 1128-1135.

Kaetzel, C. S., Rao, C. K. and Lamm, M. E., Biochem.J., 241 (1987) 39-47.

Kagawa, T. and Bruno, P. L., Arch.Biochem.Biophys., 260 (1988) 674-695.

Kalies, I., Kalden, J. R., Heinz, F., Janzen, R. W. C. and Lachenmayer, L., Klin.Wochenschr., 57 (1979) 875-881.

Kallajoki, M., Malmi, R., Virtanen, I. and Suominen, J., Cell Biol.Int.Rep., 9 (1985) 151-164.

Kalpaxis, D. L., J.Chromatogr., 350 (1985) 227-236.

Kamada, S., Maeda, M., Tsuji, A., Umezawa, Y. and Kurahashi, T., J.Chromatogr., 239 (1982) 773-783.

Kambayashi, J., Sakon, M., Ohno, H. and Kosaki, G., Thromb.Res., 21 (1981) 129-135.

Kamisaki, Y., Saheki, S., Nakane, M., Palmiery, J. A., Kuno, T. and Chang, B. Y., J.Biol.Chem., 261 (1986) 7236-7241.

Kamps, K. M. P., De Grip, W. J. and Daemen, F. J. M., Biochim.Biophys.Acta, 687 (1982) 296-302.

Kamrath, F. J., Dodt, G., Debuch, H. and Uhlenbruck, G., Hoppe-Seyler's Z.Physiol.Chem., 365 (1984) 539-547.

Kanamori, A., Seno, N. and Matsumoto, I., J.Chromatogr., 363 (1986) 231-242.

Kanda, F., Matsui, S. I., Sykes, D. E. and Sandberg, A. A., Biochem.Biophys.Res.Commun., 122 (1984) 1296-1306.

Kanoun, S., Amourache, L., Krishnan, S. and Vijayalakshmi, M. A., J.Chromatogr., 376 (1986) 259-267.

Kant, K. and Hallen, R. M., Anal.Biochem., 147 (1985) 455-457.

Kaplan, L. J., Barr, F. G., Daims, M., Nelson, D. and Tanner, T. B., Prep.Biochem., 14 (1984) 149-161.

Kapoor, C. L. and Cho-Chung, Y. S., J.Immunol.Methods, 57 (1983) 215.

Kardana, A., Squires, M., Adam, T., Goka, G. K. and Bagshawe, K. D., J.Immunol.Methods, 30 (1979) 47-53.

Kari, B. E. and Gray, G. R., J.Biol.Chem., 254 (1979) 3354-3357.

Karkkainen, T., Mol.Immunol., 22 (1985) 589-595.

Karlsson, K. and Marklund, S. L., Biochem.J., 242 (1987) 55-59.

Karlstam, B. and Nilsson, B., J.Immunol.Methods, 54 (1982) 119-128.

Karnas, P., Moreno-Lopez, J. and Kristiansen, T., J.Chromatogr., 266 (1983) 643-649.

Karsten, W. E., Hunsley, J. R. and Viola, R. E., Anal.Biochem., 147 (1985) 336-341.

Karube, I., Ishimori, Y., Suzuki, S. and Sato, T., Biotechnol.Bioeng., 20 (1978) 1775-1783.

Káš, J., Marek, M. and Beranová, J., J.Chromatogr., 376 (1986) 269-272.

Kasai, N., Fukuhara, K. and Murao, S., Agr.Biol.Chem. Tokyo, 48 (1984) 1533-1538.

Kasche, V. and Büchtmann, I., Hoppe-Seyler's Z.Physiol.Chem., 362 (1981) 1007-1012.

Kasche, V., Buchholz, K. and Galunsky, B., J.Chromatogr., 216 (1981) 169-174.

Kasher, M. S., Pintel, D. and Ward, D. C., Moll.Cell.Biol., 6 (1986) 3117-3127.

Kasper, L. H., Crabb, J. H. and Pfefferkorn, E. R., J.Immunol., 130 (1983) 2407-2412.

Katagiri, Y., Yamamoto, K., Tsuji, T. and Osawa, T., Carbohyd.Res., 129 (1984) 257-265.

Katagiri, K., Takeuchi, T., Taniguchi, K. and Sasaki, M., Anal.Biochem., 86 (1978) 159-165.

Kataoka, K., Sakurai, Y., Hanai, T., Maruyama, A. and Tsuruta, T., Biomaterials, 9 (1988) 218-224.

Katinakis, P. K. and Burdon, R. H., Biochim.Biophys.Acta, 653 (1981) 27-38.

Kato, M. and Nozawa, Y., Anal.Biochem., 143 (1984) 361-367.

Kato, Y., Iwase, H. and Hotta, K., Anal. Biochem., 138 (1984) 437-441.

Kato, Y., Seita, T., Hashimoto, T. and Shimizu, A., J.Chromatogr., 134 (1977) 204-206.

Katsuragi, T., Sakai, T. and Tonomura, K., Agr.Biol.Chem. Tokyo, 50 (1986b) 1713-1719.

Katsuragi, T., Sakai, T., Matsumoto, K. and Tonomura, K., Agr.Biol.Chem.Tokyo, 50 (1986a) 1721-1730.

Katz, R. W. and Reddi, A. H., Biochem.Biophys.Res.Commun., 157 (1988) 1253-1257.

Katzmann, J. A., Nesheim, M. E., Hibbard, L. S. and Mann, K. G., Proc.Nat.Acad.Sci. U.S., 78 (1981) 162-166.

Kawagishi, H. and Mizuno, T., FEBS Lett., 227 (1988) 99-102.

Kawai, K., Kumagai, K., Oshika, A. and Horitsu, H., J.Ferment.Technol., 61 (1983a) 647-649.

Kawai, K., Kumagai, K., Mori, M. and Horitsu, H., Agr.Biol.Chem. Tokyo, 47 (1983b) 29-32.

Kawai, K., Horitsu, H., Hirose, T. and Eguchi, Y., Agr.Biol.Chem. Tokyo, 46 (1982) 1065-1066.

Kawai, K., Saito, Y. and Eguchi, T., J.Ferment.Technol., 58 (1980) 569-571.

Kawata, S., Trzaskos, J. M. and Gaylor, J. L., J.Biol.Chem., 261 (1986) 3790-3799.

Kay, W. W., Can.J.Microbiol., 27 (1981) 795-800.

Kedersha, N. L. and Berg, R. A., Collagen Rel.Res., 1 (1981) 345-353.

Kee, Y., Lee, Y. S., Chung, C. H., Waxman, L. and Goldberg, A. L., J.Chromatogr., 428 (1988) 345-351.

Kegoya, Y., Setoguchi, M., Yokohiki, K. and Hatanaka, C., Agr.Biol.Chem. Tokyo, 48 (1984) 1055-1060.

Keilová, H. and Tomášek, V., Collect.Czech.Chem.Commun., 41 (1976) 489-497.

Keilová, H., Salvetová, A. and Kostka, V., Collect.Czech.Chem.Commun., 40 (1975) 580-584.

Kelleher, F. M., Dubbs, S. B. and Bhavanandan, V. P., Arch.Biochem.Biophys., 263 (1988) 349-354.

Kelleher, D. J., Rashidbaigi, A., Ruoho, A. E. and Johnson, G. L., J.Biol.Chem., 258 (1983) 12881-12885.

Kellermann, O., Tonetti, H., Brevet, A., Mirande, M., Pailliez, J. P., Waller, J.P., J.Biol.Chem., 257 (1982) 11041-11048.

Kennedy, J. F., Barnes, J. A. and Matthews, J. B., Clin.Chim.Acta, 129 (1983) 251-261.

Kennedy, J. F., Keep, P. A. and Catty, D., J.Immunol.Methods, 50 (1982) 57-75.

Kennedy, J. F. and Barnes, J. A., J.Chromatogr., 212 (1981) 179-186.

Kent, W. M., Funderburg, F. M. and Culp, L. A., Mech.Ageing Dev., 33 (1986) 115-137.

Keradjopoulos, D. and Holldorf, A. W., Biochim.Biophys.Acta, 570 (1979) 1-10.

Keski-Oja, J. and Yamada, K. M., Biochem.J., 193 (1981) 615-620.

Kessey, J. K., Bigelis, R. and Fink, G. R., J.Biol.Chem., 254 (1979) 7427-7433.

Kessner, A., Hodgins, L. T. and Troll, W., Anal.Biochem., 92 (1979) 383-389.

Khamlichi, S., Yu, X. J., Muller, D., Jozefonvicz, J., Roeland, S. and Fuks, R., Makromol.Chem.,Macromol.Symp., 19 (1988) 155-161.

Khan, I. U. and Polgar, L., Biochim.Biophys.Acta, 760 (1983) 350-356.

Khrapunov, S. N., Kadura, S. N. and Berdyshev, G. D., Biokhimiya, 45 (1980) 35-41.

Khurana, A. L. and Ho, C. T., J.Liq.Chromatogr., 12 (1989) 563-570.

Kido, H., Vita, A. and Horecker, B. L., Anal.Biochem., 106 (1980) 450-454.

Kidson, S. H. and Fabian, B. C., Biochim.Biophys.Acta, 824 (1985) 40-44.

Kieran, M. W. and Longenecker, B. M., J.Immunol.Methods, 66 (1984) 349-356.

Kilpatrick, D. C., Schmidt, J. A. and Stirling, J. L., FEMS Microbiol.Lett., 4 (1978) 67-70.

Kim, M. Y., Ikeda, S. and Ives, D. H., Biochem.Biophys.Res.Commun., 156 (1988) 92-98.

Kim, S., Nochumson, S., Chin, W. and Paik, W. K., Anal.Biochem., 84 (1978) 415-422.

Kim, Y. S., Kolattukudy, P. E. and Boss, A., Arch.Biochem.Biophys., 196 (1979) 543-551.

Kimura, Y., Hirata, M., Yamaguchi, K. and Koga, T., Arch.Biochem.Biophys., 257 (1987) 363-369.

Kimura, A., Wigzell, H., Holmquist, G., Ersson, B. and Carlsson, P., J.Exp.Med., 149 (1979) 473-484.

Kincaid, R. L., Metods Enzymol., 159 (1988) 627-652.

Kincaid, R. L. and Coulson, C. C., Biochem.Biophys.Res.Commun., 133 (1985) 256-264.

Kincaid, R. L. and Vaughhan, M., Biochemistry, 22 (1983) 826-830.

Kincaid, R. L. and Vaughan, M., Proc.Nat.Acad.Sci. U.S., 76 (1979) 4903-4907.

Kinkel, J. N., Anspach, B., Unger, K. K., Wieser, R. and Brunner, G., J.Chromatogr., 297 (1984) 167-177.

Kirchberger, J., Seidel, H. and Kopperschlager, G., Biomed.Biochim.Acta, 46 (1987) 653-663.

Kiss, L., Tar, A., Gal, S., Toth-Martinez, B. L. and Hernadi, F. J., J.Chromatogr., 448 (1988) 109-116.

Kitagawa, Y. and Okuhara, E., Anal.Biochem., 115 (1981) 102-108.

Kitlar, T., Morrison, A. I., Kinne, R. and Deutscher, J., FEBS Lett., 234 (1988) 115-119.

Kitson, T. M., J.Chromatogr., 234 (1982) 181-186.

Kivirikko, K. I. and Myllyla, R., Methods Enzymol., 144 (1987) 96-114.

Klee, C. B. and Krinks, M. H., Biochemistry, 17 (1978) 120-126.

Klinger, M. M., Margolis, R. U. and Margolis, R. K., J.Biol.Chem., 260 (1985) 4082-4090.

Klyashchitsky, B. A., Mitina, V. K., Morozevich, G. E. and Yakubovskaya, R. I., J.Chromatogr., 210 (1981) 67-76.

Klyashchitsky, B. A., Pozdnev, V. B., Mitina, V. K., Voskoboyev, A. I. and Chernikevich, I. P., Bioorg.Khim., 6 (1980) 1572-1579.

Klyashchitsky, B. A., Khim.Prir.Soedin. SSSR, (1979) 709-712.

Kminkova, M. and Kucera, J., J.Chromatogr., 244 (1982) 166-168.

Knowles, P. P. and Thorpe, P. E., Anal.Biochem., 160 (1987) 440-443.

Knuver, J. and Rohm, K. H., Z.Naturforsch.Sect.C, 37 (1982) 845-848.

Kobayashi, H., Kusakabe, I. and Murakami, K., Anal.Biochem., 122 (1982) 308-312.

Kobayashi, K. and Murakami, K., Agr.Biol.Chem. Tokyo, 42 (1978) 2227-2231.

Kocha, T., Fukuda, T., Isobe, T. and Okuyama, T., Chem.Pharm.Bull. Tokyo, 34 (1986) 3791-3802.

Kochibe, N. and Furukawa, K., Biochemistry, 19 (1980) 2841-2846.

Koehler, G. and Milstein, C., Nature, 256 (1975) 495-497.

Kohno, H., Kanda, S. and Kanno, T., J.Biol.Chem., 261 (1986) 10744-10748.

Koida, M., Aono, J., Takenaga, K., Yoshimoto, T., Kimura, T. and Sakakibara, S., J.Neurochem., 33 (1979) 1233-1237.

Koiš, P., Rosenberg, I. and Holý, A., Collect.Czech.Chem.Commun., 45 (1980) 2839-2846.

Kolhouse, J. F. and Allen, R. H., Anal.Biochem., 84 (1978) 486-490.

Koller, F., and Koller,E., J.Chromatogr., 283 (1984) 191-197.

Kominami, E., Hoffschulte, H. and Holzer, H., Biochim.Biophys.Acta, 661 (1981) 124-135.

Komiyama, T., Aoyagi, T., Takeuchi, T. and Umezawa, H., Biochem.Biophys.Res.Commun., 65 (1975) 352-377.

Konami, Y., Tsuji, T., Matsumoto, I. and Osawa, T., Hoppe-Seyler's Z.Physiol.Chem., 362 (1981) 983-989.

Konečný, P., Smrž, M., Borák, J. and Slováková, S., J.Chromatogr., 398 (1987) 387-390.

Kopetzki, E. and Entian, K. D., Anal.Biochem., 121 (1982) 181-185.

Koren, E., McConathy, W. J. and Alaupovic, P., Biochemistry, 21 (1982) 5347-5351.

Korman, A. J., Knudsen, P. J., Kaufman, J. F. and Strominger, J. L., Proc.Nat.Acad.Sci. U.S., 79 (1982) 1844-1848.

Kornfeld, K., Reitman, M. L. and Kornfeld, R., J.Biol.Chem., 256 (1981) 6633-6640.

Korpela, T. K. and Saarinen, R., J.Chromatogr., 318 (1985) 333-341.

Koshiba, T. and Minamikawa, T., Plant Cell Physiol., 2 (1981) 979-987.

Kouvonen, I. and Grasbeck, R., Biochem.Biophys.Res.Commun., 86 (1979) 358-364.

Koyama, I., Miura, M., Matsuzaki, H., Sakagishi, Y. and Komoda, T., J.Chromatogr., 413 (1987) 65-78.

Koyama, I., Sakagishi, Y. and Komoda, T., J.Chromatogr., 374 (1986) 51-59.

Kozik, A. and Zak, Z., Anal.Biochem., 121 (1982) 224-226.

Kraehenbuhl, J. P. and Bonnard, C., Methods Enzymol., 184 (1990) 629-641.

Kraus, J. P. and Rosenberg, L. E., Proc.Nat.Acad.Sci. U.S., 79 (1982) 4015-4019.

Krietsch, W. K. G., Simm, F. C., Hertenberger, B., Kuntz, G. W. K. and Wachter, E., Anal.Biochem., 128 (1983) 213-216.

Kristensen, T. and Holtlund, J., J.Chromatogr., 192 (1980) 494-499.

Kristensen, T. and Holtlund, J., Eur.J.Biochem., 88 (1978) 495-501.

Kritsi, Z. I., Theoharides, T. C., Baumgarten, A., Bondy, P. K. and Canellakis, Z. N., Prep.Biochem., 12 (1982) 445-460.

Kubota, S. I., Suzuki, K. and Imahori, K., Biochem.Biophys.Res.Commun., 100 (1981) 1189-1194.

Kučera, J., J.Chromatogr., 213 (1981) 352-254.

Kučera, J., Collect.Czech.Chem.Commun., 44 (1979) 804-807.

Kučerová, Z., Pohl, J. and Korbová, L., J.Chromatogr., 376 (1986) 409-412.

Kukongviriyapan, V., Poopyruchpong, N. and Ratanabanangkoon, K., J.Immunol.Methods, 49 (1982) 97-104.

Kula, M. R., Hatef-Haghi, D., Tauber-Finkelstein, M. and Shaltiel, S., Biochem.Biophys.Res.Commun., 69 (1976) 389-396.

Kulbe, K. D. and Schuer, R., Anal.Biochem., 93 (1979) 46-51.

Kumar, A. and Ali, R., J.Biosciences, 10 (1986) 235-242.

Kumazaki, T., Terasawa, K. and Ishii, S., J.Biochem.Tokyo, 102 (1987) 1539-1546.

Kunapuli, S. P. and Vaidyanathan, C. S., Biochem.Int., 7 (1983) 647-653.

Kunicki, T. J., Russell, N., Nurden, A. T., Aster, R. H. and Caen, J. P., J.Immunol., 126 (1981) 398-402.

Kuo, C. H. and Wells, W. W., J.Biol.Chem., 253 (1978) 3550-3556.

Kuroki, G. W. and Conn, E. E., Arch.Biochem.Biophys., 260 (1988) 616-621.

Kurowska, E. and Szewczuk, A., Arch.Immunol.Ther.Exp., 36 (1988) 7-13.

Kusov, Y. Y., Shibaev, V. N., Kalinchuk, N. A., Kuprijanov, V. V., Goguilashvili, L. M. and Kochetkov, N. K., Bioorg.Khim., 5 (1979) 438-448.

Kusser, W. and Schwarz, U., Eur.J.Biochem., 103 (1980) 277-281.

Kuwana, T., Sugita, O. and Yakata, M., Clin.Chem., 35 (1989) 179.

Kvasyuk, E. I., Kulak, T. I., Zaitseva, G. V., Mikhailopulo, I. A. and Pfleiderer, W., Bioorg.Khim., 10 (1984) 506-514.

Kyosaka, S., Yamashiro, S. and Tanaka, M., Chem.Pharm.Bull., 28 (1980) 2658-2664.

La Creta, F. P., Olszewski, J. J. and Tew, K. D., J.Chromatogr., 434 (1988) 83-93.

Lacko, A. G. and Chen, T. F., J.Chromatogr., 130 (1977) 446-450.

Ladipo, J. L. and de Zoeten, G. A., Virology, 46 (1971) 567-574.

Lagercrantz, C., Larsson, T. and Denfors, I., Comp.Biochem.Physiol.Pt.C, 69 (1981) 375-378.

Laidler, P. M., Waheed, A. and van Etten, R. L., Biochim.Biophys.Acta, 827 (1985) 73-83.

Laine, R. A., Fisher, S. J. and Zhu, B. C. R., Methods Enzymol., 144 (1987) 420-429.

Lamed, R., Oplatka, A. and Reisler, E., Biochim.Biophys.Acta, 427 (1976) 688-695.

Lamond, A. I., Konarska, M. M., Grabowski, P. J. and Sharp, P. A., Proc.Nat.Acad.Sci. U.S., 85 (1988) 411-415.

Landt, M., Boltz, S. C. and Butler, L. G., Biochemistry, 17 (1978) 915-919.

Lang, B., Vincent, A. and Newson-Davis, J., J.Immunol.Methods, 51 (1982) 371-381.

Langone, J. J., J.Immunol.Methods, 55 (1982) 277-296.

Lanzillo, J. J., Polsky-Cynkin, R. and Fanburg, B. L., Anal.Biochem., 103 (1980) 400-407.

Larson, D. E., Calton, G. J., Little, S. F., Leppla, S. H. and Burnett, J. W., Toxicon, 26 (1988) 913-921.

Lascu, I., [illegible] M. and Cristea, A., Anal.Biochem., 113 (1981) 207-211.

Latham, K. R., Apriletti, J. W., Eberhardt, N. L. and Baxter, J. D., J.Biol.Chem., 256 (1981) 12088-12093.

Lau, H. K. F., Biochem.J., 241 (1987) 567-572.

Lau, P. P., Yu, S. H., Spring, T. G. and Gray, H. B. J., Biochim.Biophys.Acta, 563 (1979) 313-319.

Lauber, M., Camier, M. and Cohen, P., Proc.Nat.Acad.Sci. U.S., 76 (1979) 6004-6008.

Laubli, U. K., Baier, W., Binz, H., Celio, M. R. and Humbel, R. E., FEBS Lett., 149 (1982) 109-112.

Lavrenova, G. I., Borovikova, V. P. and Stepanov, V. M., Biokhimiya, 44 (1979) 1657-1662.

Lawrence, G., Brown, R., Bates, J., Saul, A., Davis, M., Spark, R. and Anian, G., J.Appl.Bacteriol., 57 (1984) 333-338.

Lazarovici, P., Tayot, J. L. and Yavin, E., Toxicon, 22 (1984) 401-413.

Le Deaut, J. Y., Egly, J. M., Leding, M. and Mandel, P., Biochim.Biophys.Acta, 525 (1978) 438-445.

Le Gledic, S., Perin, J. P., Bonnet, F. and Jolles, P., Biochem.Biophys.Res.Commun., 104 (1982) 1298-1305.

Le Goffic, F. and Moreau, N., FEBS Lett., 29 (1973) 289-291.

Leah, J. M., Billett, E. E. and Palmer, T., Anal.Biochem., 170 (1988) 495-501.

Leatherbarrow, R. J. and Dwek, R. A., FEBS Lett., 164 (1983) 227-230.

Leatherbarrow, R. J. and Dean, P. D. G., Biochem.J., 189 (1980) 27-34.

Lee, C. -., Charles, D. and Bronson, D., J.Biol.Chem., 254 (1979) 6375-6381.

Lee, C. Y., Charles, D. and Bronson, D., J.Biol.Chem., 254 (1979) 6375-6381.

Lee, C. Y., Lazarus, L. H., Kabakoff, D. S., Russell, P. J., Laver, M. and Kaplan, N. O., Arch. Biochem. Biophys., 178 (1977) 8-18.

Lee, J. C., Int.J.Biochem., 16 (1984) 1143-1147.

Lee, S. M., Gustafson, M. E., Pickle, D. J., Flickinger, M. C., Muschik, G. M. and Morgan, J. A. C., J.Biotechnol., 4 (1986) 189-204.

Leflar, C. C., Freytag, J. W., Powell, L. M., Strahan, J. C., Wadsley, J. J., Tyler, C. A. and Miller, W. K., Clin.Chem., 30 (1984) 1809-1811.

Leger, C., Alessandri, J. M., Kann, G., Charles, M., Corring, T. and Flanzy, J., Biochim.Biophys.Acta, 713 (1982) 208-221.

Leger, R. J. S., Charnley, A. K. and Cooper, R. M., Arch.Biochem.Biophys., 253 (1987) 221-232.

Legler, G. and Beiberich, E., Arch.Biochem.Biophys., 260 (1988) 427-436.

Lehmann, F. G., Biochim.Biophys.Acta, 616 (1980) 41-59.

Lei, M. G. and Reeck, G. R., J.Chromatogr., 363 (1986) 315-321.

Leicht, W., Werber, M. M. and Eisenberg, H., Biochemistry, 17 (1978) 4004-4010.

Leissing, N. and McGuinness, E. T., Biochim.Biophys.Acta, 524 (1978) 254-261.

Leith, D. K. and Baseman, J. B., J.Bacteriol., 157 (1984) 678-680.

Leonardi, C. L. and Rubin, R. W., Anal.Biochem., 118 (1981) 58-64.

Leone, A., Shatkin, A. J. and Cancedda, R., FEBS Lett., 100 (1979) 103-106.

Leonil, J., Langrene, S., Sicsic, S. and Le Goffic, F., J.Chromatogr., 347 (1985) 316-319.

Leonowicz, A. and Malinowska, M., Acta Biochim.Pol., 29 (1982) 219-226.

Levitsky, K., Chandrasekaran, K., Mora, P. T. and Simmons, D. T., Int.J.Cancer., 32 (1983) 597-602.

Lewis, J. G. and Elder, P. A., J.Steroid Biochem., 29 (1988) 191-195.

Lewis, J. G. and Elder, P. A., J.Steroid Biochem., 22 (1985) 387-390.

Liedgens, W., Gruetzmann, R. and Schneider, H. A. W., Z.Naturforsch., 35 (1980) 958-962.

Liguri, G., Nassi, P., Camici, G., Manao, G., Cappugi, G., Stefani, M., Berti, A. and Ramponi, G., Biochem.J., 217 (1984) 499-505.

Limbach, B. and Schmidt, H. L., J.Chromatogr., 285 (1984) 457-466.

Lin, C. W., Kirley, S. D. and Pierre, M. S., Oncodev.Biol.Med., 2 (1981b) 267-280.

Lin, J. T., Da Cruz, M. E. M., Riedel, S. and Kinne, R., Biochim.Biophys.Acta, 640 (1981a) 43-54.

Lincoln, T. M., Dills, W. L. and Corbin, J. D., J.Biol.Chem., 252 (1977) 4263-4275.

Lindberg, U., Methods Enzymol., 34 (1974) 517-520.

Linden, C. D., Dedman, J. R., Chafouleas, J. G., Means, A. R. and Roth, T. F., Proc.Nat.Acad.Sci. U.S., 78 (1981) 308-312.

Lingwood, C. A., J.Lipid Res., 25 (1984) 1010-1012.

Linhardt, R. J., Merchant, Z. M. and Persinger, D. W., Int.J.Biochem., 17 (1985) 1179-1183.

Link, R. P., Perlman, K. L., Pierce, E. A., Schnoes, H. K. and De Luca, H. F., Anal.Biochem., 157 (1986) 262-269.

Lis, H., Joubert, F. J. and Sharon, N., Phytochemistry, 24 (1985) 2803-2809.

Lis, H. and Sharon, N., J.Chromatogr., 215 (1981) 361-372.

Liscia, D. S. and Vonderhaar, B. K., Proc. Nat. Acad. Sci. U.S., 79 (1982) 5930-5934.

Lisman, J. J. W. and Overdijk, B., Hoppe-Seyler's Z.Physiol.Chem., 359 (1978) 1019-1022.

Litman, B. J., Methods Enzymol., 81 (1982) 150-153.

Liu, D. Y., David, J. R. and Remold, H. G., Nature, 296 (1982) 78-80.

Liu, F., Zinnecker, M., Hamaoka, T. and Katz, D. H., Biochemistry, 18 (1979) 690-697.

Ljungquist, S., J.Biol.Chem., 252 (1977) 2808-2814.

Lobarzewski, J., Fiedurek, J., Ginalska, G. and Wolski, T., Biochem.Biophys.Res.Commun., 131 (1985) 666-674.

Lobarzewski, J., Int.J.Biol.Macromol., 3 (1981) 77-81.

Lobb, R. R., Wade Harper, J. and Fett, W. J., Anal.Biochem., 154 (1986) 1-14.

Lobl, T. J., Deibel, M. R. J. and Yem, A. W., Anal.Biochem., 170 (1988) 502-511.

Lofdahl, S., Guss, B., Uhlen, M., Philipson, L. and Lindberg, M., Proc.Nat.Acad.Sci. U.S., 80 (1983) 697-701.

Logeat, F., Pamphile, R., Loosfelt, H., Jolivet, A., Fournier, A. and Milgrom, E., Biochemistry, 24 (1985) 1029-1035.

Lombes, M., Claire, M., Lustenberger, P., Michaud, A. and Rafestin-Oblin, M. E., J.Biol.Chem., 262 (1987) 8121-8127.

Longacre, S. S. and Mach, B., J.Biol.Chem., 253 (1978) 7500-7507.

Lotersztajn, S., Hanoune, J. and Pecker, F., J.Biol.Chem., 256 (1981) 11209-11215.

Louie, A., Masuda, E., Yoder, M. and Jurnak, F., Anal.Biochem., 141 (1984) 402-408.

Lowe, C. R., Glad, M., Larsson, P. O., Ohlson, S., Small, D. A. P., Atkinson, T. and Mosbach, K., J. Chromatogr., 215 (1981) 303-316.

Lowe, C. R., Hans, M., Spibey, N. and Drabble, W. T., Anal.Biochem., 104 (1980) 23-28.

Lowe, C. R., Harvey, M. J., Craven, D. B., Kerfoot, M. A., Hollows, M. E. and Dean, P. D. G., Biochem.J., 133 (1973) 507-513.

Lucacchini, A., Martini, C., Segnini, D. and Ronca, G., Experientia, 35 (1979) 1148-1149.

Lukacovic, M. F., Feinstein, M. B., Sha'Afi, R. I. and Perrie, S., Biochemistry, 20 (1981) 3145-3151.

Luna, E. J., Goodloe-Holland, C. M. and Ingalls, H. M., J.Cell Biol., 99 P1 (1984) 58-70.

Luna, E. J., Wang, Y. L., Voss, E. W., Branton, D. and Taylor, D. L., J.Biol.Chem., 257 (1982) 13009-13100.

Lund, K., Merrill, D. K. and Guynn, R. W., Arch.Biochem.Biophys., 254 (1987) 319-328.

Lundblad, A., Schroer, K. and Zopf, D., J.Immunol.Methods, 68 (1984) 227-234.

Lutter, L. C. and Faulstich, H., Biochem.Biophys.Res.Commun., 119 (1984) 42-48.

Lyon, M. and Phelps, C. F., Biochem.J., 199 (1981) 419-426.

Lyublinskaya, L. A., Vaganova, T. I., Ivanova, N. M., Yusupova, M. P., Oksenoit, E. S., Yakusheva, L. D. and Stepanov, V. M., Bioorg.Khim., 10 (1984a) 188-194.

Lyublinskaya, L. A., Yusupova, M. P., Vaganova, T. I., Ivanova, N. M. and Stepanov, V. M., Bioorg.Khim., 10 (1984b) 1490-1495.

MacDonald, E. M. S. and Morris, R. O., Methods Enzymol., 110 (1985) 347-358.

Machuga, E. J., Calton, G. J. and Burnett, J. W., Toxicon, 24 (1986) 187-195.

Mack, J. P. G. and Slaytor, M. B., J.Chromatogr., 157 (1978) 153-159.

MacKay, B. J., Iacono, V. J., Zuckerman, J. M., Osserman, E. F. and Pollock, J. J., Eur.J.Biochem., 129 (1982) 93-98.

Madar, D. A., Hall, T. J., Hiskey, R. G. and Koehler, K. A., Biochem.J., 193 (1981) 411-418.

Madar, D. A., Hall, T. J., Reisner, H. M., Hiskey, R. G. and Koehler, K. A., J.Biol.Chem., 255 (1980) 8599-8605.

Madaras, F., Bell, W. R. and Castaldi, P. A., Haemostasis, 7 (1978) 321-331.

Madelian, V. and Warren, W. A., Anal.Biochem., 64 (1975) 517-520.

Madhav, R., Coetzee, M. L. and Ove, P., Arch.Biochem.Biophys., 200 (1980) 99-107.

Madhok, T. C. and Leung, B. S., J.Steroid Biochem., 15 (1981) 299-305.

Maestas, R. R., Prieto, J. R., Kuehn, G. D. and Hageman, J. H., J.Chromatogr., 189 (1980) 225-231.

Magnani, M., Stocchi, V., Serafini, G., Chiarantini, L. and Fornaini, G., Arch.Biochem.Biophys., 260 (1988) 388-399.

Magous, R., Lecou, C. and Bali, J. P., Biochem.Biophys.Res.Commun., 85 (1978) 1453-1459.

Mahe, N., Leger, C. and Alessandri, J. M., Reprod.Nutr.Develop., 26 (1986) 1195-1196.

Mahuran, D. and Lowden, J. A., Can.J.Biochem., 58 (1980) 287-294.

Majumdar, T. and Surolia, A., Prep.Biochem., 8 (1978) 119-131.

Maliarik, M. J., Roberts, D. D. and Goldstein, I. J., Arch. Biochem. Biophys., 255 (1987) 194-200.

Malik, N., Smulson, M. and Bustin, M., J.Biol.Chem., 259 (1984) 699-702.

Malik, N., Miwa, M., Sugimura, T., Thraves, P. and Smulson, M., Proc.Nat.Acad.Sci. U.S., 80 (1983) 2554-2558.

Malinoff, H. L. and Wicha, M. S., J.Cell.Biol., 96 (1983) 1475-1479.

Mallik, T. K., Wilber, J. F. and Pegues, J., J.Clin.Endocrinol.Metab., 54 (1982) 1194-1198.

Malmstrom, A., Roden, L., Feingold, D. S., Jacobsson, I., Backstrom, G. and Lindahl, U., J.Biol.Chem., 255 (1980) 3878-3883.

Malthouse, J. P. G. and Brocklehurst, K., Biochem.J., 159 (1976) 221-234.

Maly, P. and Luthi, C., Biochem.Biophys.Res.Commun., 137 (1986) 695-701.

Manabe, T., Higuchi, N., Okuyama, T. and Mukaiyama, Y., J.Chromatogr., 431 (1988) 45-54.

Manak, R. C. and Vos Jr., E. W., Immunochemistry, 15 (1978) 643-651.

Manz, B., Heubner, A., Kohler, I., Grill, H. and Pollow, K., Eur. J. Biochem., 131 (1983) 333-338.

Manz, B., Grill, H. J., Kohler, I., Heubner, A. and Pollow, K., Eur.J.Biochem., 128 (1982) 249-255.

Marcel, Y. L., Vezina, C., Emond, D., Verdery, R. B. and Milne, R. W., J.Lipid.Res., 22 (1981) 1198-1205.

Marcel, Y. L., Vezina, C., Emond, D. and Suzue, G., Proc.Nat.Acad.Sci. U.S., 77 (1980) 2969-2973.

Marciniak, R. A., Wands, J. R., Bruns, R. R., Malchesky, P. S., Nose, Y. and Haber, E., Proc.Nat.Acad.Sci. U.S., 80 (1983) 3821-3825.

Marcipar, A. J., Lentwojt, E., Segard, E., Afchain, D., Fruit, J. and Capron, A., Parasite Immunol., 4 (1982) 109-115.

Marcus, S. L., Methods Enzymol., 34 (1974) 377-385.

Maresh, G. A., Chernoff, E. A. G. and Culp, L. A., Arch.Biochem.Biophys., 233 (1984) 428-437.

Marinkovic, D. V. and Marinkovic, J. N., Prep.Biochem., 7 (1977) 9-18.

Markelonis, G. J. and Oh, T. H., J.Neurochem., 37 (1981) 95-99.

Marks, N. and Berg, M. J., Arch.Biochem.Biophys., 259 (1987) 131-143.

Marossy, K., Hauck, M. and Elodi, P., Biochim.Biophys.Acta, 615 (1980) 237-245.

Marquez, A. J., de la Rosa, M. A. and Vega, J. M., J.Chromatogr., 235 (1982) 435-443.

Marquis, J. K., Hilt, D. C. and Mautner, H. G., J.Neurochem., 34 (1980) 1071-1076.

Marshak, D. R., Lukas, T. J., Watterson, D. M. and Cohen, C. M., in H.Hidaka and D.J.Hartshorne (Editors), Calmodulin Antagonists and Cellular Physiology, Academic Press, Orlando, 1985, pp.495-510.

Marshall, J. J. and Woloszczuk, W., Carbohyd.Res., 61 (1978) 407-417.

Marshall, J. J. and Humphreys, J. D., J.Chromatogr., 137 (1977) 468-470.

Martin, L. N., J.Immunol.Methods, 50 (1982) 319-329.

Martini, C., Lucacchini, A. and Ronca, G., Prep.Biochem., 11 (1981) 487-499.

Maruo, T., Cohen, H., Segal, S. J. and Koide, S. S., Proc.Nat.Acad.Sci. U.S., 76 (1979) 6622-6626.

Marynen, P., Van Leuven, F., Cassiman, J. J. and Van den Berghe, H., J.Biol.Chem., 259 (1984) 7075-7079.

Mas-Oliva, J., Williams, A. J. and Nayler, W. G., Anal.Biochem., 103 (1980) 222-226.

Masuda, T., Sakamoto, M., Nishizaki, I., Hayashi, H., Yamamoto, M. and Wada, H., J.Biochem.Tokyo, 101 (1987) 643-652.

Masuda, T., Ozaki, H. and Tai, A., Agr.Biol.Chem. Tokyo, 50 (1986) 2763-2769.

Mata, A., Pinto, M. C. and Lopez-Barea, J., Z.Naturforsch., 39c (1984) 908-915.

Matsuda, H. and Hirayama, O., Agr.Biol.Chem. Tokyo, 43 (1979) 463-469.

Matsuda, H., Tanaka, G., Morita, K. and Hirayama, O., Agr.Biol.Chem. Tokyo, 43 (1979) 563-570.

Matsuda, Y., Shimura, K., Shiraki, H. and Nakagawa, H., Biochim.Biophys.Acta, 616 (1980) 340-350.

Matsumoto, U. and Shibusawa, Y., J.Chromatogr., 356 (1986) 27-36.

Matsumoto, U., Ban, M. and Shibusawa, Y., J.Chromatogr., 285 (1984) 69-79.

Matsumoto, I., Ito, Y. and Seno, N., J.Chromatogr., 239 (1982) 747-754.

Matsumoto, I., Kitagaki, H., Akai, Y., Ito, Y. and Seno, N., Anal.Biochem., 116 (1981) 103-110.

Matsumoto, I., Seno, N., Golovtchenko-Matsumoto, A. M. and Osawa, T., J.Biochem. Tokyo, 87 (1980) 535-540.

Matsuzawa, T. and Ishiguro, I., Biochim.Biophys.Acta, 616 (1980) 381-383.

Mattiasson, B. and Olsson, U., J.Chromatogr., 370 (1986) 21-28.

Matuo, Y., Sano, R., Tosa, T. and Chibata, I., Anal.Biochem., 68 (1975) 349-357.

Matuszewska, B. and Borchardt, R. T., Arch.Biochem.Biophys., 256 (1987) 50-55.

May, W. S. and Ihle, J. N., Biochem.Biophys.Res.Commun., 135 (1986) 870-879.

Mayer, R. T. and Durrant, J. L., J.Biol.Chem., 254 (1979) 756-761.

Mayhew, S. G. and Strating, M. J. J., Eur.J.Biochem., 59 (1975) 539-544.

Maylie-Pfenninger, M. F. and Jamieson, J. D., J.Cell.Biol., 80 (1979) 69-76.

Mazzini, M. N., Cerezo, A. S. and de Cerezo, J. M. S., J.Chromatogr., 173 (1979) 365-371.

Mazzoni, M. R., Martini, C., Segnini, D., Ciompi, M. L. and Lucacchini, A., Appl.Biochem.Biotech., 7 (1982) 189-194.

Mc Cleary, B. V., Phytochemistry, 17 (1978) 651-653.

Mc Conathy, W. J., Koren, E., Wieland, H., Campos, E. M., Lee, D. M., Kloer, H. U. and Alaupovic, P., J.Chromatogr., 342 (1985) 47-66.

Mc Farthing, K. G., Angal, S. and Dean, P. D. G., Anal.Biochem., 122 (1982) 186-193.

Mc Ginnis, J. F., Biochem.Biophys.Res.Commun., 116 (1983) 689-695.

Mc Illhinney, R. A. J. and Patel, S., J.Biol.Chem., 260 (1985) 489-494.

Mc Lennan, B. D. and Raney, J. P., Anal.Biochem., 148 (1985) 490-497.

Mc Lennan, B. D. and Raney, J. P., J.Biol.Chem., 258 (1983) 7592-7596.

Mc Loughlin, D. J., Terry, D. and Mac Quarrie, R., Prep.Biochem., 10 (1980) 549-559.

McCarthy, A. D., Walker, J. M. and Tipton, K. F., Biochem.J., 191 (1980) 605-611.

McClard, R. W., Black, M. J., Livingstone, L. R. and Jones, M. E., Biochemistry, 19 (1980) 4699-4706.

McGinnis, J. F. and De Vellis, J., J.Biol.Chem., 253 (1978) 8483-8492.

McKinney, R. M., Thacker, L., Wong, M. C. and Hebert, G. A., J.Immunol.Methods, 21 (1978) 1-10.

McVicar, J. P., Kunitake, S. T., Hamilton, R. L. and Kane, J. P., Proc.Nat.Acad.Sci. U.S., 81 (1984) 1356-1360.

Mei-Hao, H., Ai, W. and Hui-Fen, H., Anal.Biochem., 125 (1982) 1-5.

Melendy, T. and Ray, D. S., J.Biol.Chem., 264 (1989) 1870-1876.

Melvin, W. T., Milne, H. B., Slater, A. A., Allen, H. J. and Keir, H. M., Eur.J.Biochem., 92 (1978) 373-379.

Mendel, R. R. and Alikulov, Z. A., J.Chromatogr., 267 (1983) 409-413.

Mendes, M. J., Karmali, A. and Brown, P., Biochemie, 70 (1988) 1369-1371.

Mendicino, J., Sivakami, S., Davila, M. and Chandrasekaran, V., J.Biol.Chem., 257 (1982) 3987-3994.

Mendonca, M. H. and Zancan, G. T., Arch.Biochem.Biophys., 252 (1987) 507-514.

Merrill, A. H. and McCormick, D. B., Methods Enzymol., 66 (1980a) 338-344.

Merrill, A. H. J. and McCormick, D. B., J.Biol.Chem., 255 (1980b) 1335-1338.

Merrill, A. H. and McCormick, D. B., Anal.Biochem., 89 (1978) 87-102.

Merskey, C., Johnson, A. J., Harris, J. U., Wang, M. T. and Swain, S., Brit.J.Haematol., 44 (1980) 655-670.

Mertens, R., Stuning, M. and Weiler, E. W., Naturwissenschaften, 69 (1982) 595-596.

Messerschmidt, G. L., Henry, D. H., Snyder, H. W., Bertram, J., Mittelman, A., Ainsworth, S., Fiore, J., Viola, M. V., Louie, J., Ambinder, E., Mac Kintosh, R., Higby, D. J., O'Brien, P., Kiprov, D., Hamburger, M., Balint, J. P., Fisher, L. D., Perkins, W., Pinsky, C. M. and Jones, F. R., J.Clin.Oncol., 6 (1988) 203-212.

Methews, H. L., Brunda, M. J. and Minden, P., J.Immunol., 124 (1980) 1141-1147.

Metrione, R. M., Biochim.Biophys.Acta, 526 (1978) 531-536.

Michalski, J. C., Corfield, A. P. and Schauer, R., Hoppe-Seyler's Z.Physiol.Chem., 363 (1982) 1097-1102.

Michl, J., Pieczonka, M. M., Unkeless, J. C., Bell, G. I. and Silverstein, S. C., J.Exp.Med., 157 (1983) 2121-2139.

Micklem, K. J. and Sim, R. B., Biochem.J., 231 (1985) 233-236.

Middle, F. A., Bannister, A., Bellingham, A. J. and Dean, P. D. G., Biochem.J., 209 (1983) 771-779.

Mikhailov, V. I., Semenyuk, Y. P. and Wiederschain, G. Y., Bioorg.Khim., 11 (1985) 211-218.

Milhaud, P., Blanchard, J. M. and Jeanteur, P., Biochimie, 60 (1978) 1343-1346.

Miller, J. B. and Toft, D. O., Biochemistry, 17 (1978) 173-177.

Miller, A. L., Biochim.Biophys.Acta, 522 (1978) 174-186.

Mills, A., Demoliou-Mason, C. D. and Barnard, E. A., J.Biol.Chem., 263 (1988) 13-16.

Minaga, T., McLick, J., Pattabiraman, N. and Kun, E., J.Biol.Chem., 257 (1982) 11942-11945.

Mingari, M. C., Gerosa, F., Moretta, A., Zubler, R. H. and Moretta, L., Eur.J.Immunol., 15 (1985) 193-196.

Minobe, S., Sato, T., Tosa, T. and Chibata, I., J.Chromatogr., 262 (1983) 193-198.

Mintz, K. P. and Brimijoin, S., J.Neurochem., 44 (1985) 225-232.

Miron, T. and Wilchek, M., Appl.Biochem.Biotechnol., 11 (1985) 445-456.

Miron, T. and Wilchek, M., J.Chromatogr., 215 (1981) 55-63.

Miroshnichenko, O. I., Naroditskii, B. S., Khilko, S. N., Platonova, T. N., Gruber, I. M. and Tikhonenko, T. I., Biokhimiya, 47 (1982) 686-694.

Mitamura, T., J.Biochem. Tokyo, 91 (1982) 25-29.

Mitina, V. K., Frantsuzova, N. A., Kutsenko, N. G., Zolotov, N. N. and Klyashchitsky, B. A., Bioorg.Khim., 11 (1985) 173-179.

Mitra, D., Sarkar, M. and Allen, A. K., Biochem.J., 242 (1987) 331-338.

Mitra, G., Hall, E. and Mitra, I., Biotechnol.Bioeng., 28 (1986) 217-222.

Miura, K., Inoue, Y., Hashimoto, Y., Inoue, A. and Ueda, T., Chem.Pharm.Bull. Tokyo, 32 (1984) 4054-4060.

Miyata, K., Yamamoto, Y., Ueda, M., Kawade, Y., Matsumoto, K. and Kubota, I., J.Biochem. Tokyo, 99 (1986) 1681-1688.

Mizuno, K., Koyama, M. and Shibaoka, H., J.Biochem. Tokyo. 89 (1981) 329-332.

Mizuochi, T., Hamako, J. and Titani, K., Arch.Biochem.Biophys., 257 (1987) 387-394.

Moczar, E., Laurent, M. and Courtois, Y., Biochim.Biophys.Acta, 719 (1982) 396-405.

Modak, M. J. and Marcus, S. L., J.Biol.Chem., 252 (1977) 11-19.

Mohan, S., Bishayee, S. and Bachhawat, B. K., Indian J.Biochem.Biophys., 18 (1981) 177-181.

Mohindru, A., Chenet, A. and Rhoads, A. R., Biochemistry, 17 (1978) 3297-3304.

Mol, J. A., Van Der Berg, T. P. and Visser, T. J., Mol.Cell.Endocrinol., 55 (1988) 159-166.

Molday, R. S. and Molday, L. L., FEBS Lett., 170 (1984) 232-238.

Molday, R. S. and Mac Kenzie, D., J.Immunol.Methods, 52 (1982) 353-367.

Momsen, W. E. and Brockman, H. L., J.Lipid.Res., 19 (1978) 1032-1037.

Moncharmont, B., Su, J. L. and Parikh, I., Biochemistry, 21 (1982) 6916-6921.

Montelaro, R. C., West, M. and Issel, C. J., J.Virol.Meth., 6 (1983) 337-346.

Montelius, J., Carlsson, S., Lindstedt, J., Galaris, D., Hojeberg, B. and Rydstrom, J., J.Steroid Biochem., 11 (1979) 1551-1559.

Moore, R. M. and Walters, R. R., J.Chromatogr., 384 (1987) 91-103.

Moore, W. M. and Spilburg, C. A., Biochemistry, 25 (1986) 5189-5195.

Morelli, A., Benatti, U., Gaetani, G. F. and De Flora, A., Proc.Nat.Acad.Sci. U.S., 75 (1978) 1979-1983.

Moreno-Lopez, J., Kristiansen, T. and Karsnas, P., J.Virol.Meth., 2 (1981) 293-300.

Morgan, B. P., Daw, R. A., Siddle, K., Luzio, J. P. and Campbell, A. K., J.Immunol.Methods, 64 (1983) 269-281.

Morgan, M. R. A., Kerr, E. J. and Dean, P. D. G., J.Steroid Biochem., 9 (1978) 767-770.

Morgenthaler, J. J., Baillod, P. and Friedli, H., Vox Sang., 47 (1984) 41-46.

Mori, Y., J.Chromatogr., 189 (1980) 428-432.

Morinaga, T., Kitamikado, M., Iwase, H., Li, S. C. and Li, Y. T., Biochim.Biophys.Acta, 749 (1983) 211-213.

Moriyama, T., Tozawa, T., Yamashita, H., Onodera, S., Nabuoka, M. and Makino, M., J. Chromatogr., 571 (1991) 61-72.

Moroi, M., Goetze, A., Dubay, E., Wu, C., Hasitz, M. and Jamieson, G. A., Thromb.Res., 28 (1982) 103-114.

Mosley, R. L., Murphy, J. W. and Cox, R. A., Infec.Immunity, 51 (1986) 844-850.

Mosolov, V. V., Fedurkina, N. V. and Valueva, T. A., Biochim.Biophys.Acta, 522 (1978) 187-194.

Mossner, E., Boll, M. and Pfleiderer, G., Hoppe-Syler's Z.Physiol.Chem., 361 (1980) 543-549.

Moudgil, V. K., Cikalo, M., Samokyszyn, V. M., Eessalu, T. E. and Singh, V. B., J.Steroid Biochem., 22 (1985) 747-751.

Moudgil, V. K. and John, J. K., Biochem.J., 190 (1980) 809-818.

Mount, C. D., Lukas, T. J. and Orth, D. N., Arch.Biochem.Biophys., 255 (1987) 1-7.

Mouton, C. A., Pang, D., Natraj, C. V. and Shafer, J. A., Arch.Biochem.Biophys., 218 (1982) 101-108.

Muller, D., Yu, X. J., Fischer, A. M., Bros, A. and Jozefonvicz, J., J.Chromatogr., 359 (1986) 351-357.

Murgier, M., Pelissier, C., Lazdunski, A., Bernadac, A. and Lazdunski, C., Eur.J.Biochem., 74 (1977) 425-433.

Murooka, Y., Yim, M. H., Yamada, T. and Harada, T., Biochim.Biophys.Acta, 485 (1977) 134-140.

Murti, T. R. and Pandit, M. W., J.Chromatogr., 260 (1983) 216-222.

Myatt, L., Elder, M. G., Neethling, C. and Lim, L., Biochem.J., 202 (1982) 203-209.

Myllyla, R., Anttinen, H., Risteli, L. and Kivirikko, K. I., Biochim.Biophys.Acta, 480 (1977) 113-121.

Myohanen, T. A., Bouriotis, V. and Dean, P. D. G., Biochem.J., 197 (1981) 683-688.

Nader, H. B., Takahashi, H. K., Guimaraes, J. A., Dietrich, C. P., Bianchini, P. and Ozima, B., Int.J. Biol.Macromol., 3 (1981) 356-360.

Nadin-Davis, S. and Mezl, V. A., J.Biochem.Biophys.Meth., 11 (1985) 185-189.

Nadin-Davis, S. and Mezl, V. A., Can.J.Biochem., 61 (1983) 353-362.

Nagamatsu, Y., Okamoto, U., Tsuda, Y. and Okada, Y., Thromb.Haemost., 51 (1984) 243-247.

Nagase, H. and Barret, A. J., Biochem.J., 193 (1981) 187-192.

Naguib, A. M. F., Bansal, J., Calton, G. J. and Burnett, J. W., Toxicon, 26 (1988) 387-394.

Nair, B. C., Johnson, D. E., Majeska, R. J., Rodkey, J. A., Bennett, C. D. and Rodan, G. A., Arch. Biochem. Biophys., 254 (1987) 28-34.

Nakagawa, H., Kotani, T., Ohtaki, S., Nakamura, M. and Yamazaki, I., Biochem.Biophys.Res.Commun., 127 (1985) 8-14.

Nakamura, K., Toyoda, K. and Kato, Y., J.Chromatogr., 445 (1988) 234-238.

Nakane, M. and Deguchi, T., Biochim.Biophys.Acta, 525 (1978) 275-285.

Nakano, N. I., Shimamori, Y. and Yamaguchi, S., J.Chromatogr., 188 (1980) 347-356.

Nakano, K., Fukui, T. and Matsubara, H., J.Biochem. Tokyo, 87 (1980) 919-927.

Nakata, H. and Fujisawa, H., Eur.J.Biochem., 122 (1982) 41-47.

Nakata, H. and Fujisawa, H., J.Biochem. Tokyo, 90 (1981) 567-569.

Nakayama, H., Shikano, H., Aoyama, T., Amano, T. and Kanaoka, Y., FEBS Lett., 208 (1986) 278-282.

Nakayama, H., Withy, R. M. and Raftery, M. A., Proc.Nat.Acad.Sci. U.S., 79 (1982) 7575-7579.

Nakayasu, H., Mihara, K. and Sato, R., Biochem.Biophys.Res.Commun., 83 (1978) 697-703.

Nandedkar, U. N., Sawhney, S. Y., Bhide, S. V. and Kale, N. R., J.Chromatogr., 396 (1987) 363-368.

Napier, M. A., Lipari, M. T., Courter, R. G. and Cheng, C. H. K., Arch.Biochem.Biophys., 259 (1987) 296-304.

Narahashi, Y., Hattori, K. and Murakoshi, H., Agr.Biol.Chem. Tokyo, 44 (1980) 1661-1662.

Naseem, I. and Hadi, S. M., Arch.Biochem.Biophys., 255 (1987) 437-445.

Nealon, D. E. and Cook, R. A., Biochemistry, 18 (1979) 3616-3622.

Necessary, P. C., Humphrey, P. A., Mahajan, P. B. and Ebner, K. E., J.Biol.Chem., 259 (1984) 6942-6946.

Nedospasov, A. A., Khomutov, R. M., Gambaryan, A. S., Morosov, I. A. and Venkstern, T. V., Molekulyarnaya Biologiya, 12 (1978) 1105-1111.

Nelson, T. J. and Kaufman, S., Arch.Biochem.Biophys., 257 (1987) 69-84.

Nelson, W. J. and Traub, P., J.Cell.Sci., 57 (1982) 25-49.

Nesheim, M., Blackburn, M. N., Lawler, C. M. and Mann, K. G., J.Biol.Chem., 261 (1986) 3214-3221.

Neumaier, M., Fenger, U. and Wagener, C., J.Immunol., 135 (1985) 3604-3609.

Neurath, A. R., Prince, A. M. and Giacalone, J., Experientia, 34 (1978) 414-415.

Newman, J. D. and Harrison, L. C., Biochem.Biophys.Res.Commun., 132 (1985) 1059-1065.

Nexo, E., Hock, R. A. and Hollenberg, M. D., J.Biol.Chem., 254 (1979) 8740-8743.

Ngo, T. T., Bio/Technology, 4 (1986) 134-137.

Nicolas, M. and Crouzet, J., Phytochemistry, 19 (1980) 15-18.

Nicolas, J. C., Methods Enzymol., 34 (1974) 552-554.

Nicolas, J. C., Pons, M., Descomps, B. and Crastes de Paulet, A., FEBS Lett., 23 (1972) 175-179.

Nicollet, I., Lebreton, J. P., Fontaine, M. and Hiron, M., Biochim.Biophys.Acta, 668 (1981) 235-245.

Nicotra, J., De Bari, V. A. and Needle, M. A., Immunol.Lett., 4 (1982) 249-252.

Nielsen, L. S., Hansen, J. G. Andreasen, P. A., Skriver, L., Dano, K. and Zeuther, J., EMBO J., 2 (1983) 115-119.

Nielsen, T. B., Downs Jr., R. W. and Spiegel, A. M., Biochem.J., 190 (1980) 439-443.

Nilsson, B., Abrahmsen, L. and Uhlen, M., EMBO J., 4 (1985) 1075-1080.

Nilsson, B., J.Chromatogr., 276 (1983) 413-417.

Nilsson, K. and Larsson, P. O., Anal.Biochem., 134 (1983) 60-72.

Nilsson, K. and Mosbach, K., FEBS Lett., 118 (1980) 145-150.

Nilsson, R., Myhre, E., Kronvall, G. and Sjogren, H. O., J.Immunol.Methods, 62 (1983) 241-245.

Nilsson, R., Myhre, E., Kronvall, G. and Sjogren, H. O., Mol.Immunol., 19 (1982) 119-126.

Nishi, S. and Yamazaki, H., Ann.N.Y.Acad.Sci., 417 (1983) 452-458.

Nishikata, M., J.Biochem. Tokyo, 93 (1983) 73-79.

Nishikata, M., Kasai, K. I. and Ishii, S. I., Biochim.Biophys.Acta, 660 (1981) 256-261.

Nishimori, K., Kawaguchi, Y., Uozumi, T. and Beppu, T., Agr.Biol.Chem. Tokyo, 48 (1984) 1091-1092.

Nishimoto, Y., Takeuchi, F. and Shibata, Y., J.Chromatogr., 169 (1979) 357-364.

Nishino, N. and Powers, J. C., J.Biol.Chem., 255 (1980) 3482-3486.

Nogues, M. V., Guasch, A., Alonso, J. and Cuchillo, C. M., J.Chromatogr., 268 (1983) 255-264.

Nordstrom, B., Randahl, H., Slaby, I. and Holmgren, A., J.Biol.Chem., 256 (1981) 3112-3117.

Norman, R. I., Scmid, A., Lombet, H., Barhanin, J. and Lazdunski, M., Proc.Nat.Acad.Sci. U.S., 80 (1983) 4164-4168.

Novick, D., Eshhar, Z., Fischer, D. G., Friedlander, J. and Rubinstein, M., EMBO J., 2 (1983a) 1527-1530.

Novick, D., Eshhar, Z., Gigi, O., Marks, Z., Revel, M. and Rubinstein, M., J.Gen.Virol., 64 (1983b) 905-910.

Nowak, T. P., Kobiler, D., Roel, L. E. and Barondes, S. H., J.Biol.Chem., 252 (1977) 6026-6030.

Nummi, M., Niku-Paavola, M. L., Enari, T. M. and Raunio, V., Anal.Biochem., 116 (1981) 137-141.

Nygren, H. and Stenberg, M., J.Immunol.Methods, 79 (1985) 7-11. O'Neil, E. A. and Kelly, T. J., J. Biol. Chem., 263 (1988) 931-937.

O'Sullivan, M. J., Gnemmi, E., Chieregatti, G., Morris, D., Simmonds, A. D., Simmons, M., Bridges, J. W. and Marks, V., J.Immunol.Methods, 30 (1979) 127-137.

Ochi, Y., Fujiyama, Y., Hosoda, S., Hamazu, M., Kajita, Y., Myazaki, T., Hachiya, T. and Ishida, M., J.Immunol.Methods, 52 (1982a) 213-221.

Ochi, Y., Fujiyama, Y., Hosoda, S., Miyazaki, T., Hachiya, T. and Kajita, Y., Clin.Chim.Acta, 122 (1982b) 145-160.

Ochiya, T., Baba, T., Mizushima, A., Onozaki, K. and Yaoita, H., Cell Immunol., 71 (1982) 346-352.

Ochoa, J. L., Kempf, J. and Egly, J. M., Int.J.Biol.Macromol., 2 (1980) 33-38.

Ochoa, N., Agundis, C. and Cordoba, F. A., Prep.Biochem., 17 (1987) 337-347.

Oda, Y., Kasai, K. and Ishii, S., J.Biochem., 89 (1981) 285-296.

Oestreicher, A. B., Van Dongen, C. J., Zwiers, H. and Gispen, W. H., J.Neurochem., 41 (1983) 331-340.

Ogunyemi, E., Pittner, F. and Hoffmann-Ostenhof, O., Hoppe-Seyler's Z.Physiol.Chem., 359 (1978) 613-616.

Ohlson, S., Hansson, L., Glad, M., Mosbach, K. and Larsson, P. O., Trends Biotechnol., 7 (1989) 179-186.

Ohlson, S., Gudmundsson, B. M., Wikstrom, P. and Larsson, P. O., Clin. Chem., 34 (1988a) 2039-2043.

Ohlson, S., Lundblad, A. and Zopf, D., Anal.Biochem., 169 (1988b) 204-208.

Ohlson, S. and Wieslander, J., J.Chromatogr., 397 (1987) 207-212.

Ohlson, S., Hansson, L., Larsson, P. O. and Mosbach, K., FEBS Lett., 93 (1978) 5-9.

Ohta, T., Hattori, S., Murakami, M., Horiuchi, S., Nishiyama, S. and Matsuda, I., Clin.Chim.Acta, 179 (1989) 183-190.

Ohtsuru, M., Tang, J. and Delaney, R., Int.J.Biochem., 14 (1982) 925-932.

Oji, Y., Miki, Y. and Okamoto, S., Plant Cell Physiol., 23 (1982) 1025-1031.

Oka, K., Kato, T., Sugimoto, T., Matsuura, S. and Nagatsu, T., Biochim.Biophys.Acta, 661 (1981) 45-53.

Oka, S., Ikeda, K., Kawasaki, T. and Yamashina, I., Arch.Biochem.Biophys., 260 (1988) 257-266.

Okada, M., Kawashima, S. and Imahori, K., J.Biochem. Tokyo, 85 (1979) 1225-1233.

Okada, Y., Nagase, H. and Harris, E. D., J.Biol.Chem., 261 (1986) 14245-14255.

Okada, Y., Tsuda, Y., Nagamatsu, Y. and Okamoto, U., Chem.Pharm.Bull. Tokyo, 30 (1982) 1528-1530.

Okai, Y., Yukioka, M. and Inoue, A., FEBS Lett., 141 (1982) 102-105.

Okayama, H., Ueda, K. and Hayaishi, O., Proc.Nat.Acad.Sci. U.S., 75 (1978) 1111-1115.

Okuda, H., Tavoloni, N., Kiang, C. L., Jones, M. J. T. and Berk, P. D., Biochem.Biophys.Res.Commun., 117 (1983) 406-412.

Olsen, K. W., Anal.Biochem., 109 (1980) 250-254.

Olsson, U. and Mattiasson, B., J.Chromatogr., 370 (1986) 29-37.

Olsson, R. A., J.Chromatogr., 176 (1979) 239-241.

Omori, K., Takemura, S., Omori, K., Mega, T. and Tashiro, Y., J.Biochem. Tokyo, 94 (1983) 1857-1866.

Ong, H., Adam, A., Perreault, S., Marleau, S., Bellemare, M., Du Souich, P. and Beaulieu, N., J.Chromatogr., 497 (1989) 213-221.

Onozaki, K., Homma, Y. and Hashimoto, T., Experientia, 35 (1979) 1556-1557.

Ooshima, H., Sakata, M. and Harano, Y., Biotechnol.Bioeng., 25 (1983) 3103-3114.

Oray B and Norton S.J., Biochim.Biophys.Acta, 611 (1980) 168-173.

Oren, M., Winocour, E. and Prives, C., Proc. Nat. Acad. Sci. U.S., 77 (1980) 220-224.

Orikasa, M., Iwase, I., Kozima, K. and Shimizu, F., J.Gen.Microbiol., 130 (1984) 1757-1763.

Orlowski, M., Flick, M. R., Rand, J. and Lesser, M., Arch.Biochem.Biophys., 254 (1987) 156-169.

Oroszlan, S., Long, C. W. and Gilden, R. V., Virology, 72 (1976) 523-526.

Osborne, W. R. A., J.Biol.Chem., 255 (1980) 7089-7092.

Osiecki-Newman, K. M., Fabbro, D., Dinur, T., Boas, S., Gatt, S., Legler, G., Desnick, R. J. and Grabowski, G. A., Enzyme, 35 (1986) 147-153.

Ostapenko, O. V., Evstratova, N. G., Serebrennikova, G. A. and Evstigneeva, R. P., Bioorg.Khim., 10 (1984) 1639-1644.

Ostro, M. and Fondy, T. P., J.Biol.Chem., 252 (1977) 5575-5583.

Ototani, N., Kikuchi, M. and Yosizawa, Z., Carbohyd.Res., 88 (1981) 291-303.

Ototani, N. and Yosizawa, Z., Carbohyd.Res., 70 (1979) 295-306.

Pace, B. and Pace, N. R., Anal.Biochem., 107 (1980) 128-135.

Packman, L. C. and Shaw, W. V., Biochem.J., 193 (1981) 541-552.

Pai, S. B., Biochem.Biophys.Res.Commun., 110 (1983) 412-416.

Pajula, R. L., Raina, A. and Kekoni, J., FEBS Lett., 90 (1978) 153-156.

Pak, K. Y., Blaszczyk, M., Steplewski, Z. and Koprowski, H., Mol.Immunol., 20 (1983) 1369-1377.

Palatnik, C. M., Storti, R. V. and Jacobson, A., J.Mol.Biol., 150 (1981) 389-398.

Palmer, F. B. S. C., J.Lipid.Res., 22 (1981) 1296-1300.

Pantoliano, M. W., Holmquist, B. and Riordan, J. F., Biochemistry, 23 (1984) 1037-1042.

Paoletti, F. and Aldinucci, D., Arch.Biochem.Biophys., 245 (1986) 212-219.

Papsidero, L. D., Groghan, G. A., Johnson, E. A. and Chu, T. M., Mol.Immunol., 21 (1984) 955-960.

Pardoe, I. U. and Burness, A. T. H., J.Gen.Virol., 57 (1981) 239-243.

Parham, P., Methods Enzymol., 92 (1983) 110-138.

Parkers, H. C., Stirling, J. L. and Calvo, P., Biochem.J., 219 (1984) 1009-1015.

Parrinello, N. and Canicatti, C., Develop.Comp.Immunol., 6 (1982) 53-64.

Pastore, E. J., Plante, L. T. and Kisliuk, R. L., Methods Emzymol., 34 (1974) 281-288.

Paszczynski, A., Miedziak, R., Lobarzewski, J., Kochmanska, J. and Trojanowski, J., FEBS Lett., 149 (1982) 63-66.

Patel, T. R., Jackman, D. M. and Bartlett, F. M., Appl.Environ.Microbiol., 46 (1983) 333-337.

Pattinson, N. R., Biochim.Biophys.Acta, 667 (1981a) 70-76.

Pattinson, N., Anal.Biochem., 115 (1981b) 424-427.

Pattinson, N., Collins, D. and Campbell, B., J.Chromatogr., 187 (1980) 409-412.

Patton, J. S. and Andersson, L., FEBS Lett., 86 (1978) 179-182.

Paul, A., Picard, J., Mergey, M., Veissiere, D., Finkbeiner, W. E. and Basbaum, C. B., Arch. Biochem. Biophys., 260 (1988) 75-84.

Pazur, J. H., Kelly-Delcourt, S. A., Miskiel, F. J., Burdett, L. and Docherty, J. J., J.Immunol.Methods, 89 (1986) 19-25.

Pazur, J. H., Erikson, M. S., Tay, M. E. and Allen, P. Z., Carbohyd.Res., 124 (1983) 253-263.

Pazur, J. H., Dreher, K. L., Kubrick, R. L. and Erikson.M.S., Anal.Biochem., 126 (1982) 285-294.

Peanasky, R. J., Bentz, Y., Paulson, B., Graham, D. L. and Babin, D. R., Arch.Biochem.Biophys., 232 (1984) 127-134.

Pedersen, N. S., Petersen, C. S., Axelsen, N. H., Birch-Andersen, A. and Hovind-Hougen, K., Scand.J.Immunol., 14 (1981) 137-144.

Pekonen, F., Williams, D. M. and Weintraub, B. D., Endocrinology, 106 (1980) 1327-1332.

Pelaez, F., De Herreros, A. G. and De Haro, C., Arch.Biochem.Biophys., 257 (1987) 328-338.

Pen, J., Van Beeumen, J. and Beintema, J. J., Biochem.J., 238 (1986) 691-699.

Penke, B., Zarandi, M., Varga, J. R., Toth, G. K., Kovacs, K. and Szajani, B., J.Chromatogr., 376 (1986) 307-314.

Pereira, M. E. A. and Kabat, E. A., J.Cell.Biol., 82 (1979) 185-194.

Perriard, J. C., Caravatti, M., Perriard, E. R. and Eppenberger, H. M., Arch.Biochem.Biophys., 191 (1978) 90-100.

Petit-Glatron, M. F., Benyahia, F. and Chambert, R., Eur.J.Biochem., 163 (1987) 379-387.

Petruzelli, L., Herrera, R. and Rosen, O. M., Proc.Nat.Acad.Sci. U.S., 81 (1984) 3327-3331.

Pfadenhauer, E. H. and Tong, S. D., J.Chromatogr., 162 (1979) 585-590.

Pfeiffer, F., Graham, D. and Betz, H., J.Biol.Chem., 257 (1982) 9389-9393.

Pfeuffer, E., Dreher, R. M., Metzger, H. and Pfeuffer, T., Proc.Nat.Acad.Sci. U.S., 82 (1985) 3086-3090.

Pfeuffer, T., Gaugler, B. and Metzger, H., FEBS Lett., 164 (1983) 154-160.

Pfeuffer, T. and Metzger, H., FEBS Lett., 146 (1982) 369-375.

Philip, M., Tsai, I. and Bender, M. L., Biochemistry, 18 (1979) 3769-3773.

Phillips, T. M., in J.C.Venter and L.C.Harrison (Editors), The Use of HPLC in Receptor Purification and Characterization, Alan R. Liss, Inc., New York, 1989, pp. 129-154.

Phillips, T. M. and Frantz, S. C., J.Chromatogr., 444 (1988) 13-20.

Phillips, T. M., More, N. S., Queen, W. D. and Thompson, A. M., J.Chromatogr., 327 (1985) 205-211.

Phillips, L. A., Pang, R. H. L., Park, J. J., Holis, V. W. J. and Famuyiwa, F., Prep.Biochem., 10 (1980) 11-26.

Phillips, N. C., Winchester, B. G. and Jolly, R. D., Biochem.J., 163 (1977) 269-277.

Pick, K. H. and Wober, G., Prep.Biochem., 9 (1979) 293-302.

Pierschbacher, M. D., Hayman, E. G. and Ruoslahti, E., Cell, 26 (1981) 259-267.

Pike, J. W., Sleator, N. M. and Haussler, M. R., J.Biol.Chem., 262 (1987) 1305-1311.

Pike, L. J., Eakes, A. T. and Krebs, E. G., J.Biol.Chem., 261 (1986) 3782-3789.

Pillion, D. J., Carter-Su, C. A., Pilch, P. F. and Czech, M. P., J.Biol.Chem., 255 (1980) 9168-9176.

Pischel, K. D. and Little, J. R., Mol.Immunol., 17 (1980) 305-312.

Pittalis, S., Martinez di Montemuros, F., Tavazzi, D. and Fiorelli, G., J. Chromatogr., 573 (1992) 29-34.

Plevani, P., Foiani, M., Valsasnini, P., Badaracco, G., Cheriathundam, E. and Chang, L. M. S., J.Biol.Chem., 260 (1985) 7102-7107.

Plummer, T. H. and Hurwitz, M. Y., J.Biol.Chem., 253 (1978) 3907-3912.

Pochon, F. and Bieth, J. G., J.Biol.Chem., 257 (1982) 6683-6685.

Podhradský, D., Toth, G., Suchár, G., Kristian, P. and Antalík, M., J.Chromatogr., 328 (1985) 402-408.

Pohl, G., Jornvall, H., Kok, P. and Wallen, P., FEBS Lett., 205 (1986) 92-96.

Pohl, J., Zaoral, M., Jindra, A. J. and Kostka, V., Anal.Biochem., 139 (1984) 265-271.

Pohl, J., Bureš, L. and Slavík, K., Collect.Czech.Chem.Commun., 46 (1981) 3302-3313.

Poirier, J. C., Combarnous, Y. and Blanc, M. R., Acta Endocrinol., 106 (1984) 38-42.

Poliquin, L. and Shore, G. C., Anal.Biochem., 109 (1980) 460-465.

Pollard, K. M. and Webb, J., J.Immunol.Methods, 54 (1982) 81-94.

Porter, T. D., Wilson, T. E. and Kasper, C. B., Arch.Biochem.Biophys., 254 (1987) 353-367.

Porumb, T., Porumb, H., Lascu, I. and Proinov, I., J.Chromatogr., 319 (1985) 218-221.

Poulsen, F. M. and Johansen, J. T., Carlsberg Res.Commun., 42 (1977) 397-405.

Povoa, G., Wennberg, G. and Hall, K., Biochem.Biophys.Res.Commun., 136 (1986) 253-259.

Pratt, M. L., Maher, J. F. and Roche, T. E., Eur.J.Biochem., 125 (1982) 349-355.

Prestwich, G. D. and Hammock, B. D., Proc.Nat.Acad.Sci. U.S., 82 (1985) 1663-1667.

Prigent, M. J., Blanchard, D. and Cartron, J. P., Arch.Biochem.Biophys., 222 (1983) 231-244.

Prigent, M. J., Lombart, C. and Asseraf, A., FEBS Lett., 150 (1982) 85-88.

Prigent, A. F., Nemoz, G. and Pacheco, H., Biochem.Biophys.Res.Commun., 95 (1980) 1080-1089.

Prisyazhnoy, V. S., Fusek, M. and Alakhov, Y. B., J. Chromatogr., 424 (1988) 243-253.

Pujar, B. G. and Ribbons, D. W., Indian J.Biochem.Biophys., 20 (1983) 112-114.

Puma, P., Buxser, S. E., Watson, L., Kelleher, D. J. and Johnson, G. L., J.Biol.Chem., 258 (1983) 3370-3375.

Pusztai, A., Grant, G., Stewart, J. C. and Watt, W. B., Anal.Biochem., 172 (1988) 108-112.

Pyne, N. J., Cooper, M. E. and Houslay, M. D., Biochem.J., 242 (1987) 33-42.

Pytela, R., Pierschbacher, M. D., Argraves, S., Suzuki, S. and Ruoslahti, E., Methods Enzymol., 144 (1987) 475-489.

Quarles, R. H., Mc Intyre, L. J. and Pasnak, C. F., Biochem.J., 183 (1979) 213-221.

Quinn, J. M. and Etzler, M. E., Arch.Biochem.Biophys., 258 (1987) 535-544.

Raba, R., Aaviksaar, A., Raba, M. and Siigur, J., Eur.J.Biochem., 96 (1979) 151-158.

Rabier, J., Vijayalakshimi, M. A. and Lambert, C., J.Chromatogr., 295 (1984) 215-219.

Rabinovitch, M. L., Klyosov, A. A., Grigorash, S. Y., Kalnina, I. A. and Chernogalzov, V. M., Bioorg.Khim., 8 (1982) 84-95.

Racois, A. and Boschetti, E., Biochimie, 60 (1978) 193-196.

Rademacher, B. E. and Steele, W. J., Anal.Biochem., 160 (1987) 119-126.

Radojkovic, J. and Ureta, T., Biochem.J., 242 (1987) 895-903.

Radparvar, S., Houghton, P. J. and Houghton, J. A., Arch.Biochem.Biophys., 260 (1988) 342-350.

Raftell, M. and Blomberg, K., Arch.Biochem.Biophys., 199 (1980) 165-171.

Rajgopal, S. and Vijayalakshmi, M. A., J.Chromatogr., 280 (1983) 77-84.

Rajgopal, S. and Vijayalakshmi, M., J.Chromatogr., 243 (1982) 164-167.

Rakonczay, Z., Mallol, J., Schenk, H., Vincendon, G. and Zanetta, J. P., Biochim.Biophys.Acta, 657 (1981) 243-256.

Ramadoss, C. S., Steczko, J., Uhlig, J. W. and Axelrod, B., Anal.Biochem., 130 (1983) 481-484.

Ramwani, J. and Mishra, R. K., J.Biol.Chem., 261 (1986) 8894-8898.

Randall, R. E., J.Immunol.Methods, 60 (1983) 147-165.

Randle, B. J., Morgan, A. J., Stripp, S. A. and Epstein, M. A., J.Immunol.Methods, 77 (1985) 25-36.

Rao, G. S. J., Savithri, H. S., Seethalakshmi, S. and Rao, N. A., Anal.Biochem., 95 (1979) 401-405.

Rao, L. V. M., Thromb.Res., 51 (1988) 373-384.

Rao, L. V. M. and Rapaport, S. I., Anal.Biochem., 165 (1987) 365-370.

Rase, B., Bartfai, T. and Ernster, L., Arch.Biochem.Biophys., 172 (1976) 380-386.

Rasilo, M. L., Wartiovaara, J. and Renkonen, O., Can.J.Biochem., 58 (1980) 384-393.

Rassi, Z. E., Horvath, C., Yu, R. K. and Ariga, T., J.Chromatogr., 488 (1989) 229-236.

Rassi, Z. E., Truei, Y. and Horvath, C., Makromol.Chem.,Macromol.Symp., 17 (1988) 305-319.

Rathelot, J., Bosc-Bierne, I., Guy-Crotte, O., Delori, P., Rochat, H. and Sarda, L., Biochim.Biophys.Acta, 744 (1983a) 115-118.

Rathelot, J., Delori, P. and Sarda, L., Biochim.Biophys.Acta, 742 (1983b) 39-48.

Ratnam, S., Delcamp, T. J., Hynes, J. B. and Freisheim, J. H., Arch.Biochem.Biophys., 255 (1987) 279-289.

Rauterberg, E. W., Schieck, C., Kreft, H. and Romer, W., Immunobiology, 166 (1984) 439-445.

Ray, P. K., Saha, S., Bassett, J. G. and Cooper, D. R., Fed.Proc., 40 (1981) 1039.

Ray, S., Salman, M., Ruiz, A. A., Stotter, P. L. and Chamness, G. C., J.Steroid Biochem., 24 (1986) 1111-1115.

Reagan, M. E., Robb, M., Bornstein, I. and Niday, E. G., Thromb.Res., 40 (1985) 1-9.

Reavil, C. A., Wooster, M. S. and Plummer, D. T., Biochem.J., 173 (1978) 851-856.

Reczek, P. R. and Villee, C. A., Biochem.Biophys.Res.Commun., 107 (1982) 1158-1165.

Reddy, C. C., Tu, C. P., Burgess, J. R., Ho, C. -., Scholz, R. W. and Massaro, E. J., Biochim. Biophys. Res.Commun., 101 (1981) 970-978.

Reddy, M. K., Heda, G. D. and Reddy, J. K., Biochem.J., 242 (1987) 681-687.

Reddy, M. V. and Jacob, T. M., Indian J.Biochem.Biophys., 20 (1983a) 183-187.

Reddy, M. V. and Jacob, T. M., Indian J.Biochem.Biophys., 20 (1983b) 321-326.

Redshaw, J. C. and Loten, E. G., FEBS Lett., 123 (1981) 261-264.

Reeber, A., Zanetta, J. P. and Vincendon, G., Biochim.Biophys.Acta, 801 (1984) 444-455.

Reeck, G. R., Walsh, K. A. and Neurath, H., Biochemistry, 10 (1971) 4690-4698.

Reeve, A. E. and Huang, R. C., Anal.Biochem., 130 (1983) 14-18.

Reeves, M. W., Arko, R. J., Chandler, F. W. and Bridges, N. B., Infec.Immunity, 51 (1986) 431-439.

Reeves, H. C., Heeren, R. and Malloy, P., Anal.Biochem., 115 (1981) 194-196.

Regan, J. W., Barden, N., Lefkowitz, R. J., Caron, M. G., Demarinis, R. M., Krog, A. J., Holden, K. G., Matthews, W. D. and Hieble, J. P., Proc.Nat.Acad.Sci. U.S., 79 (1982) 7223-7227.

Regnault, V., Rivat, C. and Stoltz, J. F., J.Chromatogr., 432 (1988) 93-102.

Reh, E., J.Chromatogr., 433 (1988) 119-130.

Reichen, J. and Berk, P. D., Biochem.Biophys.Res.Commun., 91 (1979) 484-489.

Reidl, L. S., Campagnoni, C. W. and Campagnoni, A. T., J.Neurochem., 37 (1981) 373-380.

Reinberg, D., Arenas, J. and Hurwitz, J., J.Biol.Chem., 260 (1985) 6088-6097.

Reindeau, D., Falgueyret, J. P., Nathaniel, D. J., Rokach, J., Ueda, N. and Yamamoto, S., Biochem.Pharmacol., 38 (1989) 2313-2321.

Reiss, U. and Sacktor, B., Arch.Biochem.Biophys., 209 (1981) 342-348.

Relton, J. M., Gee, N. S., Matsas, R., Turner, A. J. and Kenny, A. J., Biochem.J., 215 (1983) 519-523.

Relyveld, E. H., Ann.Immunol.-Int.Pasteur., C132 (1981) 365-374.

Renauer, D., Oesch, F., Kinkel, J., Unger, K. K. and Wieser, R. J., Anal.Biochem., 151 (1985) 424-427.

Rendell, M., Kao, G., Mecherikunnel, P., Petersen, B., Duhaney, R., Nierenberg, J., Rasbold, K., Klenk, D. and Smith, P. K., Clin.Chem., 31 (1985) 229-234.

Renoir, J. M., Mester, J., Buchou, T., Catelli, M. G., Tuohimaa, P., Binart, N., Joab, I., Radanyi, C. and Baulieu, E. E., Biochem.J., 217 (1984) 685-692.

Repaske, M. G., Nunnari, J. M. and Limbird, L. E., J.Biol.Chem., 262 (1987) 12381-12386.

Retzinger, G. S., Meredith, S. C., Lau, S. H., Kaiser, E. T. and Kezdy, F. J., Anal.Biochem., 150 (1985) 131-140.

Rexová-Benková, L., Filka, K. and Kocourek, J., J.Chromatogr., 376 (1986) 413-420.

Rexová-Benková, L., Omelková, J., Filka, K. and Kocourek, J., Carbohyd.Res., 122 (1983) 269-281.

Rexová-Benková, L., Markovič, O. and Foglietti, M. J., Collect Czech.Chem.Commun., 42 (1977) 1736-1741.

Rey-Campos, J., Rey-Mendez, M. and Freire, M., Int.J.Biochem., 15 (1983) 155-157.

Reyes, P. and Sandquist, R. B., Anal.Biochem., 88 (1978) 522-531.

Rhoads, R. E. and Hellmann, G. M., J.Biol.Chem., 253 (1978) 1687-1693.

Ricard-Blum, S., Hartmann, D. J., Herbage, D., Payen-Meyran, C. and Ville, G., FEBS Lett., 146 (1982) 343-347.

Ricardo, M. J., Trouy, R. L. and Grimm, D. T., J.Immunol., 127 (1981) 946-951.

Rich, D. H., Brown, M. A. and Barrett, A. J., Biochem.J., 235 (1986) 731-734.

Richardson, P. J., J.Neurochem., 46 (1986) 1251-1255.

Richardson, P. J. and Luzio, J. P., Appl.Biochem.Biotech., 13 (1986) 133-145.

Richardson, P. J., Siddle, K. and Luzio, J. P., Biochem.J., 219 (1984) 647-654.

Richet, E. and Kohiyama, M., J.Biol.Chem., 253 (1978) 7490-7495.

Ricquier, D., Gervais, C., Kader, J. C. and Hemon, P., FEBS Lett., 101 (1979) 35-38.

Rieke, E., Barry, S. and Mosbach, K., Eur.J.Biochem., 100 (1979) 203-212.

Riesen, W. F., Jaross, W., Descoeudres, C., Mordasini, R., Koban, F., Thulin, H., Fisher, S. and Bergmann, S., Ann.Biol.Clin.Paris, 46 (1988) 787-791.

Riggio, G., Hopff, W. H., Hofmann, A. A. and Waser, P. G., Helv.Chim.Acta, 63 (1980) 488-497.

Riisom, K., Diederichsen, H. and Andersen, I., J.Clin.Pathol., 35 (1982) 750-753.

Risley, J. M. and Van Etten, R. L., Arch.Biochem.Biophys., 258 (1987) 404-412.

Risteli, L., Biochem.J., 169 (1978) 189-196.

Riva, E., Ferry, N., Cometti, A., Cuisnaud, G., Gallo, G. G. and Sassard, J., J.Chromatogr., 421 (1987) 99-110.

Rivera-Marrero, C. A. and Hillyer, G. V., J.Parasitol., 71 (1985) 547-555.

Robbins, J. B. and Schneerson, R., Methods Enzymol., 34 (1974) 703-722.

Rocher, M. A., Martin, M. P., Toro, M. J. and Montoya, E., J.Chromatogr., 368 (1986) 462-467.

Rodahl, E. and Maeland, J. A., Acta Pathol.Microbiol.Immun.C, 92 (1984) 247-254.

Rode, W., Ciesla, J., Zielinski, Z. and Kedzierska, B., Int.J.Biochem., 18 (1986) 361-368.

Rogers, M. E., Adlard, M. W., Saunders, G. and Holt, G., J.Chromatogr., 326 (1985) 163-172.

Rogers, L. and Kolattukudy, P. E., Anal.Biochem., 137 (1984) 444-448.

Rogers, K. M., White, G. F. and Dodgson, K. S., Biochim.Biophys.Acta, 527 (1978) 70-85.

Rohde, W., Arch.Virol., 71 (1982) 169-176.

Rohm, K. H., Hoppe-Seyler's Z.Physiol.Chem., 368 (1982) 641-649.

Romain, P. L. and Schlossman, S. F., Methods Enzymol., 108 (1984) 148-153.

Römer, W. and Rauterberg, E.W., J.Immunol. Methods, 38 (1980) 239-250.

Ronin, C., Fenouillet, E., Hovsepian, S., Fayet, G. and Fournet, B., J.Biol.Chem., 261 (1986) 7287-7293.

Roque-Barreira, M. C. and Campos-Neto, A., J.Immunol., 134 (1985) 1740-1743.

Rose, K., Priddle, J. D. and Offord, R. E., J.Chromatogr., 210 (1981) 301-309.

Roseman, B., Lough, J., Houkom, E. and Herman, T., Biochem.Biophys.Res.Commun., 137 (1986) 474-479.

Rosemeyer, H. and Seela, F., Carbohyd.Res., 90 (1981) 53-60.

Rosemeyer, H. and Seela, F., J.Med.Chem., 22 (1979a) 1545-1547.

Rosemeyer, H. and Seela, F., Carbohyd.Res., 74 (1979b) 117-125.

Rosenberg, M., Gazit, E. and Sharon, N., Hum.Immunol., 7 (1983) 67-77.

Rosenberg, I. H. and Saini, P. K., Methods Enzymol., 66 (1980) 667-670.

Rosenfeld, P. J. and Kelly, T. J., J.Biol.Chem., 261 (1986) 1398-1408.

Roth, W. W. and Srinivasan, V. R., Prep.Biochem., 8 (1978) 57-71.

Rotman, A. and Linder, S., Biochim.Biophys.Acta, 641 (1981) 114-121.

Rotondi, A. and Auricchio, F., Biochem.J., 178 (1979) 581-587.

Roulleau, M. F., Boschetti, E., Burnouf, T., Kirzin, J. M. and Saint-Blancard, J., in T.C.J.Gribnau, J.Visser and R.J.F.Nivard (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982, pp.323-331.

Rousset, B. and Wolff, J., J.Biol.Chem., 255 (1980) 11677-11681.

Roy, S. K., Mc Gregor, W. C. and Orichowskyj, S. T., J.Chromatogr., 327 (1985) 189-192.

Roy, S. K., Weber, D. V. and McGregor, W. C., J.Chromatogr., 303 (1984) 225-228.

Roy, S. K. and Kundu, S. K., Anal.Biochem., 98 (1979) 238-241.

Roy, S. K. and Nishikawa, A. H., Biotechnol.Bioeng., 21 (1979) 775-785.

Ruan, K., Hashida, S., Yoshitake, S., Ishikawa, E., Wakisaka, O., Yamamoto, Y., Ichioka, T. and Nakajima, K., Clin.Chim.Acta, 147 (1985) 167-172.

Ruiz-Carillo, A., Methods Enzymol., 34 (1974) 547-552.

Runswick, M. J. and Harris, J. I., FEBS Lett., 92 (1978) 365-367.

Ruoslahti, E., Hayman, E. G., Pierschbacher, M. and Engvall, E., Methods Enzymol., 82 (1982) 803-831.

Rupprecht, K. M., Sonenberg, N., Shatkin, A. J. and Hecht, S. M., Biochemistry, 20 (1981) 6570-6577.

Russel, C. S. and Pollack, S. E., J.Chromatogr., 166 (1978) 632-636.

Russell, S. H., Carter, R. and Firkin, F. C., J.Immunol.Methods, 66 (1984) 323-326.

Russell, D. W., Yamamoto, T., Schneider, W. J., Slaughter, C. J., Brown, M. S. and Goldstein, J. L., Proc.Nat.Acad.Sci. U.S., 80 (1983) 7501-7505.

Russell, R. R. B., FEMS Microbiol.Lett., 11 (1981) 279-282.

Russell, M. W., Zanders, E. D., Bergmeier, L. A. and Lehner, T., Infec.Immunity, 29 (1980) 999-1006.

Russo, A. J., Cobbs, C. S., Ishay, J. S., Calton, G. J. and Burnett, J. W., Toxicon, 21 (1983) 166-169.

Ryan, R. L. and McClure, W. O., Biochemistry, 18 (1979) 5357-5365.

Ryzlak, M. T. and Pietruszko, R., Arch.Biochem.Biophys., 255 (1987) 409-418.

Sadler, J. E., Rearick, J. I. and Hill, R. L., J.Biol.Chem., 254 (1979) 5934-5941.

Šafařík, I., J.Chromatogr., 284 (1984) 515-517.

Saini, M. S. and Van Etten, R. L., Arch.Biochem.Biophys., 191 (1978) 613-624.

Saini, P. K. and Rosenberg, I. H., J.Biol.Chem., 249 (1974) 5131-5134.

Saint-Blancard, J., Kirzin, J. M., Riberon, P., Petit, F., Fourcart, J., Girot, P. and Boschetti, E., in T.C.J.Gribnau, J.Visser and R.J.F.Nivard (Editors), Affinity Chromatography and Related Techniques, Elsevier, Amsterdam, 1982,pp.305-312.

Sairam, M. R., Ranganathan, M. R. and Lamothe, P., J.Endocrinol., 84 (1980) 17-23.

Sairam, M. R., Arch.Biochem.Biophys., 194 (1979) 63-70.

Saklatvala, J., Bond, J. S. and Barret, A. J., Biochem.J., 193 (1981) 251-259.

Salier, J. P., Vercaigne, D., Sesboue, R. and Martin, J. P., J.Immunol.Methods, 47 (1981) 239-248.

Salter, D. N., Scott, K. J., Slade, H. and Andrews, P., Biochem.J., 193 (1981) 469-476.

Saltiel, A. R., Fox, J. A., Sherline, P., Sahyoun, N. and Cuatrecasas, P., Biochem.J., 241 (1987) 759-763.

Samejima, K. and Yamanoha, B., Arch.Biochem.Biophys., 216 (1982) 213-222.

Samuel, D. J., Amlot, P. L., Shepherd, P. and Lachmann, P. J., Clin.Exp.Immunol., 65 (1986) 458-464.

Sanchez-Chiang, L., Ponce, O., Landsberger, E. and Enriquez, S., Comp.Biochem.Physiol.Pt.B, 85 (1986) 81-87.

Sancho, J., Peleato, M. L., Gomez-Moreno, C. and Edmondson, D. E., Arch.Biochem.Biophys., 260 (1988) 200-207.

Sanjanwala, M., Sun, G. Y. and Mac Quarrie, R. A., Arch.Biochem.Biophys., 258 (1987) 299-306.

Santa-Coloma, T. A., Garraffo, H. M., Gros, E. G. and Charreau, E. H., J.Chromatogr., 415 (1987) 297-304.

Santoro, S. A. and Frazier, W. A., Methods Enzymol., 144 (1987) 438-446.

Santos, E. and Kaback, H. R., Methods Enzymol., 126 (1986) 370-377.

Santos, E., Tahara, S. M. and Kaback, H. R., Biochemistry, 24 (1985) 3006-3011.

Saraceno, H. and De Robertis, E., Biochem.Biophys.Res.Commun., 69 (1976) 555-561.

Sarantoglou, V., Imbault, P. and Weil, J. H., Biochem.Biophys.Res.Commun., 93 (1980) 134-140.

Sarbakanova, S. T., Dunaevsky, Y. E., Belozersky, M. A. and Rudenskaya, G. N., Biokhimiya, 52 (1987) 1263-1269.

Sarkar, M. and Mandal, C., J.Immunol.Methods, 83 (1985) 55-60.

Sarngadharan, M. G., Kalyanaraman, V. S., Rahman, R. and Gallo, R. C., J.Virol., 35 (1980) 555-559.

Sasaki, H., Hayashi, A., Kitagaki-Ogawa, H., Matsumoto, I. and Seno, N., J.Chromatogr., 400 (1987) 123-132.

Sasaki, M., Minakata, K., Yamamoto, H., Niwa, M., Kato, T. and Ito, N., Biochem.Biophys.Res.Commun., 76 (1977) 917-924.

Sato, H., Fukunaga, K., Araki, S., Ohtsuki, I. and Miyamoto, E., Arch.Biochem.Biophys., 260 (1988) 443-451.

Sato, N., Yabuki, Y., Toh, K., Ishii, Y. and Kikuchi, K., Immunology, 36 (1979) 421-426.

Scawen, M. D., Hammond, P. M., Comer, M. J. and Atkinson, T., Anal.Biochem., 132 (1983) 413-417.

Scawen, M. D., Darbyshire, J., Harvey, M. J. and Atkinson, T., Biochem.J., 203 (1982) 699-705.

Schabort, J. C., J.Chromatogr., 73 (1972) 253-256.

Schachtele, K. H., Schlitz, E. and Palm, D., Eur.J.Biochem., 92 (1978) 427-435.

Schacht, J., J.Lipid.Res., 19 (1978) 1063-1967.

Schack, P. and Kaarsholm, N. C., Biochem.J., 219 (1984) 727-733.

Schaeg, W., Kranz, T., Bruckler, J. and Blobel, H., Zbl.Bakt.Hyg. A, 259 (1985) 71-77.

Schafer, D. A. and Hultquist, D. E., Biochem.Biophys.Res.Commun., 95 (1980) 381-387.

Scheiner, O., Stemberger, H., Kraft, D. and Wiedermann, G., J.Immunol.Methods, 21 (1978) 355-364.

Schell, H. D. and Arsene, M. L., Rev.Roum.Biochem., 19 (1982) 63-68.

Schilf, W. and Martin, H. H., Eur.J.Biochem., 105 (1980) 361-370.

Schlimme, E., Boos, K. S. and Weise, M., J.Clin.Chem.Biochem., 19 (1981) 55-60.

Schluter, S. F. and Ey, P. L., J.Immunol.Methods, 66 (1984) 89-97.

Schmidt, C. N. G. and Jervis, L., Anal.Biochem., 104 (1980) 127-129.

Schmidt, R., Wiegand, H. and Reichert, U., Eur.J.Biochem., 93 (1979) 355-361.

Schmitt, S., Muller, R. and Lingens, F., J.Immunol.Methods, 68 (1984) 263-268.

Schmitz, G., Bottcher, A., Kahl, H. G. and Bruning, T., J.Chromatogr., 431 (1988) 327-342.

Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U. and Greaves, M. F., J.Biol.Chem., 257 (1982) 10766-10769.

Schneider, R. J., Kulczycki, A., Law, S. K. and Atkinson, J. P., Nature (London), 290 (1981) 789-792.

Schneider, W. and Gschwendt, M., Biochim.Biophys.Acta, 633 (1980) 105-113.

Schrempf-Decker, G. E., Baron, D. and Wernet, P., J.Immunol.Methods, 32 (1980) 285-296.

Schroder, U., Segren, S., Gemmefors, G., Hedlung, G., Jansson, B., Sjongren, H. O. and Borrebaeck, C. A. K., J.Immunol.Methods, 93 (1986) 45-53.

Schroder, C., Weinblum, D., Zahn, R. K. and Dose, K., Hoppe-Seyler's Z.Phys.Chem., 360 (1979) 473-476.

Schuler, G., Aubock, J. and Linert, J., J.Immunol., 130 (1983) 2008-2010.

Schwartzman, M. L., Pagano, P. J., McGift, J. C. and Abraham, N. G., Arch.Biochem.Biophys., 252 (1987) 635-645.

Schwarting, G. A., Gajewski, A., Carroll, P. and De Wolf, W. C., Arch.Biochem.Biophys., 256 (1987) 69-77.

Schwartz, S. A., Bioscience Rep., 1 (1981) 107-118.

Schwartz, M., Lancet, D., Tarrab-Hazdai, R. and Fuchs, S., Mol.Immunol., 16 (1979) 483-487.

Schwartz, N. B. and Roden, L., Carbohyd.Res., 37 (1974) 167-180.

Scopes, R. K., Anal.Biochem., 136 (1984) 525-529.

Scott, J. D. and Fothergill, J. E., Biochem.J., 205 (1982) 575-580.

Scott, M. G., Hoffmann, J. W., Meltzer, V. N., Siegfried, B. A. and Chan, K. M., Clin.Chem., 30 (1984) 896-898.

Scouten, W. H., Methods Enzymol., 34 (1974) 288-294.

Seagrave, J. and Reyes, P., Anal.Biochem., 149 (1985) 169-176.

Seargeant, L. E. and Stinson, R. A., J.Chromatogr., 173 (1979) 101-108.

Secher, D. S. and Burke, D. C., Nature, 285 (1980) 446-450.

Seeling, G. F. and Meister, A., J.Biol.Chem., 257 (1982) 5092-5096.

Seganti, L., Mastromarino, P., De Stasio, A., Sinibaldi, L., Valenti, P. and Orsi, N., Acta Virol., 24 (1980) 311-316.

Sehmi, D., Williams, L. G. and Williams, D. J., FEBS Lett., 197 (1986) 335-338.

Seibert, G. and Zahn, R. K., J.Chromatogr., 161 (1978) 324-326.

Seidl, M. and Hormann, H., Hoppe-Seyler's Z.Physiol.Chem., 364 (1983) 83-92.

Sekar, M. C., Web, G. and Roufogalis, B. D., Biochim.Biophys.Acta, 613 (1980) 420-428.

Sekura, R. D., Fish, F., Manclark, C. R., Meade, B. and Yan-Ling Zhang, J.Biol.Chem., 258 (1983) 14647-14651.

Sela, B. A., Wang, J. L. and Edelman, G. M., Proc.Nat.Acad.Sci. U.S., 72 (1975) 1127-1131.

Selhub, J., Darcy-Vrillon, B. and Fell, D., Anal.Biochem., 168 (1988) 247-251.

Selhub, J., Ahmad, O. and Rosenberg, I. H., Methods Enzymol., 66 (1980) 686-690.

Senapathy, P., Jayabaskaran, C. and Jacob, M. T., Nucl.Acid.Res., 13 (1985) 5833-5842.

Sene, C., Girot, P., Boschetti, E., Plassat, J. L., Bloch, J. and Egly, J. M., J.Chromatogr., 248 (1982) 441-445.

Serafini-Fracassini, A., Field, J. M., Rodger, G. W. and Spina, M., Biochim.Biophys.Acta, 386 (1975) 80-86.

Serrano, A. and Rivas, J., Anal.Biochem., 126 (1982) 109-115.

Seydel, F., Lill, H. R. and Hartmann, G. R., Eur.J.Biochem., 117 (1981) 87-91.

Shadduck, R. K., Waheed, A., Pigoli, G., Boegel, F. and Higgins, L., Blood, 53 (1979) 1182-1190.

Shafit-Zagardo, B. and Turner, B. M., Biochim.Biophys.Acta, 659 (1981) 7-14.

Shaginyan, K. A., Izotova, L. S., Strongin, A. Y. and Stepanov, V. M., Biokhimiya, 45 (1980) 695-703.

Shanker, J. and Datta, K., Arch.Biochem.Biophys., 248 (1986) 652-657.

Shapiro, S. L. and Young, J. R., J.Biol.Chem., 256 (1981) 1495-1498.

Shapiro, L. J., Hall, C. W., Leder, I. G. and Neufeld, E. F., Arch.Biochem.Biophys., 172 (1976) 156-161.

Sharma, K. K. and Pattabiraman, T. N., Indian J.Biochim.Biophys., 19 (1982) 54-56.

Sharma, S. D., Araujo, F. G. and Remington, J. S., J.Immunol., 133 (1984) 2818-2820.

Sharma, S. K., Bradford, S., Babitch, J. A. and Couch, E. F., Eur.J.Cell Biol., 40 (1986) 44-52.

Sharma, S. K. and Babitch, J. A., J.Chromatogr., 312 (1984) 435-442.

Sharma, S. K. and Mahendroo, P. P., J.Chromatogr., 184 (1980) 471-499.

Sharma, S. K., Garrett, J. M. and Brown, S. A., Z.Naturforsch.Sect.C, 34 (1979) 387-391.

Sharma, S. K. and Brown, S. A., Can.J.Biochem., 57 (1979) 986-994.

Sharma, R. K., Desai, R., Thompson, T. R. and Wang, J. H., Can.J.Biochem., 56 (1978) 598-604.

Sharma, R. K., Tam, S. W., Waisman, D. M. and Wang, J. H., J.Biol.Chem., 255 (1980) 11102-11105.

Shen, R. F. and Tai, H. H., J.Biol.Chem., 261 (1986) 11585-11591.

Shepherd, H. A., Priddle, J. D., Jenkins, W. J. and Jewell, D. P., Clin.Chim.Acta, 139 (1984) 155-165.

Sherwood, R. F., Melton, R. G., Alwan, S. M. and Hughes, P., Eur.J.Biochem., 148 (1985) 447-453.

Sheth, J. J., Moodbidri, S. B., Sheth, A. R., Rao, S. S. and Panthaki, M. H., Indian J.Biochem.Biophys., 21 (1984) 89-92.

Shibata, S., Peters, B. P., Roberts, D. D., Goldstein, I. J. and Liotta, J. A., FEBS Lett., 142 (1982) 194-198.

Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B. and Peumans, W. J., Arch.Biochem.Biophys., 254 (1987) 1-8.

Shick, V. V., Belyavsky, A. V. and Mirzabekov, A. D., J.Mol.Biol., 185 (1985) 329-339.

Shieh, J. H., Cini, J. K., Wu, M. C. and Yunis, A. A., Arch.Biochem.Biophys., 253 (1987) 205-213.

Shigeoka, S., Onishi, T., Nakano, Y. and Kitaoka, S., Biochem.J., 242 (1987) 511-515.

Shimizu, F., Kist, M. and Vogt, A., Res.Exp.Med., 172 (1978) 231-238.

Shimura, K., Kasai, K. I. and Ishii, S. I., J.Chromatogr., 350 (1985) 265-272.

Shimura, K. and Kasai, K. I., J.Chromatogr., 315 (1984) 161-166.

Shimura, K., Kazama, M. and Kasai, K. I., J.Chromatogr., 292 (1984) 369-382.

Shinagawa, T., Do, Y. S., Tam, H. and Hsueh, W. A., Biochem.Biophys.Res.Commun., 139 (1986) 446-454.

Shing, Y., J.Biol.Chem., 263 (1988) 9059-9062.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrun, M., Science, 223 (1984) 1296-1299.

Shioi, Y., Nagamine, M., Kuroki, M. and Sasa, T., Biochim.Biophys.Acta, 616 (1980) 300-309.

Shivaraj, B. and Pattabiraman, T. N., Biochem.J., 193 (1981) 29-36.

Shkumatov, V. M., Gilevich, S. N., Chashchin, V. L. and Akhrem, A. A., Bioorg.Khim., 9 (1983) 1231-1236.

Shulgin, M. N., Valueva, T. A., Kestere, A. Y. and Mosolov, V. V., Biokhimiya, 46 (1981) 473-480.

Shulman, S., Pretorius, E. and Keane, T., Amer.J.Reprod.Immunol.Microb., 9 (1985) 62-66.

Siddell, S. G., Eur.J.Biochem., 92 (1978) 621-629.

Sidler, W. and Zuber, H., European J.Appl.Microbiol.Biotechnol., 10 (1980) 197-209.

Siegel, T. W., Ganguly, S., Jacobs, S., Rosen, O. M. and Rubin, C. S., J.Biol.Chem., 256 (1981) 9266-9273.

Sigel, E., Mamalaki, C. and Barnard, E. A., FEBS Lett., 147 (1982) 45-48.

Silva, A., De Landazuri, M. O., Alvarez, J. and Kreisler, J. M., J.Immunol.Methods, 23 (1978) 303-313.

Silver, R. E., Adamany, A. M. and Blumenfeld, O. O., Arch.Biochem.Biophys., 256 (1987) 285-294.

Simmons, B. M. and Russell, J. H., Anal.Biochem., 146 (1985) 206-210.

Simonianová, E. and Patáková, M., Collect.Czech.Chem.Commun., 44 (1979) 626-630.

Singh, U. S., Rao, V. K. M. and Ahmad, A., Indian J.Biochim.Biophys., 18 (1981) 152-154.

Sjogren, H. O., Nilsson, K., Malmstrom, P. and Axelsson, B., J.Immunol.Methods, 56 (1983) 285-294.

Sjöström, H., Norén, O., Jeppesen, L., Staun, M., Svensson, B. and Christiansen, L., Eur.J.Biochem., 88 (1978) 503-511.

Skipper, P. L., Trudel, L. J., Tannenbaum, S. R. and Wogan, G. N., Proc.Am.Assoc.Cancer Res., 28 (1987) 128.

Skjak-Braek, G. and Larsen, B., Carbohyd.Res., 103 (1982) 137-140.

Skovbjerg, H., Sjösröm, H. and Noren, O., Eur.J.Biochem., 114 (1981) 653-661.

Slankard-Chahinian, M. and Siskind, G. W., Immunology, 38 (1979) 145-156.

Slater, E. E. and Strout, H. V., J.Biol.Chem., 256 (1981) 8164-8171.

Slavík, K. and Slavikova, V., Methods Enzymol., 66 (1980) 709-723.

Slavík, K., Žižkovský, V., Slavíková, V. and Fořt, P., Biochem. Biophys. Res.Commun., 59 (1974) 1173-1184.

Sliwkowski, M. X., Sliwkowski, M. B., Horton, H. R. and Swaisgood, H. E., Biochem.J., 209 (1983) 731-739.

Slowinska, R. and Szewczuk, A., Acta Biochem.Pol., 26 (1979) 229-238.

Small, D. A. P., Atkinson, T. and Lowe, C. R., J.Chromatogr., 266 (1983) 151-156.

Smith, C. A. and Brew, K., J.Biol.Chem., 252 (1977) 7294-7299.

Smith, G. K., Schray, K. J. and Schaffer, S. W., Anal.Biochem., 84 (1978) 406-414.

Smith, G. D., Roberts, D. V. and Daday, A., Biochem.J., 165 (1977a) 121-126.

Smith, K. and Sundaram, T. K., Bioscience Rep., 3 (1983) 1035-1043.

Smith, L. T. and Kaplan, N. O., Anal.Biochem., 95 (1979) 2-7.

Smith, L. D., Emerson, R. L. and Knight, G. D., Int.J.Biochem., 8 (1977b) 883-887.

Smith, R. G., D'Istria, M. and Van, N. T., Biochemistry, 20 (1981) 5557-5565.

Smith, W. O. and Daniels, S. M., Plant Physiol., 68 (1981) 443-446.

Soliman, A., Nordlund, S., Johansson, B. C. and Baltscheffsky, H., Acta Chem.Scand.Ser.B, 35 (1981) 63-64.

Solomon, B., Moav, N., Pines, G. and Katchalski-Katzir, E., Mol.Immunol., 21 (1984) 1-11.

Som, K. and Nayak, D. P., Anal.Biochem., 87 (1978) 527-539.

Someno, T., Saino, T., Katoh, K., Miyazaki, H. and Ishii, S., J.Biochem. Tokyo, 97 (1985) 1493-1500.

Sondergaard, I. and Weeke, B., Allergy, 39 (1984) 473-479.

Song, P. S., Kim, I. S. and Hahn, T. R., Anal.Biochem., 117 (1981) 32-39.

Sorensen, S. and Trentemoller, S., Oncodev.Biol.Med., 4 (1983) 351-361.

Sorenson, J. C. and Scandalios, J. G., Biochem.Biophys.Res.Commun., 63 (1975) 239-246.

Speek, A. J., Odink, J., Schrijver, J. and Schreurs, W. H. P., Clin.Chim.Acta, 128 (1983) 103-111.

Spettoli, P., Nuti, M. P. and Zamorani, A., Appl.Environ.Microbiol., 48 (1984) 900-901.

Spivak, J. L., Small, D. and Hollenberg, M. D., Proc.Nat.Acad.Sci. U.S., 74 (1977) 4633-4635.

Spolarics, Z. and Bond, J. S., Arch.Biochem.Biophys., 260 (1988) 469-479.

Sportsman, J. R. and Wilson, G. S., Anal.Chem., 52 (1980) 2013-2018.

Springer, T. A., J.Biol.Chem., 256 (1981) 3833-3839.

Spurll, G. M. and Owen, F. L., J.Immunol.Methods, 39 (1980) 335-341.

Srivastava, P. N. and Farooqui, A. A., Biochem.J., 183 (1979) 531-537.

Staehelin, T., Hobbs, D. S., Kung, H. F., Lai, C. Y. and Pestka, S., J.Biol.Chem., 256 (1981) 9750-9754.

Stalla, G. K., Krassnigg, F. and Muller, O. A., Fresenius Z.Anal.Chem., 311 (1982) 458-459.

Stanley, C., Lew, A. M. and Steward, M. W., J.Immunol.Methods, 64 (1983) 119-132.

Staples, M. A. and Houston, L. L., Biochim.Biophys.Acta, 613 (1980) 210-219.

Stapleton, S. R. and Porter, J. W., Biochem.J., 226 (1985) 653-659.

Stark, B. C. and Chooi, W. Y., J.Chromatogr., 319 (1985) 367-375.

Staroscik, K., Wasniowska, K., Syper, D. and Lisowska, E., Arch.Immunol.Ther.Exp., 31 (1983) 127-133.

Stauber, G. B., Aiyer, R. A. and Aggarwal, B. B., J.Biol.Chem., 263 (1988) 19098-19104.

Staudenbauer, W. L. and Orr, E., Nucl.Acid Res., 9 (1981) 3589-3603.

Steele, J. K., Stammers, A. T. and Levy, J. G., J.Immunol., 135 (1985) 1201-1206.

Stein, B. S. and Sussman, H. H., J.Biol.Chem., 258 (1983) 2668-2673.

Stella, A. M. and del Batlle, A. M. C., Int.J.Biochem., 8 (1977) 353-358.

Stemberger, A. and Hormann, H., Hoppe-Seyler's Z.Physiol.Chem., 356 (1975) 341-348.

Stenman, U. H., Sutinen, M. L., Selander, R. K., Tontti, K. and Schroder, J., J.Immunol.Methods, 46 (1981) 337-345.

Stepanov, V. M., Rudenskaya, G. N., Yanonis, V. V., Ostoslavskaya, V. I., Gonchar, M. V., Kotlova, E. K. and Strongin, A. Y., Bioorg.Khim., 4 (1978) 1256-1263.

Stephenson, J. R., Lee, J. M. and Wilton-Smith, P. D., Anal.Biochem., 142 (1984) 189-195.

Steup, M., Biochim.Biophys.Acta, 659 (1981) 123-131.

Steven, F. S., Griffin, M. M., Wong, T. L. H. and Itzhaki, S., J.Enzyme Inhibition, 1 (1986) 127-137.

Stevens, A. and Augusteyn, R. C., Exp. Eye Res., 46 (1988) 499-505.

Stewart, K.K. and Doherty, R.F., Proc. Nat.Acad. Sci. U.S., 70 (1973) 2850-2852.

Stiller, J. M. and Nielsen, K. H., J.Clin.Microbiol., 17 (1983) 323-326.

Sting, R., Schmitt, M. and Blobel, H., J.Chromatogr., 497 (1989) 258-262.

Stolar, M. W., Baumann, G., Vance, M. L. and Thorner, M. O., J.Clin.Endocrinol.Metab., 59 (1984) 235-239.

Strader, C. D., Lazarides, E. and Raftery, M. A., Biochem.Biophys.Res.Commun., 92 (1980) 365-373.

Strand, M., McMillan, A. and Pan, X. Q., Exp.Parasitol., 54 (1982) 145-156.

Strasberg, P. M., Lowden, J. A. and Mahuran, D., Can.J.Biochem., 60 (1982) 1025-1031.

Strittmatter, S. M., Thiele, E. A., Kapiloff, M. S. and Snyder, S. H., J.Biol.Chem., 260 (1985) 9825-9832.

Strohsacker, M. W., Minnich, M. D., Clark, M. A., Shorr, R. G. L. and Crooke, S. T., J.Chromatogr., 435 (1988) 185-192.

Štrop, P., Borvák, J., Kašička, V., Prusík, Z. and Morávek, L., J.Chromatogr., 214 (1981) 317-325.

Stults, L. W., Moshiri, F. and Maier, R. J., J.Bacteriol., 166 (1986) 795-800.

Subbaramaiah, K. and Sharma, R., J.Chromatogr., 408 (1987) 385-387.

Subramanian, V., Liu, T. N., Yeh, W. K. and Gibson, D. T., Biochem.Biophys.Res.Commun., 91 (1979) 1131-1139.

Subramanian, M., Koppikar, S. V. and Fatterpaker, P., Indian J.Biochem.Biophys., 15 (1978) 214-218.

Suda, H., Yamamoto, K., Aoyagi, T. and Umezawa, M., Biochim.Biophys.Acta, 616 (1980) 60-67.

Suenaga, R., Evans, M., Hatfield, M. and Abdou, N. I., J.Immunol.Methods, 93 (1986) 131-140.

Sugiura, M., Kawasaki, T. and Yamashina, I., J.Biol.Chem., 257 (1982) 9501-9507.

Sugiyama, T. and Yamano, T., FEBS Lett., 52 (1975) 145-148.

Sugumaran, M. and Vaidyanethan, C. S., FEMS Microbiol.Lett., 4 (1978) 343-347.

Sul, H. S., Wise, L. S., Brown, M. L. and Rubin, C. S., J.Biol.Chem., 259 (1984) 1201-1205.

Suleiman, S. A. and Stevens, J. B., Arch.Biochem.Biophys., 258 (1987) 219-225.

Sullivan, R. and Klagsbrun, M., J.Biol.Chem., 260 (1985) 2399-2403.

Summaria, L. and Robbins, K. C., J.Biol.Chem., 251 (1976) 5810-5813.

Sundaram, P. V., Ann.N.Y.Acad.Sci., 413 (1983) 297-299.

Sundberg, B., Sandberg, G. and Crozier, A., Phytochemistry, 25 (1986) 295-298.

Sundberg, L. and Hoglund, S., FEBS Lett., 37 (1973) 70-73.

Suri, B. S., Targ, M. E. and Robinson, D. S., Atherosclerosis, 53 (1984) 195-205.

Suryanarayana, T., Biochem.Int., 7 (1983) 719-725.

Susz, J. P. and Dawson, G., J.Neurochem., 32 (1979) 1009-1013.

Suzuki, Y., Takii, Y. and Taguchi, H., Eur.J.Appl.Microbiol.Biotech., 18 (1983) 254-256.

Suzuki, F., Nakamura, Y., Nagata, Y., Ohsawa, T. and Murakami, K., J.Biochem. Tokyo, 89 (1981) 1107-1112.

Svanas, G. W. and Weiner, H., Anal.Biochem., 124 (1982) 314-319.

Svenson, A. and Hynning, P. A., Prep.Biochem., 11 (1981) 99-108.

Svensson, B., Danielsen, M., Staun, M., Jeppesen, L., Noren, O. and Sjostrom, H., Eur.J.Biochem., 90 (1978) 489-498.

Swaisgood, H. E. and Chaiken, I. M., J. Chromatogr., 327 (1985) 193-204.

Swamy, G. S. and Pillay, D. T. N., Z.Pflanzenphysiol., 93 (1979) 403-410.

Swartz, J. R., Pace, G. W., Solomon, B. A., Colton, C. K. and Archer, M. C., Prep.Biochem., 8 (1978) 479-502.

Sweet, C., Stephen, J. and Smith, H., Immunochemistry, 11 (1974) 295-304.

Swierczynski, J., Biochim.Biophys.Acta, 616 (1980) 10-21.

Swoveland, P. T., J.Virol.Meth., 13 (1986) 333-341.

Sygusch, J., Lehoux, L. and Beaudry, D., Biochem.Biophys.Res.Commun., 123 (1984) 1069-1075.

Syu, W. J., Wu, S. H. and Wang, K. T., J.Chromatogr., 262 (1983) 346-351.

Szamel, M., Goppelt-Strube, M., Bessler, W., Wiesmueller, K. H. and Resch, K., Biochim.Biophys.Acta, 899 (1987) 247-257.

Szewczyk, B., Kur, J. and Taylor, A., FEBS Lett., 139 (1982) 97-100.

Tabary, F., Font, J. and Bourrillon, R., Arch.Biochem.Biophys., 259 (1987) 79-88.

Taguchi, J. I. and Kuriyama, K., Brain Res., 323 (1984) 219-226.

Tahir, S. and Hider, R. C., Anal.Biochem., 135 (1983) 332-334.

Taira, H., Mizutani, S., Kurauchi, O., Narita, O. and Tomoda, Y., J.Chromatogr., 339 (1985) 339-403.

Takada, Y., Hiwada, K. and Kokubu, T., J.Chromatogr., 421 (1987) 350-354.

Takagahara, I., Suzuki, Y., Fujita, T., Yamauti, J., Fujii, K., Yamashita, J. and Horio, T., J.Biochem. Tokyo, 83 (1978) 585-597.

Takahara, H., Okamoto, H. and Sugawara, K., J.Biochem. Tokyo, 99 (1986) 1417-1424.

Takahashi, N., Takahashi, Y., Heiny, M. E. and Putnam, F. W., J.Chromatogr., 326 (1985) 373-385.

Takahashi, K., Tavassoli, M. and Jacobsen, D. W., Nature, 288 (1980) 713-715.

Takahashi, T., Sugahara, T. and Ohsaka, A., Biochim.Biophys.Acta, 351 (1974) 155-171.

Takeuchi, T., Clin.Chem., 25 (1979) 1406-1410.

Taki, T., Abe, M. and Matsumoto, M., J.Biochem. Tokyo, 91 (1982) 1813-1816.

Taki, T., Hirabayashi, Y., Takagi, K., Kamada, R., Kojima, K. and Matsumoto, M., J.Biochem. Tokyo, 89 (1981) 503-510.

Tamura, G. S., Dailey, M. O., Gallatin, W. M., McGrath, M. S., Weissman, I. L. and Pillemer, E. A., Anal.Biochem., 136 (1984) 458-464.

Tanaka, H., Sasaki, I., Yamashita, K., Miyazaki, K., Matuo, Y., Yamashita, J. and Horio, T., J.Biochem. Tokyo, 88 (1980) 797-806.

Tanaka, Y. and Uritani, I., J.Biochem. Tokyo, 81 (1977) 963-970.

Taniguchi, N., Yokosawa, N., Ono, M., Kinoshita, K. and Makita, A., J.Chromatogr., 339 (1985) 394-398.

Tanimoto, K., Nishimoto, T., Saitoh, F. and Yamaguchi, H., J.Biochem. Tokyo, 99 (1986) 601-604.

Tappeser, B., Wellnitz, D. and Klambt, D., Z.Pflanzenphysiol., 101 (1981) 295-302.

Tarkowski, A., Czerkinsky, C., Nilsson, L. A., Nygren, H. and Ouchterlony, O., J.Immunol.Methods, 72 (1984) 451-459.

Tarng, S. F., Huang, S. Y. and Perez, J. C., Toxicom, 24 (1986) 567-573.

Tarnowski, S. J. and Liptak, R. A., in A.Mizrahi and A.L. van Wezel (Editors), Advances in Biotechnical Processes,Vol.2, Alan R.Liss, Inc., New York, 1983, pp.271-287., (1983).

Taylor, R. F., in R.F. Taylor (Editor), Protein Immobilization, Fundamentals and Applications, Marcel Dekker, New York, 1991, pp. 139-160.

Taylor, R. F., Anal.Chim.Acta, 172 (1985) 241-248.

Taylor, J. L., Allmond, W. K. and Himel, C. M., J.Chromatogr., 257 (1983) 275-284.

Taylor, S. S., Lee, C. Y., Swain, L. and Stafford, P. H., Anal.Biochem., 76 (1976) 45-52.

Tayot, J. L., Holmgren, J., Svennerholm, L., Lindblad, M. and Tardy, M., Eur.J.Biochem., 113 (1981) 249-258.

Teisner, B., Davey, M. W. and Grudzinskas, J. G., Clin.Chim.Acta, 127 (1983) 413-417.

Tengblad, A., Biochem.J., 199 (1981) 297-305.

Tengblad, A., Biochim.Biophys.Acta, 578 (1979) 281-289.

Tercero, J. C. and Diaz-Maurino, T., Anal.Biochem., 174 (1988) 128-136.

Ternynck, T. and Avrameas, S., FEBS Lett., 23 (1972) 24-28.

Then, R. L., Anal.Biochem., 100 (1979) 122-128.

Thomae, R., Beck, S. and Pohl, F. M., Proc.Nat.Acad.Sci. U.S., 80 (1983) 5550-5553.

Thomas, T. E. and Lansdorp, P. M., J.Immunol.Metods, 112 (1988) 219-226.

Thomas, H. G. and Richmond, A., Arch.Biochem.Biophys., 260 (1988) 719-724.

Thomas, K. A. and Schechter, A. N., Anal.Biochem., 91 (1978) 209-223.

Thornburg, W., Kieras, R. and Lindell, T. J., Prep.Biochem., 8 (1978) 133-146.

Thorne, K. J. I. and Franks, D., Clin.Exp.Immunol., 56 (1984) 464-472.

Thorpe, R., Spitz, L., Spitz, M. and Austin, B. M., FEBS Lett., 151 (1983) 105-110.

Tigyi, G. J., Balazs, L., Monostori, E. and Ando, I., Mol.Immunol., 21 (1984) 889-894.

Tischer, A. and Gerike, E., Acta Virol., 30 (1986) 373-380.

Titanji, V. P. K. and Pahlman, S., Biochim.Biophys.Acta, 525 (1978) 380-391.

Titovets, E. P., Biokhimiya, 47 (1982) 276-283.

Tláskalová-Hogenová, H., Větvička, V., Pospíšil, M., Fornůsek, L., Prokešová, L., Čoupek, J., Frydrychová, A., Kopeček, J., Fiebig, H., Brochier, J. and Mančal, P., J.Chromatogr., 376 (1986) 401-408.

Tláskalová, H., Tučková, L., Křivaková, M., Rejnek, J. and Čoupek, J., Immunochemistry, 12 (1975) 801-805.

Tobe, H., Kojima, F., Aoyagi, T. and Umezawa, U., Biochim.Biophys.Acta, 613 (1980) 459-468.

Tokuda, M., Kamei, M., Yui, S. and Koyama, F., J.Chromatogr., 323 (1985) 434-438.

Toll, J. B. C., Wikberg, J. E. S. and Andersson, R. G. G., Allergy, 36 (1981) 411-417.

Tollefsen, S. E., Thompson, K. and Petersen, D. J., J.Biol.Chem., 262 (1987) 16461-16469.

Tomono, T., Igarashi, S. and Sawada, E., Thromb.Res., 35 (1984) 467-473.

Toraya, T. and Fukui, S., J.Biol.Chem., 255 (1980) 3520-3525.

Torreilles, J., Guerin, M. C. and Descomps, B., Biochimie, 68 (1986) 565-568.

Torres, J. L., Guzman, R., Carbonell, R. G. and Kilpatrick, P. K., Anal.Biochem., 171 (1988) 411-418.

Torres, B. V. and Smith, D. F., Anal.Biochem., 170 (1988) 209-219.

Tosa, T., Sato, T., Sano, R., Yamamoto, K., Matuo, Y. and Chibata, I., Biochim.Biophys.Acta, 334 (1974) 1-11.

Totani, K., Harumiya, S., Nanjo, F. and Usui, T., Agr.Biol.Chem. Tokyo, 47 (1983) 1159-1162.

Touraille, S., Briand, Y., Druand, R., Bonnafous, J. C. and Mani, J. C., FEBS Lett., 128 (1981) 142-144.

Toyoda, Y., Miwa, I. and Okuda, J., Chem.Pharm.Bull. Tokyo, 30 (1982) 2880-2884.

Traas, D. W., Hoegee-de Nobel, B. and Nieuwenhuizen, W., Thromb.Haemost., 52 (1984) 347-349.

Trayer, I. P., Trayer, H. R., Small, D. A. P. and Bottomley, R. C., Biochem.J., 139 (1974) 609-623.

Trevillyan, J. M. and Pall, M. L., J.Biol.Chem., 257 (1982) 3978-3986.

Trommer, W. E. and Becker, G., Biochim.Biophys.Acta, 422 (1976) 1-7.

Tsai, T. S., Anal.Biochem., 151 (1985) 137-141.

Tsang Lee, M. Y. W. and Whyte, W. A., Anal.Biochem., 138 (1984) 291-297.

Tsao, D. and Kim, Y. S., J.Biol.Chem., 253 (1978) 2271-2278.

Tseng, Y. M., Harris, B. G. and Jacobson, M. K., Biochim.Biophys.Acta, 568 (1979) 205-214.

Tsuge, H., Nakano, Y., Onishi, H., Futamura, Y. and Ohashi, K., Biochim.Biophys.Acta, 614 (1980) 274-284.

Tsutsui, K., Methods Enzymol., 123 (1986) 331-338.

Tsutsui, K. and Mueller, G. C., Anal.Biochem., 121 (1982) 244-250.

Tucker, R. F., Babul, J. and Stellwagen, E., J.Biol.Chem., 256 (1981) 10993-10998.

Turini, P., Sabatini, S., Befani, O., Chimenti, F., Casanova, C., Riccio, P. L. and Mondovi, B., Anal.Biochem., 125 (1982) 294-298.

Turková, J., Beneš, M. J., Kuhn, M., Stepanov, V. M. and Lyublinskaya, L. A., in J.Chaloupka and V.Krumphanzl (Editors),Extracellular Enzymes of Microorganisms, Plenum Press, New York, London, 1987, pp.99-103.

Turková, J., Fusek, M., Maksimov, J. J. and Alakhov, Y. B., J.Chromatogr., 376 (1986) 315-321.

Turkova, J., Blaha, K. and Adamova, K., J.Chromatogr., 236 (1982) 375-383.

Turková, J., Bláha, K., Horáček, J., Vajčner, J., Frydrychová, A. and Čoupek, J., J.Chromatogr., 215 (1981) 165-179.

Turková, J., Affinity Chromatography, Elsevier, Amsterdam, 1978, pp. 319-322.

Turková, J. and Seifertová, A., J.Chromatogr., 148 (1978) 293-297.

Turková, J., Bláha, K., Valentová, O., Čoupek, J. and Seifertová, A., Biochim.Biophys.Acta, 427 (1976a) 586-593.

Turková, J., Valentová, O. and Čoupek, J., Biochim.Biophys.Acta, 420 (1976b) 309-315.

Turková, J., Vavreinová, S., Křivaková, M. and Čoupek, J., Biochim.Biophys.Acta, 386 (1975) 503-508.

Turner, A. J. and Hryszko, J., Biochim.Biophys.Acta, 613 (1980) 256-265.

Turpeinen, U., Syvanen, A. C. and Hamberg, U., FEBS Lett., 131 (1981) 137-142.

Twining, S. S. and Atassi, M. Z., J.Immunol.Methods, 30 (1979) 139-151.

Uchida, T. and Shibata, Y., J.Biochem. Tokyo, 90 (1981) 463-471.

Uchida, K., Niimobe, M., Kato H, ;. S., Biochim.Biophys.Acta, 614 (1980) 501-510.

Uchida, T. and Nagai, Y., J.Biochem. Tokyo, 87 (1980) 1829-1841.

Ueda, N., Kaneko, S., Yoshimoto, T. and Yamamoto, S., J.Biol.Chem., 261 (1986) 7982-7988.

Ugelstad, J., Berge, A., Ellingsen, T., Aune, O., Kilaas, L., Nilsen, T. N., Schmid, R., Stenstad, P., Funderud, S., Kvalheim, G., Nustad, K., Lea, T., Vartdal, F. and Danielsen, H., Makromol. Chem., Macromol. Symp., 17 (1988) 177-211.

Uhlen, M., Nilsson, B., Guss, B., Lindberg, M., Gatenbeck, S. and Philipson, L., Gene, 23 (1983) 369-373.

Ulbrich, N., Wool, I. G., Ackerman, E. and Sigler, P. B., J.Biol.Chem., 255 (1980) 7010-7016.

Ullah, A. H. J. and Gibson, D. M., Arch.Biochem.Biophys., 260 (1988) 514-520.

Ullmann, A., Gene, 29 (1984) 27-31.

Ulloa, R. M., Rubinstein, C. P., Molina y Vedia, L., Torres, H. N. and Tellez-Inon, M. T., FEBS Lett., 241 (1988) 219-222.

Umekawa, H., Tanaka, T., Kimura, Y. and Hidaka, H., Biochemical Pharmacol., 33 (1984) 3339-3344.

Uotila, L. and Koivusalo, M., Arch.Biochem.Biophys., 196 (1979) 33-45.

Updyke, T. V. and Nicolson, G. L., J.Immunol.Methods, 73 (1984) 83-95.

Usov, A. I. and Miroshnikova, L. I., Carbohyd.Res., 43 (1975) 204-207.

Ussuf, K. K. and Nair, P. M., Ind.J.Biochem.Biophys., 17 (1980) 335-337.

Usui, T., Hosokawa, S., Mizuno, T., Suzuki, T. and Meguro, H., J.Biochem. Tokyo, 89 (1981a) 1029-1037.

Usui, T., Iwasaki, Y. and Mizuno, T., Carbohyd.Res., 92 (1981b) 103-114.

Vacek, A. T., Bourque, D. P. and Hewlett, N. G., Anal.Biochem., 124 (1982) 414-420.

Vallette, F. M., Marsh, D. J., Muller, F., Massoulie, J., Marcot, B. and Viel, C., J.Chromatogr., 257 (1983) 285-296.

Valoti, M., Della Corte, L., Tipton, K. F. and Sgaragli, G., Biochem.J., 250 (1988) 501-507.

Van Den Burg, B., Eijsink, V. G. H., Stulp, B. K. and Venema, G., J.Biochem.Biophys.Meth., 18 (1989) 209-220.

Van der Zee, R. and Welling, G. W., J.Chromatogr., 327 (1985) 377-380.

Van der Wall, J., Campbell, P. A. and Abel, C. A., Develop.Comp.Immunol., 5 (1981) 679-684.

Van Etten, R. L. and Waheed, A., Arch.Biochem.Biophys., 202 (1980) 366-373.

Van Overveld, F. J., Terpstra, G. K., Bruijnzeel, P. L. B., Raaijmakers, J. A. M. and Kreukniet, J., Scand.J.Immunol., 27 (1988) 1-6.

Van Tilbeurgh, H., Bhikhabhai, R., Pettersson, L. G. and Claeyssens, M., FEBS Lett., 169 (1984) 215-218.

Van Tunen, A. J. and Mol, J. N. M., Arch.Biochem.Biophys., 257 (1987) 85-91.

Van Zyl, J., Kriegler, A., Koch, H. and van der Walt, B., S.Afr.J.Sci., 84 (1988) 807-810.

Van't Riet, J., Zegers, H. C. and Abraham, P. R., FEMS Microbiol. Lett., 12 (1981) 223-228.

Vaněčková, J., Barthová, J., Barth, T., Krejčí, I. and Rychlík, I., Collect.Czech.Chem.Commun., 40 (1975) 1461-1465.

Vansteelandt, R., Beeckmans, S. and Kanarek, L., Arch.Int.Physiol.Biochim., 89 (1981) B36.

Varady, L., Kalghatgi, K. and Horvath, C., J. Chromatogr., 458 (1988) 207-215.

Varandani, P. T. and Shroyer, L. A., Arch.Biochem.Biophys., 181 (1977) 82-93.

Vasstrand, E. N. and Jensen, H. B., Scand.J.Dent.Res., 88 (1980) 219-228.

Venkatesan, S., Gershowitz, A. and Moss, B., J.Biol.Chem., 255 (1980) 2829-2834.

Verma, A. K., Penniston, J. T., Muallem, S. and Lew, V., J.Bioenerg.Biomembrane, 16 (1984) 365-378.

Vernooy-Gerritsen, M., Bos, A. L. M., Veldink, G. A. and Vliegenthart, J. F. G., Biochim.Biophys.Acta, 748 (1983) 148-152.

Vernooy-Gerritsen, M., Veldink, G. A. and Vliegenthart, J. F. G., Biochim.Biophys.Acta, 708 (1982) 330-334.

Veselský, L., Jonáková, V. and Čechová, D., J.Chromatogr., 376 (1986) 395-400.

Vidal, J., Godbillon, G. and Gadal, P., Phytochemistry, 21 (1982) 2829-2830.

Vidal, P., Nielsen, E. and Welinder, B. S., J. Chromatogr., 573 (1992) 201-206.

Vihko, P., Kontturi, M. and Korhonen, L. K., Clin.Chem., 24 (1978) 466-470.

Viktorova, L. N., Klyashchitskii, B. A. and Ramensky, E., FEBS Lett., 91 (1978) 194-197.

Villiers, C. L., Duplaa, A. M., Arlaud, G. J. and Colomb, M. G., Biochim.Biophys.Acta, 700 (1982) 118-126.

Villiers, M. B., Reboul, A., Thielens, N. M. and Colomb, M. G., FEBS Lett., 132 (1981) 49-54.

Vincent, W. S. and Goldstein, E. S., Anal.Biochem., 110 (1981) 123-127.

Vioque, A. and Altman, S., Proc.Nat.Acad.Sci. U.S., 83 (1986) 5904-5908.

Virella, G., Kilpatrick, J. M., Chenais, F. and Fudenberg, H. H., Methods Enzymol., 74 (1981) 644-663.

Viscarello, B. R., Stein, R. L., Kusner, E. J., Holsclaw, D. and Krell, R. D., Prep.Biochem., 13 (1983) 57-67.

Visser, J., van Dongen, W. and Straiting, M., FEBS Lett., 85 (1978) 81-85.

Visser, J. and Strating, M., Biochim.Biophys.Acta, 384 (1975) 69-80.

Vockley, J. and Harris, H., Biochem.J., 217 (1984) 535-541.

Vold, B. S., Longmire, M. E. and Keith, D. E. J., J.Bacteriol., 148 (1981) 869-876.

Vold, B. S., Lazar, J. M. and Gray, A. M., J.Biol.Chem., 254 (1979) 7362-7367.

Von Clausbruch, U. C. and Tschesche, H., Biol.Chem.Hoppe-Seyler, 369 (1988) 683-691.

Von Der Mark, K. and Risse, G., Methods Enzymol., 144 (1987) 490-507.

Von Dobeln, U., Biochemistry, 16 (1977) 4368-4371.

Voordouw, G., Van der Vies, S. M., Eweg, J. K., Veeger, C., Van Breeman, J. F. L. and Van Bruggen, E. F. J., Eur.J.Biochem, 111 (1980) 347-355.

Voynow, J. A., Scanlin, T. F. and Glick, M. C., Anal.Biochem., 168 (1988) 367-373.

Wachter, E. and Hochstrasser, K., Hoppe-Seyler's Z.Physiol.Chem., 362 (1981) 1351-1355.

Wadano, A., Hobus, P. A. and Liao, T. H., Biochemistry, 18 (1979) 4124-4130.

Wagener, C., Clark, B. R., Rickard, K. J. and Shively, J. E., J.Immunol., 130 (1983a) 2302-2307.

Wagener, C., Yang, Y. H. J., Crawford, F. G. and Shively, J. E., J.Immunol., 130 (1983b) 2308-2315.

Wagener, C., Schwonzen, M. and Breuer, H., J.Clin.Chem.Clin.Biochem., 20 (1982) 787-789.

Wagh, P. V., J.Chromatogr., 152 (1978) 565-568.

Waheed, A. and Shadduck, R. K., Exp.Hematol., 17 (1989) 61-65.

Wahl, A. F., Kowalski, S. P., Harwell, L. W., Lord, E. M. and Bambara, R. A., Biochemistry, 23 (1984) 1895-1899.

Waibel, R., Amado, R. and Neukom, H., J.Chromatogr., 197 (1980) 86-91.

Wallace, R. W., Lynch, T. J., Tallant, A. E. and Cheung, W. Y., J.Biol.Chem., 254 (1979) 377-382.

Walsh, K. A., Burstein, Y. and Pangburn, M. K., Methods Enzymol., 34 (1974) 435-440.

Walter, G., Hutchinson, M. A., Hunter, T. and Eckhart, W., Proc.Nat.Acad.Sci. U.S., 78 (1981) 4882-4886.

Walters, R. R., J.Chromatogr., 249 (1982) 19-28.

Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S. and Gill, G. N., Anal.Biochem., 161 (1987) 425-437.

Walzel, H., Ringel, B., Jenssen, H. L., Mix, E., Franz, H. and Ziska, P., Arch.Geschwulstforsch., 54 (1984) 451-461.

Wandzilak, T. M. and Benson, R. W., Biochemistry, 17 (1978) 426-431.

Wang, C. S., Anal.Biochem., 105 (1980) 398-402.

Wang, W. C., Clark, G. F., Smith, D. F. and Cummings, R. D., Anal.Biochem., 175 (1988) 390-396.

Ware, C. F., Wetsel, R. A. and Kolb, W. P., Mol.Immunol., 18 (1981) 521-531.

Waseem, A. and Salahuddin, A., Indian J.Biochem.Biophys., 20 (1983) 253-258.

Watanabe, Y., Adachi, T., Ito, Y., Hirano, K. and Sugiura, M., Chem.Pharm.Bull.Tokyo, 30 (1982) 3284-3287.

Watanabe, H., Pollett, M. and Mackenzie, J. S., Aust.J.Exp.Biol.Med.Sci., 57 (1979) 303-312.

Watanabe, T. and Suga, T., Anal.Biochem., 86 (1978) 357-362.

Watanabe, T., Kondo, N., Fujii, T. and Noguchi, T., Plant Cell Physiol., 18 (1977) 387-392.

Watkins, N. G., Thorpe, S. R. and Baynes, J. W., J.Biol.Chem., 260 (1985) 10629-10636.

Webb, N. R., Chari, R. V. J., de Pillis, G., Kozarich, J. W. and Rhoads, R. E., Biochemistry, 23 (1984) 177-181.

Webb, R., Baxter, G., McBride, D., Nordblom, G. D. and Shaw, M. P. K., J.Steroid Biochem., 23 (1985) 1043-1051.

Webber, S., Harzer, G. and Whiteley, J. M., Anal.Biochem., 106 (1980) 63-72.

Webber, S. and Whiteley, J. M., Biochem.Biophys.Res.Commun., 97 (1980) 17-21.

Weber, D. V. and Bailon, P., J. Chromatogr., 510 (1990) 59-69.

Weber, D. V., Keeney, R. F., Familleti, P. C. and Bailon, P., J. Chromatogr., 431 (1988) 55-63.

Weber, J. L. and Cole, R. D., J.Biol.Chem., 257 (1982) 11774-11783.

Weber, W., Bertics, P. J. and Gill, G. N., J.Biol.Chem., 259 (1984) 14631-14636.

Weber, W., Vogel, C. W. and Hilz, H., FEBS Lett., 99 (1979) 62-66.

Webster, G. D. and Jackson, J. B., Biochim.Biophys.Acta, 503 (1978) 135-154.

Weigel, P. H., Schnaar, R. L., Kuhlenschmidt, M. S., Schmell, E., Lee, R. T., Lee, Y. C. and Roseman, S., J.Biol.Chem., 254 (1979) 10830-10838.

Weis, J. J., Richards, S. A., Smith, J. A. and Fearon, D. T., J.Immunol.Methods, 92 (1986) 79-87.

Weisgraber, K. H. and Mahley, R. W., J.Lipid Res., 21 (1980) 316-325.

Weisweiler, P., J.Chromatogr., 425 (1988) 169-177.

Welch, J. S. and Dobson, C., Trans.Roy.Soc.Trop.Med.Hyg., 75 (1981) 5-14.

Welty, F. K. and Wood, H. G., J.Biol.Chem., 253 (1978) 5832-5838.

Wen, L. and Reeck, G. R., J.Chromatogr., 314 (1984) 436-444.

Weselake, R. J. and Hill, R. D., Carbohyd.Res., 108 (1982) 153-161.

West, T. P., Maldonado, I. and Kantrowitz, E. R., Biochim.Biophys.Acta, 839 (1985) 32-39.

Westcott, K. R., La Porte, D. C. and Storm, D. R., Proc.Nat.Acad.Sci. U.S., 76 (1979) 204-208.

Weston, P. A., Levy, N. L. and Koren, H. S., J.Immunol., 125 (1980) 1387-1394.

Whitaker, J. N. and Seyer, J. M., J.Neurochem., 32 (1979) 325-333.

Whiting, P. and Lindstrom, J., J.Neurosci., 6 (1986) 3061-3069.

Whiting, P. J. and Lindstrom, J. M., Biochemistry, 25 (1986) 2082-2093.

Wichman, A., Biochem.J., 181 (1979) 691-698.

Wielckens, K., Bredehors, R., Adamietz, P. and Hilz, H., Eur.J.Biochem., 117 (1981) 69-74.

Wightman, B. C., Weltman, E. A. and Culp, L. A., Biochem.J., 235 (1986) 469-479.

Wiginton, D. A., Coleman, M. S. and Hutton, J. J., J.Biol.Chem., 255 (1980) 6663-6669.

Wikstrom, P. and Larsson, P. O., J. Chromatogr., 388 (1987) 123-134.

Wilimowska-Pelc, A., Wieczorek, M., Otlewski, J., Leluk, J. and Wilusz, T., J.Chromatogr., 269 (1983) 22-27.

Wilk, H. E., Kecskemethy, N. and Schafer, K. P., Nucl.Acid.Res., 10 (1982) 7621-7633.

Wilkinson, M. L., Iqbal, M. J., Forbes, A., Corbishley, T. P. and Williams, R., Biochem.J., 240 (1986) 75-79.

Willey, D. G., Rosenthal, M. A. and Caldwell, S., Diabetologia, 27 (1984) 56-58.

Williams, G. T., Johnstone, A. P. and Dean, P. D. G., Biochem.J., 205 (1982) 167-171.

Williams, D. C., Morgan, G. S., Mc Donald, E. and Battersby, A. R., Biochem.J., 193 (1981) 301-310.

Williams, J. W. and Northrop, D. B., Biochemistry, 15 (1976) 125-131.

Williamson, J. H., Krochko, D. and Bentley, M. M., Comp.Biochem.Physiol. PT, B65 (1980) 339-343.

Wilson, B. S., Phan, S. H. and Lloyd, R. V., Regul.Peptides, 13 (1986) 207-223.

Wiman, B., Biochem.J., 191 (1980) 229-232.

Winstanley, M. A., Small, D. A. P. and Trayer, I. P., Eur.J.Biochem., 98 (1979) 441-446.

Wintzerith, M., Dietrich, A. and Mandel, P., Biochim.Biophys.Acta, 613 (1980) 191-202.

Wittig, B. and Wittig, S., Biochim.Biophys.Acta, 520 (1978) 598-611.

Wolk, M., Svennerholm, A. M. and Holmgren, J., Current Microbiology, 3 (1980) 339-344.

Wolkoff, A. W., Bhargava, M. M., Chung, C. and Gatmaitan, Z., Proc.Soc.Exp.Biol.Med., 160 (1979) 150-153.

Wong, C. H., Ho, C. L. and Wang, K. T., J.Chromatogr., 154 (1978) 25-32.

Wong, J. Y. and Switzer, R. L., Arch.Biochem.Biophys., 196 (1979) 134-137.

Wong, S. W., Paborsky, L. R., Fisher, P. A., Wang, T. S. F. and Korn, D., J.Biol.Chem., 261 (1986) 7958-7968.

Wong, W. W., Jack, R. M., Smith, J. A., Kennedy, C. A. and Fearon, D. T., J.Immunol.Methods, 82 (1985) 303-313.

Wood, C. L., Caron, M. G. and Lefkowitz, R. J., Biochem.Biophys.Res.Commun., 88 (1979) 1-8.

Wood, E., Hounsell, E. F. and Teizi, T., Carbohyd.Res., 90 (1981) 269-282.

Wood, M. R. G. and Johnson, P., Biochim.Biophys.Acta, 662 (1981) 138-144.

Wood, T. G. and Lingrel, J. B., J.Biol.Chem., 252 (1977) 457-463.

Wood, W. I., Peterson, D. O. and Bloch, K., J.Biol.Chem., 253 (1978) 2650-2656.

Woodford, T. A., Schlegel, R. and Pardee, A. B., Anal.Biochem., 171 (1988) 166-172.

Woodward, J., Maquess, H. J. and Picker, C. S., Prep.Biochem., 16 (1986) 337-352.

Wormmeester, J., Stiekema, F. and De Groot, K., J.Immunol.Methods, 67 (1984) 389-394.

Worobec, E. A., Paranchych, W., Parker, J. M. R., Taneja, A. K. and Hodges, R. S., J.Biol.Chem., 260 (1985) 938-943.

Wouters, J. and Stockx, J., Arch.Int.Physiol.Biochim., 89 (1981) B211-B212.

Wright, I. P. and Sundaram, T. K., Biochem.J., 177 (1979) 441-448.

Wright, C. L., Warsy, A. S., Holroyde, M. J. and Trayer, I. P., Biochem.J., 175 (1978) 125-135.

Wroblewska, Z., Gilden, D., Green, M., Devlin, M. and Vafai, A., J.Gen.Virol., 66 (1985) 1795-1799.

Wu, J. M. and Eslami, B., Biochem.Int., 6 (1983) 207-216.

Wu, L. N. Y., Pennington, R. M., Everett, T. D. and Fischer, R. R., J.Biol.Chem., 257 (1982) 4052-4055.

Wu, S. J. and Martin, D. L., J.Neurochem., 42 (1984) 1607-1612.

Wun, T. C., Ossowski, L. and Reich, E., J.Biol.Chem., 257 (1982) 7262-7268.

Yagi, S., Izawa, K., Nakagawa, T., Tanaka, H., Yoshitake, A. and Mohri, Z. I., J.Chromatogr., 493 (1989) 27-33.

Yagi, S., Nakagawa, T., Tanaka, H., Yoshitake, A. and Mohri, R., J.Chromatogr., 400 (1987) 279-283.

Yagi, T., Yamamoto, T. and Soda, K., Biochim.Biophys.Acta, 614 (1980) 63-70.

Yamada, H., Fukumura, T., Ito, Y. and Imoto, T., Anal.Biochem., 146 (1985) 71-74.

Yamada, Y., Goto, H. and Ogasawara, N., Biochim.Biophys.Acta, 616 (1980) 199-207.

Yamaguchi, H., Liotta, A. S. and Krieger, D. T., J.Clin.Endocrinol.Metab., 51 (1980) 1002-1008.

Yamaguchi, T. and Matsumura, Y., Biochim.Biophys.Acta, 481 (1977) 706-711.

Yamamoto, K. and Sekine, T., J.Biochem.Tokyo, 101 (1987) 519-523.

Yamamoto, K. and Degroot, L. J., J.Biochem.Tokyo, 91 (1982) 775-782.

Yamamoto, K., Katsuda, N., Himeno, M. and Kato, K., Eur.J.Biochem., 95 (1979) 459-467.

Yamamoto, R., Hattori, S., Inukai, T., Matsuura, A., Yamashita, K., Kosaka, A. and Kato, K., Clin.Chem., 27 (1981) 1721-1723.

Yamasaki, N. and Eto, T., Agr.Biol.Chem.Tokyo, 45 (1981) 2939-2941.

Yamasaki, N., Tsujita, T., Eto, T., Masuda, S., Mizuno, K. and Sakiyama, F., J.Biochem. Tokyo, 86 (1979) 1291-1300.

Yamashita, K., Totani, K., Ohkura, T., Takasaki, S., Goldstein, I. J. and Kobata, A., J.Biol.Chem., 262 (1987) 1602-1607.

Yamashita, K., Hitoi, A., Irie, M. and Kobata, A., Arch.Biochem.Biophys., 250 (1986) 263-266.

Yamashita, K., Kochibe, N., Ohkura, T., Ueda, I. and Kobata, A., J.Biol.Chem., 260 (1985) 4688-4693.

Yang, B. H., Sundaram, P. V. and Maelicke, A., Biochem.J., 199 (1981) 317-322.

Yasmin, T. and Qadri, F., J.Chromatogr., 315 (1984) 425-429.

Yatohgo, T., Izumi, M., Kashiwagi, H. and Hayashi, M., Cell Struct.Funct., 13 (1988) 281-292.

Yavarkovskaya, L. L., Osipov, A. P. and Egorov, A. M., Bioorg.Khim., 5 (1979) 1564-1569.

Yazawa, S., Furukawa, K. and Kochibe, N., J.Biochem. Tokyo, 96 (1984) 1737-1742.

Yazawa, M. and Yagi, K., J.Biochem.Tokyo, 84 (1978) 1259-1265.

Yeung, A. T., Turner, K. J., Bascomb, N. F. and Schmidt, R. R., Anal.Biochem., 110 (1981) 216-228.

Yim, J. J. and Brown, G. M., J.Biol.Chem., 251 (1976) 5087-5094.

Ymer, S. I., Stevenson, J. L. and Herington, A. C., Biochem.J., 221 (1984) 617-622.

Yokosawa, H., Sawada, H., Abe, Y., Numakunai, T. and Ishii, S. I., Biochem.Biophys.Res.Commun., 107 (1982) 451-457.

Yokosawa, H., Holladay, L. A., Inagami, T., Haas, E. and Murakami, K., J.Biol.Chem., 255 (1980) 3498-3502.

Yon, R. J. and Simmonds, R. J., Biochem.J., 151 (1975) 281-290.

Yoneyama, Y., Arch.Biochem.Biophys., 255 (1987) 168-175.

Yonezawa, S., Tanaka, T. and Miyauchi, T., Arch.Biochem.Biophys., 256 (1987) 499-508.

Yoshida, J., Takamura, S. and Suzuki, S., J.Antibiot., 42 (1989) 448-453.

Yoshida, H., Fukuda, I. and Hashiguchi, M., J.Biochem.Tokyo, 88 (1980b) 1813-1818.

Yoshida, S., Morita, T. and Saneyoshi, M., Biochim.Biophys.Acta, 607 (1980a) 527-529.

Yoshima, H., Takasaki, S., Ito-Mega, S. and Kobata, A., Arch.Biochem.Biophys., 194 (1979) 394-398.

You, K. and Roe, C. R., Anal.Biochem., 114 (1981) 177-185.

Yu, K. T., Peters, M. A. and Czech, M. P., J.Biol.Chem., 261 (1986a) 11341-11349.

Yu, S. H., Lau, P. P. L. and Spring, T. G., Biochim.Biophys.Acta, 517 (1978) 31-42.

Yu, X. J., Fischer, A. M., Muller, D., Bros, A., Tapon-Bretaudiere, J. and Jozefonvicz, J., J.Chromatogr., 376 (1986b) 429-435. .

Zechel, K., Eur.J.Biochem., 110 (1980) 343-348.

Zecher, R. and Reske, K., Mol.Immunol., 19 (1982) 1037-1044.

Zenteno, E. and Ochoa, J. L., Phytochemistry, 27 (1988) 313-317.

Zhang, Z., Fournier, A. and Tan, Y. H., J.Biol.Chem., 261 (1986) 8017-8021.

Zhernosekov, D. D., Berezin, V. A. and Reva, A. D., Ukr.Biokhim.Zk.SSSR, 57 (1985) 9-13.

Zhu, R. X., Ching, W. M., Chung, H. K., Rhee, S. G. and Stadtman, T. C., Anal.Biochem., 161 (1987) 460-466.

Ziska, P. and Franz, H., Experientia, 35 (1979) 161.

Zopf, D., Schroer, K., Hansson, G., Dakour, J. and Lundblad, A., Methods Enzymol., 138 (1987) 307-313.

Zoppi, F., Perlangeli, V. and Faglia, E., Clin.Chem., 31 (1985) 1238-1239.

Zwilling, R., Jakob, F., Bauer, H., Neurath, H. and Enfield, D. L., Eur.J.Biochem., 94 (1979) 223-229.

Zykova, N. A., Nevinskii, G. A. and Lavrik, O. I., Mol.Biol.Engl.Tr., 16 (1982) 1165-1172.

This Page Intentionally Left Blank

Chapter 10

Immobilization of enzymes by biospecific adsorption to immobilized monoclonal or polyclonal antibodies

Biospecific adsorbents can be used not only for the purification or determination of biologically active compounds, but also for their oriented immobilization. Enzymes adsorbed to immunoadsorbents prepared with monoclonal or polyclonal antibodies may serve as examples.

10.1 IMMOBILIZATION OF ENZYMES BY BIOSPECIFIC ADSORPTION TO IMMOBILIZED MONOCLONAL ANTIBODIES

The cross-linkage of a specific complex for oriented immobilization has been discussed in Section 4.3.1. If the substance immobilized is to be used under milder conditions, then it is not necessary to use the covalent cross-linkage of the complex employed for oriented immobilization. The results reported by Solomon, Koppel and Katchalski-Katzir (1984) can be summarized by way of example. Fig.10.1. demonstrates two modes for the immobilization of carboxypeptidase A (CPA) by these authors. In both cases they simply used adsorption of CPA to the antibody against the enzyme for its immobilization. The affinity constant of this complex was of the order of $10^9\ M^{-1}$. The attachment of the antibody to the solid support was performed by two different procedures. First it was again immobilized by mere adsorption, i. e. by the adsorption of the antibody to Protein A which had been covalently attached to Sepharose. Second the antibody was covalently attached to Eupergit C. Table 10.1 lists the catalytic constants for both peptide and ester hydrolysis by the two types of immobilized carboxypeptidase A and by native carboxypeptidase A. In all three cases the catalytic constants are nearly identical. The enzymatic activity of immobilized carboxypeptidase did not change after repeated incubation with the substrate in solution. The stability of the enzyme increased after immobilization.

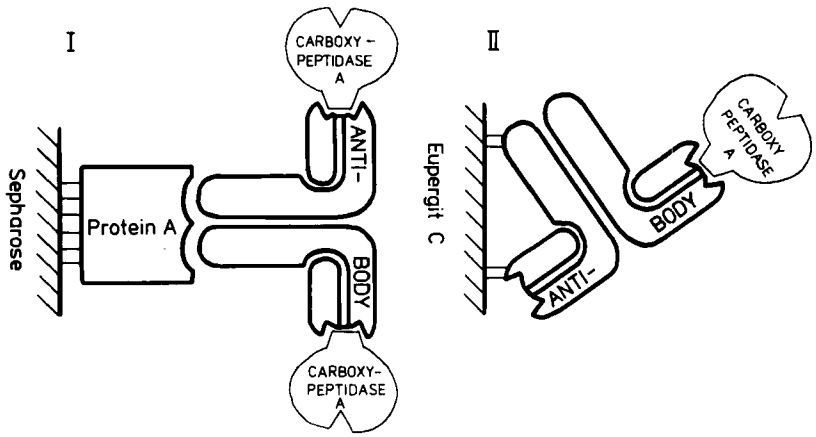


Fig. 10.1. Schematic drawing of the oriented immobilization of carboxypeptidase A by adsorption to monoclonal antibody against carboxypeptidase A (with affinity constant = $10^9 M^{-1}$) which is (I) adsorbed to Protein A-Sepharose; (II) attached to acrylic polymer Eupergit C. Covalent bonds are shown as full lines. Reproduced from J. Turková, et al., Makromol. Chem., Macromol. Symp., 17 (1988) 241-256.

The advantage of the attachment of antibodies through the carbohydrate moiety has already been discussed in Section 6.2.2. As the carbohydrate residues are mostly located on the Fc fragment of the antibody molecule remote from the binding site of the antibody, immobilization *via* this region does not usually impair the immunological activity of the antibody. Fleminger et al. (1990) used monoclonal antibodies against CPA and horse radish peroxidase (HRP) for oriented immobilization after periodate oxidation of their carbohydrate moieties on amino and hydrazide derivatives of Eupergit C. The methods described in their paper have been discussed in detail in Section 6.2.2. They immobilized monoclonal antibodies which did not inhibit the respective enzymatic activities. The oxidation and coupling reactions were optimized to achieve highly active matrix-conjugated antibodies. Thus, antibody-matrix conjugates were obtained that possessed antigen-binding activities close to the theoretical value of 2 mol antigen bound/mol immobilized antibody. A higher CPA binding activity was also achived by Hadas et al. (1990) by the direct immobilization of monoclonal antibody against CPA to epoxy-containing Eupergit C after the reversible protection of the free amino groups of monoclonal antibodies by dimethylmaleic anhydride. The activity of immobilized antibody against CPA after this treatment increased 3 - 10.7 fold.

Table 10.1. Kinetic constants for native and immobilized carboxypeptidase A (CPA)

Preparation	*Peptide hydrolysis*[a]			*Ester hydrolysis*[b]		
	Km (M) $\times 10^{-4}$	V_{max}[c] *(M/min)* $\times 10^{-5}$	*Ki (M)* $\times 10^{-5}$	*Km (M)* $\times 10^{-5}$	V_{max}[c] *(M/min)* $\times 10^{-4}$	*Ki (M)* $\times 10^{-5}$
CPA	2.6	5.5	7.0	8.8	2.0	2.0
S-(PA-Ab)-CPA	2.2	5.0	6.8	7.9	3.5	2.0
E-Ab-CPA	4.0	9.0	7.5	9.0	5.0	2.5

Sepharose-(Protein A-Antibody against CPA)-CPA = S-(PA-Ab)-CPA
Eupergit C-(Antibody against CPA)-CPA = E-Ab-CPA

[a] Using hippuryl-L-phenylalanine as substrate and phenylpropionic acid as inhibitor.

[b] Using hippuryl-DL-β-phenyl lactic acid as substrate and phenylpropionic acid as inhibitor.

[c] Calculated per mg free or bound enzyme.

Solomon et al. (1990), rather than using periodate oxidation of carbohydrate moieties of antibodies against CPA and HRP, used instead enzymatic oxidation of these antibodies by soluble or immobilized bifunctional enzyme complexes of neuraminidase and galactose oxidase. Sialic acid and galactose are terminal sugars of carbohydrate chains of immobilized antibodies. Concomitant treatment of carbohydrate moieties of the antibodies with neuraminidase and galactose oxidase generated aldehyde groups in the oligosaccharide moieties of immunoglobulins. These groups reacted selectively with hydrazide groups of Eupergit C with attached adipic dihydrazide. Neuraminidase and galactose oxidase are glycoproteins. Both enzymes, after periodate oxidation, were immobilized on Eupergit C-adipic dihydrazide. The methods used are described in detail in Section 6.2.2. The result of this immobilization was an efficient and selective system for the enzymatic oxidation of the monoclonal antibodies with no impairment of their immunological activity. Oriented immobilization of enzymatically oxidized antibodies on a hydrazide-containing carrier led to the formation of an antibody matrix.

Sepharose particles with immobilized higher plant NADH: nitrate reductase (NR,EC 1.6.6.1) were prepared by Ruoff et al. (1989) by the use of immobilized monoclonal antibody. To elucidate the question of the molecular basis of light-induced circadian

rhythms of NR activity they prepared an open system by adsorption of NR to immobilized anti-NR monoclonal antibody. They found that not all antibody-bound NR was eluted by 1 M NaCl, but that a substantial amount of enzyme remained bound to the antibodies (approximately 50%) and remained catalytically active, even after NaCl concentrations as high as 2 M were applied. They reported the first example of NADH substrate inhibition of higher plant nitrate reductase in solution and for an immobilized enzyme using a novel immobilization technique with a monoclonal antibody. The immobilized enzyme had greater thermal stability than the enzyme in solution (Fig. 10.2). This enhanced thermal stability of antibody bound enzyme might be of general interest, because many nitrate reductase extraction procedures, like the blue-Sepharose method, often result in quite unstable enzyme preparations.

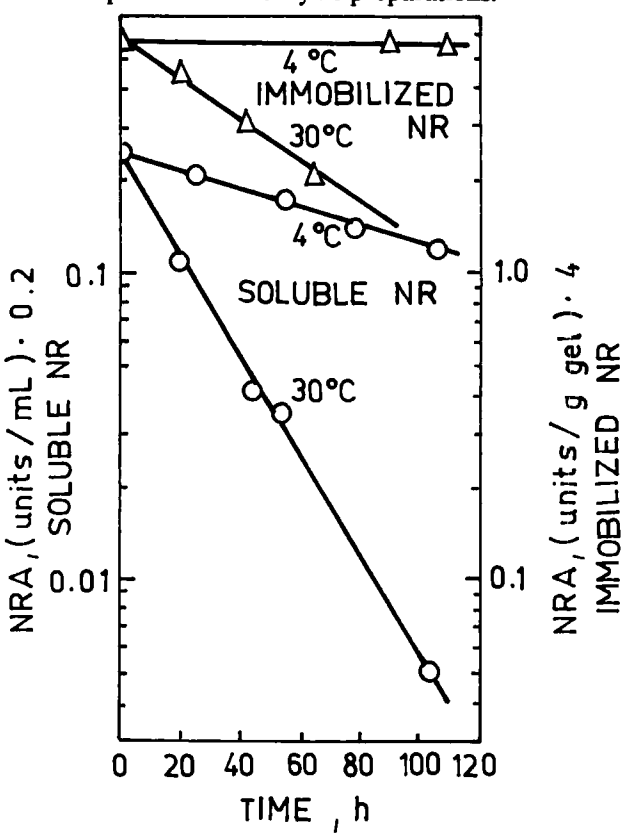


Fig. 10.2. Thermal stability of soluble and immobilized nitrate reductase (NR) preparations in 0.1 M Tris, pH 7.5. Calculated half-lifetimes for soluble NR: 4°C, 106 h; 30°C, 19 h; immobilized NR: 4°C, no significant decrease in activity observed during a six day period; 30 °C, 46 h. Reproduced with permission from P. Ruoff et al., Biochem. Biophys. Res. Commun., 161 (1989) 496-501.

10.2 MONOCLONAL ANTIBODIES IN COMPARISON WITH POLYCLONAL ANTIBODIES

Sada et al. (1986) studied adsorption equilibria in immunoaffinity chromatography using monoclonal and polyclonal antibodies. The equilibrium characteristics of polyclonal antibodies, which are mixtures of antibodies produced by a variety of lymphocyte cell lines and which possess a wide range of binding affinities, were compared with those of monoclonal antibodies which are homogeneous antibodies obtained by fusing an antibody-producing cell with a myeloma cell. The effects of pH, ionic strength, anion species, and antibody concentration on the adsorption equilibrium between immobilized antibodies and antigens were studied by the use of polyclonal antibodies against bovine and human serum albumins, and monoclonal antibody against human serum albumin coupled to CNBr-activated Sepharose 4B. The immobilized monoclonal antibody showed a homogeneous affinity of the Langmuir type. The heterogeneity of immobilized polyclonal antibodies was accounted for the assuming a normal distribution of the free energy of the antibody-antigen combination. Fig. 10.3, which has been

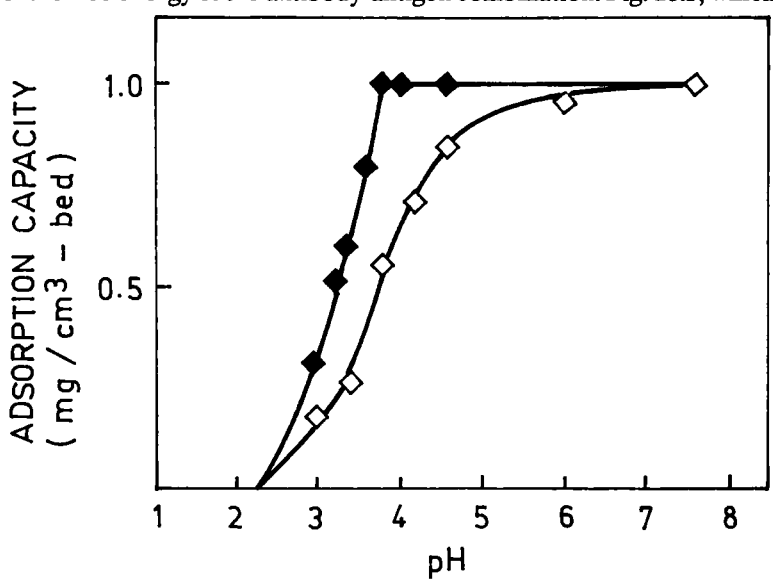


Fig. 10.3. Effect of pH on adsorption capacity of Sepharose 4B with attached monoclonal antibody against human serum albumin (HSA) (◆) and of Sepharose 4B with attached polyclonal antibodies against HSA (◇) relative to these adsorption capacities at pH 7.6. Concentration of adsorbed HSA was 7.6×10^{-7} mol/L, ionic strength 0.3 mol/kg and temperature 25°C. Reproduced with permission from E. Sada et al., Biotechnol. Bioeng., 28 (1986) 1497-1502.

adapted from their paper, shows the effects of pH on adsorption capacity of immunoadsorbent prepared from monoclonal antibody against human serum albumin (HSA) in comparison with immunoadsorbent prepared from polyclonal anti-HSA antibodies. In contrast to immunoadsorbent prepared from monoclonal antibodies, multipoint binding of the antigen to two or more different antibodies can occur on an immunoadsorbent prepared from polyclonal antibodies, resulting in such tight binding that dissociation and recovery of the antigen may be difficult (Ernst-Cabrera and Wilchek, 1988).

10.3 ORIENTED IMMOBILIZATION OF CHYMOTRYPSIN AND TRYPSIN BY ADSORPTION TO IMMUNOADSORBENTS PREPARED FROM SUITABLE POLYCLONAL ANTIBODIES

The use of cheaper pig polyclonal antibodies against DIP-chymotrypsin for oriented immobilization of enzyme was developed by Turková et al. (1988a,b) and Fusek et al. (1988).

Two types of antibodies specific for diisopropylphosphophoryl-chymotrypsin were isolated by biospecific affinity chromatography using two columns of immobilized chymotrypsin. The first, anti-chymotrypsin IgG I, was prepared by biospecific affinity chromatography on a Sepharose column to which chymotrypsin had been attached through a covalent bond between the surface part of the molecule around the active site and the immobilized naturally occurring polyvalent trypsin inhibitor, antilysine. The first procedure is shown schematically in Fig. 10. 4.

Antilysine was covalently attached to CNBr-activated Sepharose 4B. At pH 7.2 chymotrypsin was specifically and firmly bound to this biospecific adsorbent. Desorption of chymotrypsin occurred only at low pH. The covalent coupling of chymotrypsin to immobilized antilysine was achieved by crosslinking with glutaraldehyde. After this covalent bonding practically no chymotrypsin was eluted at low pH. The assay of zero chymotryptic activity with succinyl-L-phenylalanine p-nitroanilide as substrate showed that the enzyme was immobilized *via* its active site only. The Sepharose column with chymotrypsin immobilized *via* its inhibitor, antilysine, was used

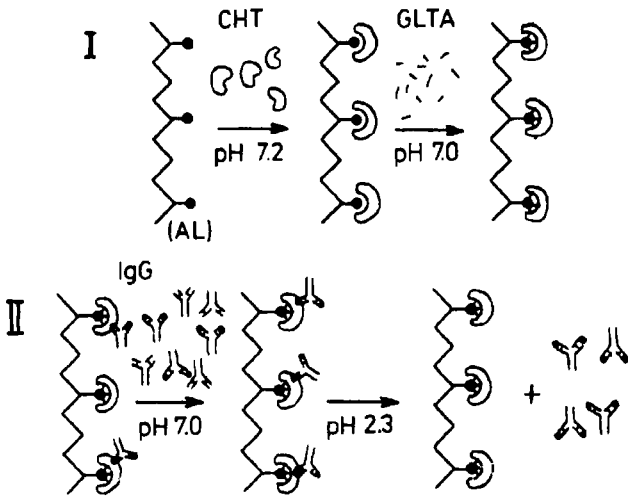


Fig. 10.4. Schematic drawing (I) of oriented immobilization of chymotrypsin (CHT) by use of covalent crosslinking of its active site to immobilized natural polyvalent trypsin inhibitor antilysine (AL) with glutaraldehyde (GLTA) and (II) of use of this column for isolation of immunoglobulin G (IgG) against DIP-chymotrypsin (with antigenic sites outside the active site of CHT). Covalent bonds are shown as full lines. Reproduced from J. Turková, et al., Makromol. Chem., Macromol. Symp., 17 (1988) 241-256.

for the isolation of anti-chymotrypsin IgG I. The effluent, which repeatedly passed unretarded through the column, was applied to an AH-Sepharose column to which chymotrypsin had been attached directly, and anti-chymotrypsin IgG II was isolated. According to the ELISA assay for the fraction of anti-chymotrypsin-IgG I, a 50% binding to chymotrypsin was achieved at a concentration of 1.33×10^{-9}M, expressed as the fraction of anti-chymotrypsin-IgG II at a concentration of 2.6×10^{-9}M. Fig. 10.5 shows differences in the effect of IgG fraction I and II on the percentage of proteolytic activity and activity determined by succinyl-L-phenylalanine p-nitroanilide (suc-L-Phe-pNA) as low molecular substrate of chymotrypsin at varying molar ratios of IgG to chymotrypsin. Unlike IgG II, there is practically no decrease of proteolytic activity with IgG I at molar ratios lower than 1:1.

This finding was utilized for the oriented immobilization of chymotrypsin based on simple biospecific adsorption to anti-chymotrypsin IgG I attached to Sepharose 4B. Table 10.2 shows the activity values of this immobilized preparation as determined with both a low molecular weight substrate, suc-L-Phe-pNA, and a high molecular weight

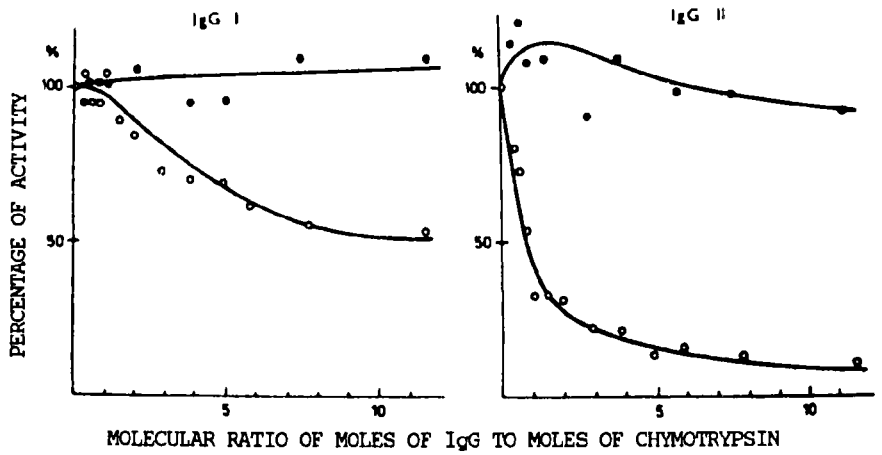


Fig. 10.5. Effect of anti-chymotrypsin IgG I and anti-chymotrypsin IgG II on the proteolytic activity of chymotrypsin (o) and activity for the low molecular substrate (●). Reproduced from M. Fusek et al., Biotechnol. Lett., 10 (1988) 85-90.

Table 10.2.

Comparison of activities of chymotrypsin (CHT) immobilized covalently directly to AH-Sepharose by use of glutaraldehyde (A) or noncovalently by biospecific sorption to Sepharose with attached anti-chymotrypsin IgG I (B)

Type of binding	*Quantity of CHT bound mg/ml*	*Relative activity of bound CHT using substrate*	
		Suc-L-Phe-pNA (%)	*Hemoglobin (%)*
A	2.1	30	8
B	1.8	110	80

substrate, hemoglobin. These activity values are compared with the activity of chymotrypsin bound covalently to glutaraldehyde-activated AH-Sepharose. The results listed in the Table 10.2 show the great advantage of immobilization of chymotrypsin by oriented adsorption.

Šťovíčková et al. (1991) described a further method for the preparation of polyclonal antibodies suitable for biospecific adsorption of trypsin. Pig polyclonal antibodies were prepared against the biospecific complex of trypsin with its natural basic pancreatic inhibitor, antilysine, wherein the active site of trypsin is buried inside the complex. The active sites of enzyme and inhibitor therefore do not elicit an antibody response. Antibodies against trypsin were isolated from prepared antiserum by bioaffinity chromatography on trypsin-bound beaded cellulose. The catalytic activity of trypsin was not affected by interaction with these antibodies, even in the presence of excess of antibody. Antibodies covalently attached to Sepharose were used for the biospecific adsorption of trypsin. Fig. 10. 6 shows a Lineweaver-Burk plot relating amidolytic reaction velocity of both free and biospecifically immobilized trypsin to substrate benzoyl-L-arginine p-nitroanilide (L-BAPNA). Table 10.3 shows kinetic data of free and biospecifically bound trypsin determined by us using L-BAPNA as a substrate at 25°C.

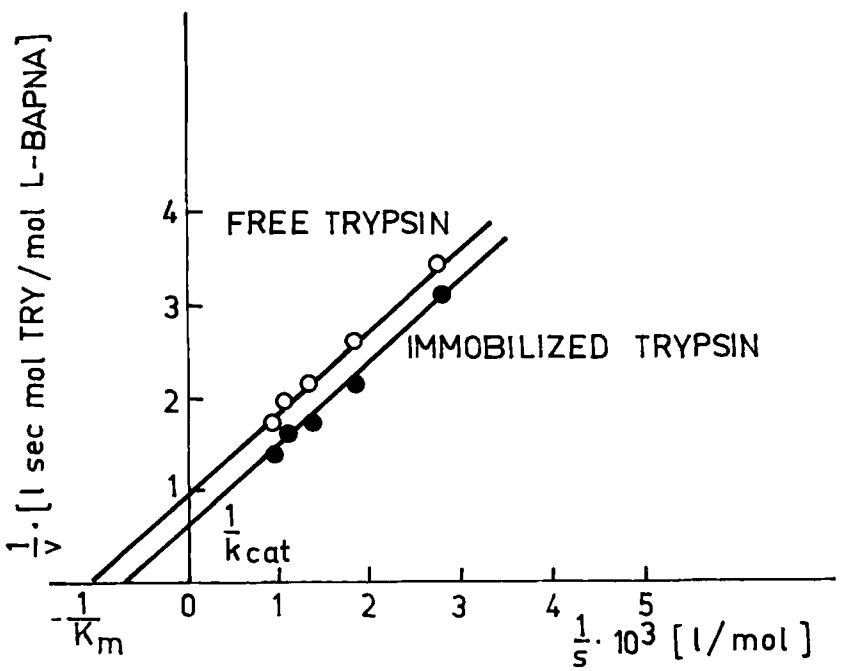


Fig. 10.6. Lineweaver-Burk plot relating amidolytic reaction velocity both of free and biospecifically immobilized trypsins to substrate (L-BAPNA). Reproduced from J. Šťovíčková et al., Biocatalysis, 5 (1991) 121-130.

Table 10.3.

Kinetic data of free and biospecifically bound trypsin using L-BAPNA as substrate at 25°C

Preparation	K_M*[mol/l]*	k_{cat}*[sec^{-1}]*	k_{cat}/K_M*[l/mol sec]*
Free trypsin	0.89×10^{-3}	1.05	1.18×10^{3}
Biospecifically bound trypsin	1.33×10^{-3}	1.58	1.19×10^{3}

Comparison of enzymatic activities of trypsin randomly bound to CNBr-activated Sepharose 4B and biospecifically immobilized trypsin is described in Table 10.4. The results show that the method used represents a general technique which is suitable for the preparation of highly active immobilized enzyme preparations for the biochemical study of enzymes naturally bound in the organelle structures, because the kinetics of metabolic processes of an organism and heterogenous catalysis of enzyme reactors are governed by the same rules.

Table 10.4.

Comparison of enzymatic activities of randomly and biospecifically bound trypsin

Preparation	*Free trypsin*	*Randomly bound trypsin*	*Biospecifically bound trypsin*
Amount[a] of bound antibody	-	-	1.40 mg
Amount of bound trypsin	-	2.6 mg	66 μg
Specific proteolytic[b] activity of trypsin	6.3 U	1.0 U	6.21 U
Specific amidolytic[c] activity of trypsin	840 nmol	585 nmol	809 nmol
Relative specific proteolytic activity	100%	15%	98.6%
Relative specific amidolytic activity	100%	70%	96.3%

[a] Amount is related to 1g of wet carrier.

[b] 1 U means increase of optical density at 280 nm for 1.0 per 1 minute.

[c] Activity is related to 1 minute and 1 mg of trypsin.

10.4 ACTIVE PROTEIN STABILIZATION BY ANTIBODIES

Non-covalent modification of the surface chemistry of the enzymes with specific antibodies can increase the stability of biologically active proteins. Their observations on stabilization by protein-protein interactions led Shami et al. (1989) to examine the immune system. The activity of a protein requires proper folding to ensure the structural and functional integrity of the active domain. This integrity can be irreversibly disrupted by forces which are either physical (heat, freezing, radiation), chemical (oxidation, reduction, solvents, metal ions, ionic strength, pH) or biological (enzymatic modification, degradation, e.g. enzymatic proteolysis). However, the immune system is a natural mechanism, which can manufacture proteins which interact specifically with many overlapping surface features of a folded target protein. Some antibodies might interact at sites where protein unfolding is initiated, or where proteolytic digestion occurs, thereby stabilizing the protein. For the average bioaffinity antibody-antigen binding (10^8 M^{-1}), the interaction could reduce the free energy of the antigen by about 10 Kcal mol^{-1}. This is sufficient to confer increased stability since the differences in free energy between the folded and unfolded states for active proteins are in the range of 5 - 15 Kcal mol^{-1}.

Sheriff et al. (1987) used X-ray crystallography to determine the three-dimensional structure of an antibody-antigen complex by using anti-lysozyme Fab and lysozyme. They concluded that the principal difference in the case of the larger antigen lies in the much greater area of the complementary surfaces that are brought into contact, with the consequent exclusion of all water molecules. In the antibody-lysozyme interaction three salt links are involved, whith ten hydrogen bonds and 74 Van der Waals' interactions. Such cross-linking, supported by hydrophobic interaction, should stabilize the folded structure of the antigen.

Shami et al. (1989) used a number of model enzymes and complexed them with specific polyclonal or monoclonal antibodies. Fig. 10.7 shows the thermostability of an antibody-α-amylase complex. There is a correlation between thermal stability and resistance to proteolysis. Thermostable enzymes from thermophilic bacteria, for

example, are more resistant to proteolysis than similar enzymes from mesophilic organisms. Therefore, processes that confer thermal stability may also confer a significant degree of protection against inactivation by proteolytic enzymes.

In order to evaluate chemical disruption, the enzymes were exposed to a low pH, or treated with oxidant (NaOCl) or alcohol. L-Asparaginase exposed to pH 3.0 lost 98% of its activity during 50 minutes, while antibody-protected enzyme retained close to 40% activity at 50 minutes and 25% for the next 17 hours. Subtilisin exposed to oxidation by 0.05% NaOCl for 30 minutes retained only 25% of its original activity, while the antibody-protected enzyme retained close to 80% of its activity. Glucoamylase preincubated with 2.5% alcohol for 25 hours retained only 10% of its original activity while the antibody-protected enzyme retained 98% of its activity.

The model system used to evaluate biological inactivation was proteolytic treatment with trypsin of the anti-leukemic enzyme L-asparaginase. This enzyme is extremely sensitive to proteolytic enzymes. Even a single monoclonal antibody affords almost

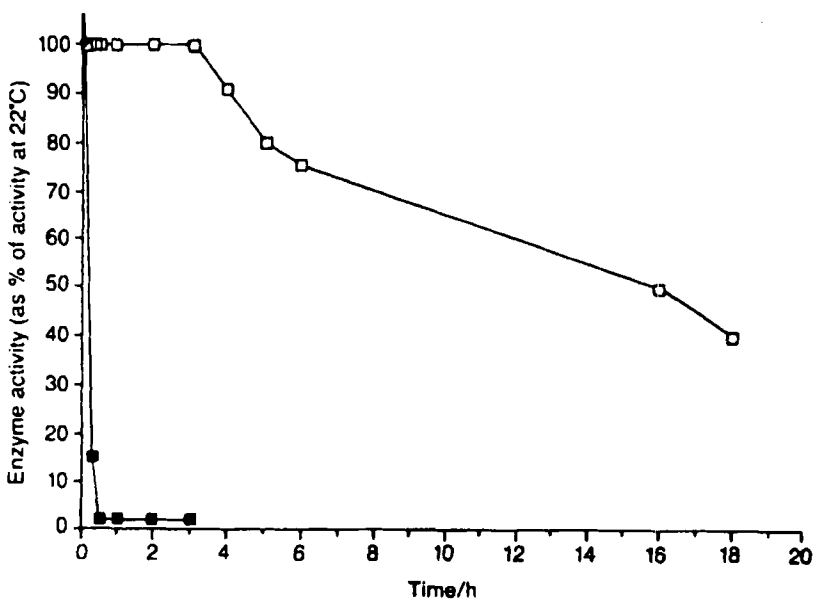


Fig. 10.7. Thermostability of an antibody-α-amylase complex. Human salivary α-amylase was complexed with specific rabbit polyclonal antibody and then placed in a water bath at 70°C. ■ , Free enzyme; ❐ , enzyme-antibody complex. Reproduced with permission from E. Y. Shami et al., Trends Biotechnol., 7 (1989) 186 -190.

complete protection. The protected trypsin-treated enzyme was virtually as active as the trypsin-untreated control, whereas the trypsin-treated unprotected enzyme displayed little activity.

REFERENCES

Ernst-Cabrera, K. and Wilchek, M., Med. Sci. Res., 16 (1988) 305-310.

Fleminger, G., Hadas, E., Wolf, T. and Solomon, B., Appl. Biochem. Biotech., 23 (1990) 123-137.

Fusek, M., Turková, J., Šťovíčková, J. and Franěk, F., Biotechnol. Lett., 10 (1988) 85-90.

Hadas, E., Koppel, R., Schwartz, F., Ravin, O. and Fleminger, G., J. Chromatogr., 510 (1990) 303-309.

Ruoff, P., Lillo, C. and Campbell, W.H., Biochem. Biophys. Res. Commun., 161 (1989) 496-501.

Sada, E., Katoh, S., Sukai, K., Tohma, M. and Kondo, A., Biotechnol. Bioeng., 28 (1986) 1497-1502.

Shami, E.Y., Rothstein, A. and Ramjeesingh, M., Trends Biotechnol., 7 (1989) 186-190.

Sheriff, S., Silverton, E.W., Padlan, E.A., Cohen, G.H., Smith-Gill, S.J., Finzel, B.C. and Davis, D.R., Proc. Nat. Acad. Sci. U.S., 84 (1987) 8075-8079.

Solomon, B., Koppel, R. and Katchalski-Katzir, E., Biotechnology, 2 (1984) 709-712.

Solomon, B., Koppel, R., Schwartz, F. and Fleminger, G., J. Chromatogr., 510 (1990) 321-329.

Šťovíčková, J., Franěk, F. and Turková, J., Biocatalysis, 5 (1991) 121-130.

Turková, J., Fusek, M., Šťovíčková, J. and Králová, Z., Makromol. Chem., Macromol. Symp., 17 (1988) 241-256.

Turková, J., Fusek, M. and Štovíčková, J., in A. Blažej and J. Zemek (Editors, Progress in Biotechnology, Vol.4, Elsevier, Amsterdam, 1988b, pp. 245-260.

This Page Intentionally Left Blank

Chapter 11

Study of the modification, mechanism of action and structure of biologically active substances using bioaffinity chromatography

Bioaffinity chromatography can be used to study the possibility of the substitution of the natural peptide chains of enzymes with various modified synthetic peptides. It can be also used for the determination of the active sites of enzymes and antibodies, for studies of the binding properties of subunits, the specificity of enzymes towards various inhibitors, the complementarity of nucleic acids, the interaction of nucleotides with peptides, the effect of the presence of various substances and microenvironment on the formation of biospecific complexes, etc.

Bioaffinity chromatography, combined with genetic approaches which allow two or more genes to be spliced together to yield fusion proteins, might provide powerful systems for protein purification. Immobilization and purification of enzymes with staphylococcal Protein A gene fusion vectors may be mentioned as an example. Other studies on the fundamentals of the course of bioaffinity chromatography will be described in connection with the mechanisms of enzymatic activities and stabilities, and with the molecular structures of fibroblast and leucocyte interferons.

11.1 SEMI-SYNTHETIC NUCLEASE AND COMPLEMENTARY INTERACTION OF NUCLEASE FRAGMENTS

Staphylococcal nuclease is an enzyme that has been studied most extensively with the use of bioaffinity chromatography. A successful isolation of nuclease on Sepharose with attached 3-(4-aminophenylphosphoryl)deoxythymidine 5'-phosphate (Sepharose-pdTp) was described as early as 1968 by Cuatrecasas et al. Using bioaffinity chromatography of a tryptic hydrolysate of nuclease labelled with (deoxythymidine--3',5'-diphosphate-aminophenyl) deoxythymidine 3'-[^{14}C]-bromoacetyl-p-aminophenylphosphate on Sepharose with attached nuclease, Wilchek (1970) obtained peptides of the active site.

When carrying out a tryptic hydrolysis of nuclease in the presence of Ca^{2+} and nucleoside 3',5'-deoxythymidine diphosphate (pdTp), Ontjes and Anfinsen (1969 a, b) obtained three fragments: a pentapeptide and two polypeptide fragments, nuclease-T - (6-48) and nuclease-T (49-149) (see Fig. 11.1). The last two fragments were inactive and disordered when alone, but they associated reversibly with the formation of a non-covalently bound complex called nuclease-T'. Nuclease-T' had approximately 8% (1620 units/mole) of the activity of the native enzyme (15,800 units/mole), and its three-dimensional structure is similar to that of nuclease. The same results were then achieved even when the native nuclease-T-(6-48) fragment was replaced with an analogous peptide prepared synthetically according to the method of Merrifield. Bioaffinity chromatography was again of considerable help during the purification of this synthetic 42 peptide. The synthetic product was purified on Sepharose with attached fragment nuclease-T-(49-149). In a preliminary experiment a small amount of native fragment nuclease-T-(6-48) was first chromatographed on this column. Sorption took place at pH 8 in the presence of calcium chloride and pdTp (necessary for the stabilization of the complex). The native fragment was then eluted with dilute acetic acid of pH 3, i.e. under conditions when dissociation of the complex takes place. On the basis of this experiment, the purification procedure for the synthetic fragment was established as follows. A 5-mg amount of synthetic peptide was applied to the column (5 x 1 cm) in 0.05 M borate buffer of pH 8, containing 0.01 M calcium chloride and 0.001 M pdTp. Using the same buffer, some peptidic material was eluted which had the same composition and mobility as the native peptide as shown by disc electrophoresis. However, it was not adsorbed on the column, even after repeated chromatography. About 7% of the total material applied was then eluted with 0.001 M acetic acid of pH 3. This fraction did not differ significantly in its amino acid composition from the starting material. This peptide fragment was then used for complementation with nuclease-T-(49-149) fragments, where it could fully replace the native peptide. In this manner the semi-synthetic nuclease was prepared.

The second example of a productive complementation was the complex of two other fragments, i.e. nuclease (1-126), prepared by limited tryptic hydrolysis of trifluo-

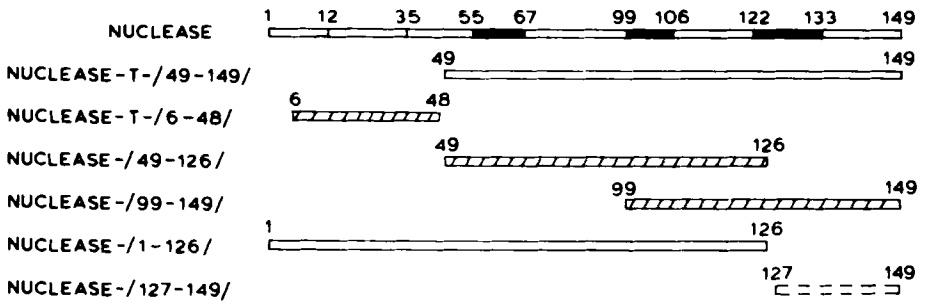


Fig 11.1. Diagram of linear relationship of the amino acid sequences of nuclease and its fragments that yield productive complementation. The X-ray crystallographic study showed β-structures between residues 12 and 35 and three α-helical parts between residues 55 and 67, 99 and 106 and 122 and 133, which are indicated on the nuclease line. Nuclease-(127 - 149) does not bind to nuclease -(1-126). Reproduced with permission from G. Andria et al., J. Biol. Chem., 246 (1971) 7421 - 7428.

roacetylated nuclease and the cyanogen bromide fragment of nuclease (99-149). This complex possessed 10 - 12 % of the enzymatic activity of native nuclease (Taniuchi, 1970). Both of the complexes mentioned were bound with Sepharose-pdTp.

A mixture of three fragments was bound on Sepharose-pdTp by Andria et al. (1971): 0.085 μmole of nuclease-T-(6-48), 0.060 μmole of nuclease-T-(49,50-126) [Nuclease-T-(49,50-126) is a mixture of the peptides nuclease-T-(49-126) and nuclease-T-(50-126)]. Amounts of 0.067 μmole of nuclease-(99-149) and 0.01 M calcium chloride in 0.2 ml of 0.1 M ammonium acetate solution of pH 8 were applied each time on a column of Sepharose-pdTp (4 x 1 cm) at 4, 10, 15 and 20°C. The amount of the complex eluted with 0.1 M acetic acid was very dependent on temperature. Fig . 11.2 shows that it was highest at 4°C and decreased with increasing temperature. Amino acid analysis and two-dimensional peptide maps of tryptic hydrolysate revealed that the material is composed of equimolar amounts of nuclease-T-(6-48), nuclease-T-(49,50-126)and nuclease-(99-149). The specific activity of the complex was 15.3 units/μmol. The decrease in the amount of the sorbed complex observed with increasing temperature may be caused by the decreasing formation of the complex or a decrease in the affinity of the affinity ligand for the complex, which is connected with the thermal instability of the

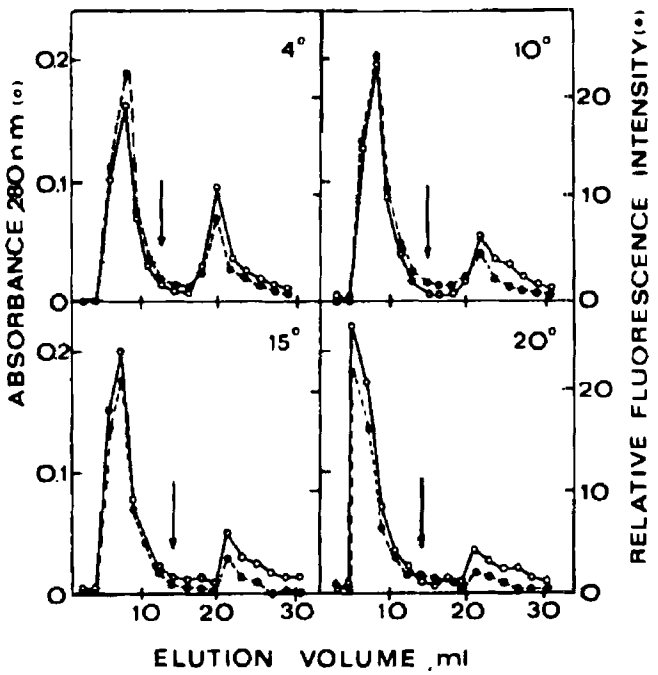


Fig. 11.2. Affinity chromatography of a mixture containing nuclease-T-(6-48), nuclease-T-(49,50-126)and nuclease-(99-149) in the presence of Ca^{2+} at various temperatures on Sepharose 4B substituted with 3'-(4'-aminophenylphosphoryl)deoxythymidine-5'-phosphate (Sepharose-pdTp). The mixture containing 0.085 μmole of nuclease-T-(6-48), 0.060 μmole of nuclease-T-(49,50-126), 0.067 μmole of nuclease-(99-149) and 0.01 M calcium chloride in 0.2 ml of 0.1 M ammonium acetate solution, pH 8, was equilibrated at a given temperature and applied to a jacketed Sepharose-pdTp column (40 x 10 mm). The elution was carried out with 0.1 M ammonium acetate-0.01 M calcium chloride solution, pH 8. The arrows indicate the points where the elution with 0.1 N acetic acid was started. The relative intensity of tryptophan fluorescence of fractions was determined in order to monitor nuclease-(99-149) with a single tryptophan residue. Reproduced with permission from G. Andria et al., J. Biol. Chem., 246 (1971) 7421-74258.

enzymatic activity of the three-fragment complex. Affinity chromatography of any combination of two of the three fragments mentioned gave no indication of a significant amount of adsorbed material, even at 4°C. At this temperature no adsorption of the three fragments was observed in the absence of calcium, nor when a mixture of nuclease fragments (1-120) and nuclease (127-149) was applied.

From these results it is evident that bioaffinity chromatography may become an important method in the study of the complementation of peptide fragments of active proteins, prepared both by isolation from natural material and by synthesis.

11.2 BIOAFFINITY CHROMATOGRAPHY IN COMBINATION WITH GENE FUSION VECTORS

Gene fusion, in which the coding sequences of two or more genes are spliced together by genetic approaches, is a technique of growing importance. Löfdahl et al. (1983) reported the cloning of the gene coding for staphylococcal Protein A in *Escherichia coli*. This protein can be used as an immunological tool due to its specific binding to the Fc part of the immunoglobulins of many species. Uhlén et al. (1983) constructed two plasmid vectors, containing the gene coding for staphylococcal Protein A and adapted for gene fusion. These vectors allow fusion of any gene to the Protein A gene, thus giving hybrid proteins which can be purified, in a one-step procedure, by IgG bioaffinity chromatography. As an example of the practical use of such vectors, the Protein A gene was fused to the *lacZ* gene of *Escherichia coli*. *E. coli* strains containing such plasmids produced hybrid proteins with both IgG binding and β-galactosidase activities. The hybrid protein was immobilized on IgG-Sepharose by its Protein A moiety with high efficiency without losing its enzymatic activity. The elution of bound β-galactosidase-Protein A fusion galactosidase protein from IgG-Sepharose was performed by phosphate-buffered saline with 0.05 % Tween 20 containing pure Protein A.

Nilsson et al. (1985) used the interaction between immunoglobulin G (IgG) and Protein A for the efficient isolation of alkaline phosphatase. Diagrammatic representation of the process of staphylococcal Protein A gene fusions is shown in Fig. 11.3. The gene of the enzyme was extended by that part of the Protein A gene which codes for the part of the protein molecule responsible for the interaction of Protein A with IgG. The lysate of *Escherichia coli* modified in this manner was chromatographed on Sepharose with immobilized IgG. As shown in Table 11.1, in one single chromatographic run these authors obtained a considerable quantity of alkaline phosphate having the specific activity of the pure enzyme. Fig 11.4 shows the elution of alkaline phosphatase-Protein A fusion protein from IgG-Sepharose with increased concentration of lithium diiodosalicylate (LIS). More than 80 % of the Protein A alkaline phosphatase hybrid protein can be eluted from IgG affinity columns with no loss of enzymatic activity.

The coding sequence for Protein A signal peptide (S), IgG-binding region (IGB) and cell wall binding region (CWB) is indicated. The promoters are staphylococcal Protein A (spa):

The gene coding for alkaline phosphatase (PhoA):

The gene coding for alkaline phosphatase (PhoA) with IgG-binding region (IGB):

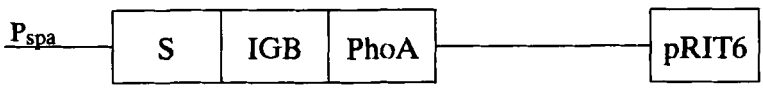


Fig. 11.3. Diagrammatic representation of the process of staphylococcal Protein A gene fusions. Reproduced with permission from B. Nilsson et al., EMBO J., 4 (1985) 1075-1080.

Table 11.1.

Content of Protein A and alkaline phosphatase in *E. coli* cells containing different plasmids

Plasmid	*Gene*	*Protein A content in cell lysate (μg/ml)*	*Activity*		*Relative activity after binding to human IgG*	
			Toluene	*Cell lysate*	*Immo-bilized*	*Superna-tant*
pSPA8	Protein A	2	0.00	0.00	nd	nd
pSKS106	β-galactosidase	< 0.1	168	165	< 1	99
pSPA13	Fusion *proA-lacZ*	0.5	0.36	0.16	94	2
pRIT1	Fusion *proA-lacZ*	25	308	505	95	< 1

Genetic approaches to protein purification has been reviewed by Uhlén et al. (1988). A gene fusion system based on the Protein A gene from *Staphylococcus aureus* has been developed to facilitate the purification of recombinant proteins, both on large and small

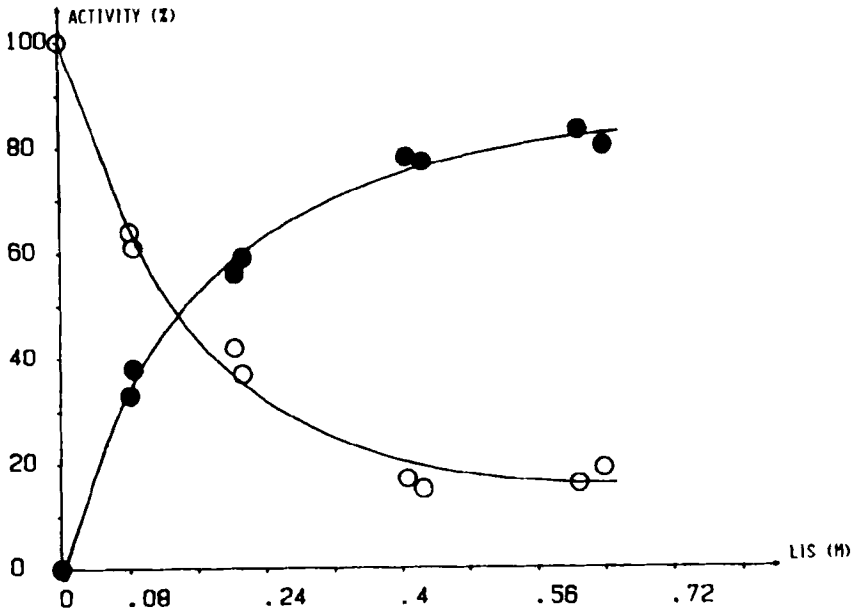


Fig. 11.4. Elution of alkaline phosphatase-Protein A fusion protein from IgG-Sepharose with increased concentrations of lithium diiodosalicylate (LIS). The relative activity of alkaline phosphatase bound to gel (o) and eluted (●) after 5 min treatment with buffer is shown. Reproduced with permission from B. Nilsson et al., EMBO J., 4 (1985) 1075 - 1080.

scales. Due to the strong interaction with IgG, it is possible to recover gene products fused to various Protein A derivatives, in a one-step procedure, with both high yield and high purity. Site-directed mutagenesis was used to introduce enzymatic and chemical cleavage sites at the fusion point between the Protein A derivative and the desired protein. The Protein A "tail" can thereby be removed from the bioaffinity purified fusion protein by the appropriate cleavage, releasing biologically active molecules. Recently, the system was improved by designing a synthetic DNA fragment encoding two IgG-binding domains derived from staphylococcal Protein A which are resistant to various chemical cleavages. The gene fusion product is secreted into the culture medium of *E. coli* and can be recovered simply by passing the clarified culture medium through an IgG Fast Flow Sepharose column. The system has been used to immobilize enzymes, to

obtain monoclonal and polyclonal antibodies, and to produce biologically active human peptide hormones in pilot plant scale.

Baneyx et al. (1990) constructed a hybrid protein consisting of Protein A and the enzyme β-lactamase using recombinant DNA techniques. The functional characteristics of the hybrid protein adsorbed on IgG-coated Sepharose matrices have been studied in detail and compared to those of (1) the hybrid protein in solution and (2) β-lactamase covalently immobilized on CNBr-activated Sepharose. Protein A-β-lactamase bound tightly and specifically to IgG-Sepharose and could be stored for at least four weeks without dissociation. The rate of penicillin G hydrolysis by the β-lactamase domain of the immobilized hybrid protein was found to depend on the amount of IgG covalently coupled to the support. For all IgG loads, higher specific activities and lower K_m values were obtained relative to covalently immobilized β-lactamase. Adsorption of the hybrid protein on the support resulted in increased stability to thermal deactivation. These results indicate that bifunctional hybrid proteins can be useful for the bioaffinity immobilization of enzymes.

An other example of the bioaffinity purification of target genes was described by Parmley and Smith (1988). They inserted foreign DNA fragments into a minor coat protein gene of filamentous phage, creating a fusion protein that is incorporated into the virion. The foreign amino acids are displayed on the surface, allowing fusion phage bearing antigenic determinants from a target gene to be purified in infectious form by affinity to antibody directed against the gene product. They introduced fusion-phage vectors that accept foreign DNA inserts with little effect on phage function. They described the bioaffinity purification of virions bearing a target determinant from a 10^8-fold excess of phage not bearing the determinant, using minute amounts of antibody. These "antibody-selectable" vectors are a promising alternative to conventional expression systems for using antibodies to clone genes.

Recognition by DNA-binding proteins of specific DNA sequences is an essential step in many cell regulating processes, including transcription, initiation of DNA replication, and site-specific recombination. Lundeberg et al. (1990) described the bioaffinity purification of specific DNA fragments using a *lac* repressor fusion protein. They

showed that the *lac*I system can be developed into a solid phase method for purification of *lac*O-containing DNA fragments as schematically outlined in Fig. 11.5. This bioaffinity purification was developed on the basis of a fusion between the *Escherichia coli lac* repressor gene (*lac*I) and the staphylococcal Protein A gene (spa). The fusion protein, expressed in *Escherichia coli*, is active both in vivo and in vitro with respect to its three functional activities: DNA binding, n-isopropyl-β-D-thio-galactopyranoside (IPTG) induction, and IgG binding. The use of the *lac* repressor as a biochemical and analytical tool has the additional advantage that the specific binding can be reversed by IPTG, which allosterically prevents the repressor from binding to the *lac*O-sequence. The recombinant protein can be immobilized in a one-step procedure with high yield and purity using the specific interaction between Protein A and the Fc-part of immunoglobulin G. The immobilized repressor can thereafter be used for bioaffinity purification of specific DNA fragments containing the *lac* operator (*lac*O) sequence.

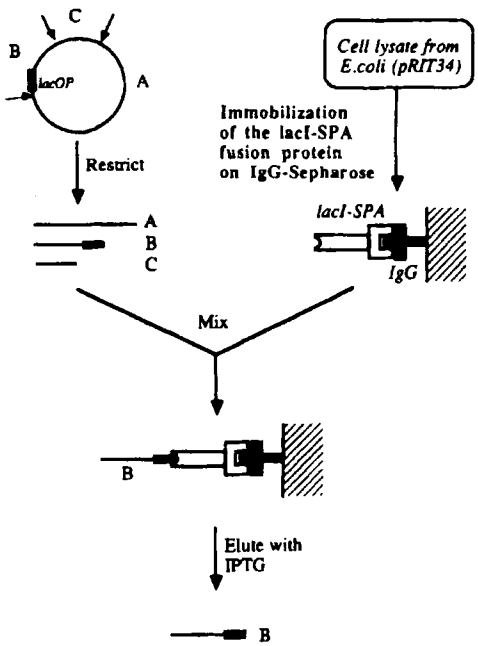


Figure 11.5. Schematic drawing outlining the concept of utilising the *Escherichia coli* lac repressor gene (lacI) and the staphylococcal Protein A gene (SPA) fusion protein to purify DNA sequences containing the lac operator sequence. Reproduced with permission from J. Lundeberg et al., Genet. Anal. Techn. Appl., 7 (1990) 47-52.

11.3 STUDY OF THE MECHANISMS OF ENZYMATIC ACTION

In order to elucidate the stages of the ATP-splitting reaction catalysed by myosin or by its enzymatically active proteolytic fragments, Oplatka et al. (1975) attached on Sepharose 2B, through adipic acid hydrazide, [γ-^{32}P]ATP, non-labelled ATP, ADP or adenosine 5'-(β,γ-imino)triphosphate [AMP-P(NH)P]. Using enzymatic digestion with papain, they used myosin to prepare enzymatically active heavy meromyosin subfragment S-1, from which they then prepared its trinitrophenyl derivative. Fig. 11.6 shows the chromatography of the native and trinitrophenylated heavy meromyosin S-1 in the presence of either Mg^{2+} or EDTA on columns of agarose-ATP, agarose-ADP and agarose-AMP-P(NH)P. It is evident that in all instances, except for agarose-ATP, the concentration of potassium chloride necessary for elution in the presence of Mg^{2+} was not higher than 0.2M and the difference in the behaviour between the native and dinitrophenylated heavy meromyosin S-1 derivative was not significant. With ATP columns, a higher concentration of potassium chloride was necessary for elution in the presence of Mg^{2+}, viz. 0.5 M for the native and even 1.6 M for the trinitrophenylated heavy meromyosin S-1. Similar results were obtained when Mg^{2+} was replaced with Ca^{2+}, with the difference that the concentration of potassium chloride necessary for elution was lower. Under all conditions the adsorption of both proteins on the column with AMP-P(NH)P was weaker than on the columns with ATP and ADP, and only part of the active protein was adsorbed in the presence of Mg^{2+} (or Ca^{2+}). Hence the presence of Mg^{2+} and Ca^{2+} significantly increases the affinity of myosin and its derivatives to agarose-ATP. However, it has no effect (in fact, it causes a slight decrease) on the affinity to ADP and AMP-P(NH)P columns (especially in the presence of Ca^{2+}). Divalent cations simultaneously accelerate the cleavage of bound ATP both with native and with trinitrophenylated heavy meromyosin S-1, which was mainly studied on agarose with attached [γ-^{32}P]ATP.

These results served as one of the important bases for the proposal of a scheme for the mechanism of cleavage of ATP with myosin. Oplatka et al. (1975) concluded that, under the conditions in which the bound ATP is split, the protein is not only adsorbed

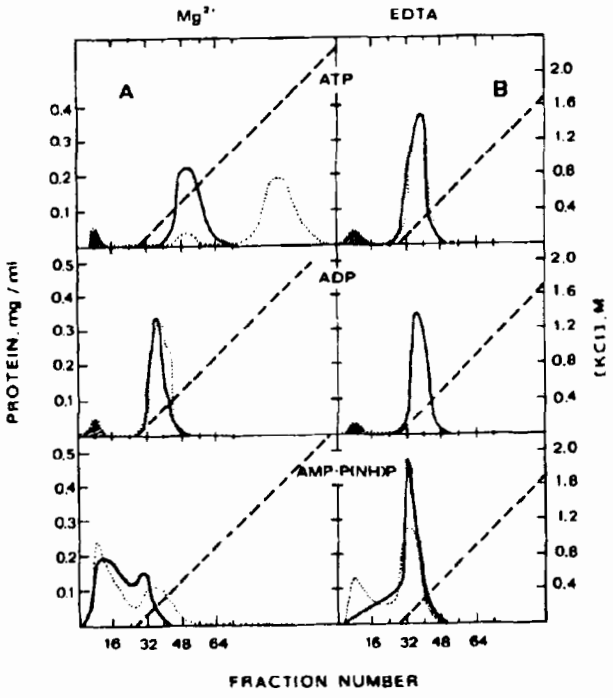


Fig. 11.6. Effect of Mg^{2+} on the behaviour of heavy meromyosin S-1 (solid line) and of trinitrophenylated heavy meromyosin S-1 (dotted line) on agarose-ATP, agarose-ADP and agarose-AMP-P(NH)P columns. Amounts of 6-10 mg of protein were loaded on columns (130 x 9 mm) filled with agarose nucleotide, previously equilibrated with 30 mM potassium chloride, 10 mM imidazole, pH 7 (or borate, pH 8, for the ATP-P(NH)P columns) and either (A) 0.5 mM EDTA + 2 mM Mg^{2+}, or (B) 1mM EDTA. Washing with the initial equilibrating solution was followed with a potassium chloride gradient (dashed line), leaving the other ingredients at the same concentration. Fractions of 2 ml were collected. Hatched areas indicate the lack of ATPase activity. Reproduced with permission from A. Oplatka et al., Biochim. Biophys. Acta, 385 (1975) 20-27.

on intact ATP, but also on the decomposition products of the latter. They used the name "dynamic" bioaffinity chromatography for this type of chromatography on an immobilized substrate under conditions favourable for an enzymatic reaction. They also assumed that the binding of enzymes under these conditions is determined by some kind of constant of the Michaelis-Menten type, rather than by the association constant of the enzyme and the intact substrate. Therefore, dynamic bioaffinity columns in which other substrates could be immobilized may evidently be useful for the detection of the

modifications of the binding sites of other enzymes and for the separation and the isolation of various isoenzymes.

The interaction between myosin and actin, which is the basis of the conversion of chemical energy into muscle, was investigated by means of bioaffinity chromatography by Bottomley and Trayer (1975). They used Sepharose with attached G-actin to follow the effect of the ionic strength on the specific and reversible binding of heavy meromyosin and myosin subfragment 1. The complexes formed between the derivatives of myosin and the immobilized G-actin can be dissociated by low concentrations of ATP, ADP and pyrophosphates, in both the presence and absence of Mg^{2+}. On the other hand, however, both Sepharose-bound and free G-actin cause only a weak increase in the Mg^{2+}-stimulated ATPase activity of myosin. This enabled a study of whether the complex formation between myosin and actin necessarily leads to the activation of ATPase to be carried out.

Similarly, affinity chromatography offers new possibilities for the study of the conversion of protein kinase into cyclic AMP-independent form by chromatography on N^6-caproyl-3',5'-cyclic adenosine monophosphate-Sepharose (Wilchek et al.,1971b). O'Carra and Barry (1972), using Sepharose with an attached oxamate derivative, proved that NADH induces the binding site for pyruvate in lactate dehydrogenase and also the formation of an "abortive" ternary enzyme-NAD^+-pyruvate complex. In an analogous manner, bioaffinity chromatography enabled Mawal et al. (1971) to detect enzyme-reactant complexes of galactosyl transferase. Doellgast and Kohlhaw (1972a,b) proved, by a study of the effect of the concentration of potassium phosphate on the binding of α-isopropylmalate synthetase onto L-leucine-Sepharose, that a high concentration of salts induces a conformational change involving the leucine binding site. Similar results were obtained by Rahimi-Laridjani et al. (1973) with biosynthetic threonine deaminase. The phosphorylation of soybean callus succinyl-CoA synthetase with immobilized adenosine triphosphate was studied by Wider de Xifra et al. (1972), while the re-naturation of agarose-bound aldolase after denaturation with 8 M urea was investigated by Chan (1970).

Bioaffinity chromatography can also be used for the isolation of enzymes that take part in certain metabolic pathways. For example, using N-(ω-aminohexyl)-L-aspartic acid-Sepharose 6B, Tosa et al. (1974), isolated enzymes involved in the metabolism of L-aspartic acid (asparaginase, aspartase and aspartate-β-decarboxylase).

11.4 MOLECULAR STRUCTURE OF FIBROBLAST AND LEUCOCYTE INTERFERONS INVESTIGATED WITH LECTIN

Human fibroblast and leucocyte interferons, both polyinosinic-polycytidylic acid-induced and virus-induced, are different from the point of view of their antigenicity. For example, antibodies formed against the fibroblast interferon are not capable of neutralizing the leucocyte interferon. The antibodies that were formed against the preparations of the leucocyte interferon partly cross-react with the fibroblast interferon, which, however, may be caused by the presence of fibroblast-type interferons in these leucocyte preparations. The sources of the difference of the antigenic behaviour of both interferons may lie both in the differences in the primary structures of the interferon polypeptide chains and in the differences in the post-translation modification (glycosylation), or in a combination of both.

In order to compare the post-translation modification of interferons, Jankowski et al. (1975) characterized both interferons on the basis of their binding properties for various immobilized lectins. The results are summarized in Table 11.2. From the lack of binding of human leucocyte interferons to lectins, which equally distinguished terminal sugar residues (horseshoe crab, *Lotus*, *Bandeiraea*) as well as internal residues (wheat germ, soybean, concanavalin A), it can be concluded that these sugar moieties are not present on interferon. Hence, it can be concluded that this interferon is not a glycoprotein. In contrast, human fibroblast interferon is a glycoprotein. On the basis of its binding to horseshoe crab lectin, it can be assumed that it contains a sialic acid residue. Further, L-fucose also exists in a form accessible for binding by specific lectins.

On the basis of the data given in Table 11.2, it seems that the differences in molecular structure can be attributed primarily to the type of cells in which the interferon was

induced. The differences based on the induction signal (virus or $rI_n.rC_n$) in the same cell seem to be of a much weaker character.

Bioaffinity chromatography was also used by Di Prisco and Casola (1975) for the detection of structural differences. Using specific immunoadsorbents they detected structural differences between nuclear and mitochondrial glutamate dehydrogenase. Wiman and Wallén (1975) used bioaffinity chromatography to investigate the structural relationship between the "glutamic acid" (A) and "lysine" (B) forms of human plasminogen, and their interaction with the NH_2-terminal activation peptide. They chromatographed the NH_2-terminal activation peptide on Sepharose with covalently bound plasminogen B, 0.27 μmole of this peptide being adsorbed on 1.0 μmole of immobilized plasminogen B. The adsorbed peptide could be eluted with a buffer containing 0.005 M 6-aminohexanoic acid. Its specific influence on the interaction can be deduced from the sufficiently low concentration. If the NH_2-terminal activation peptide was applied onto Sepharose with attached plasminogen A, only 0.10 μmole was adsorbed. In comparison with plasminogen B-Sepharose, this means a binding capacity of about 35%.

Table 11.2.

Binding of human leucocyte and fibroblast interferons on immobilized lectins

Chromatographic system	*Sugar specifity*	*Interferon source and inducer**			
		Leucocyte $rI_n.rC_n$	*Leucocyte virus*	*Fibroblast* $rI_n.rC_n$	*Fibroblast virus*
Immobilized lectins					
Horseshoe crab	N-Acetylneuraminic acid	-	-	+	+
Lotus	L-Fucose	-	-	±	+
Concanavalin A	D-Mannose	-	-	+	+
Bandeiraea simplicifolia	α- and β-D-galactose	-	-	-	-
Soyabean lectin	N-Acetyl-galactosamine	-	-	-	-
Wheatgerm lectin	N-Acetyl-glucosamine	-	-	-	-

* +, Complete binding; ±, partial binding; -, no binding.

In an effort to establish which part of the activation peptide is responsible for the specific interaction, Wiman and Wallén (1975) applied the tryptic digest of 1 μmole of the activation peptide onto a column of plasminogen-B-Sepharose. Among the five peptides formed after this tryptic hydrolysis, only the heptapeptide Ala-Phe-Glu-Tyr-His-Ser-Lys was sorbed, which is located at the 41-51 position of the total of 81 residues of the N-terminal activation peptide. Hence this peptide is responsible for the interaction, which, it seems, is of great importance for the conformational state of the plasminogen molecule.

REFERENCES

Andria, G., Taniuchi, H. and Cone, J.L., J. Biol. Chem., 246 (1971) 7421-7428.

Baneyx, F., Schmidt, C. and Georgiou, G., Enzyme Microbiol. Technol., 12 (1990) 337-342.

Bottomley, R.C. and Trayer, I.P., Biochem. J., 149 (1975) 365-379.

Chan, W.W.C., Biochem. Biophys. Res. Commun., 41 (1970) 1198-1204.

Cuatrecasas, P., Wilchek, M. and Anfinsen, C.B., Proc. Nat. Acad. Sci. U.S., 61 (1968) 636-643.

Di Prisco, G. and Casola, L., Biochemistry, 14 (1975) 4679-4683.

Doellgast, G. and Kohlhaw, G., Fed. Proc. Fed. Amer. Soc. Exp. Biol., 31 (1972a) 424.

Doellgast, G. and Kohlhaw, G., Fed. Proc. Fed. Amer. Soc. Exp. Biol., 31 (1972b) 1159.

Jankowski, W.J., Davey, M.W., O'Malley, J.A., Sulkowski, E. and Carter, W.A., J. Virol., 16 (1975) 1124-1130.

Löfdahl, S., Guss, B., Uhlén, M., Philipson, L. and Lindberg, M., Proc. Nat. Acad. Sci. U.S., 80 (1983) 697-701.

Lundeberg, J., Wahlberg, J. and Uhlén, M., Genet. Anal. Tech. Appl., 7 (1990) 47-52.

Mawal, R., Morrison, J.F. and Ebner, K.E., J. Biol. Chem., 246 (1971) 7106-7109.

Nilsson, B., Abrahmsén, L. and Uhlén, M., EMBO J., 4 (1985) 1075-1080.

O'Carra, P. and Barry, S., FEBS Lett., 21 (1972) 281-285.

Ontjes, D.A. and Anfinsen, C.B., Proc. Nat. Acad. Sci. U.S., 64 (1969a) 428-435.

Ontjes, D.A. and Anfinsen, C.B., J. Biol. Chem., 244 (1969b) 6316-6322.

Oplatka, A., Lamed, R. and Muhlrad, A., Biochim. Biophys. Acta, 385 (1975).20-27.

Parmley, S.F. and Smith, G.P., Gene, 73 (1988) 305-318.

Rahimi-Laridjani, I., Grimminger, H. and Lingens, F., FEBS Lett., 30 (1973) 185-187.

Taniuchi, H., Fed. Proc. Fed. Amer. Soc. Exp. Biol., 29 (1970) 335.

Tosa, T., Sato, T., Sano, R., Yamamoto, K., Matuo, Y. and Chibata, I., Biochim. Biophys. Acta, 334 (1974) 1-11.

Uhlén, M., Abrahmsén, L., Moks, T. and Nilsson, B., Makromol. Chem., Macromol. Symp., 17 (1988) 483-489.

Uhlén, M., Nilsson, B., Guss, B., Lindberg, M., Gatenbeck, S. and Philipson, L., Gene, 23 (1983) 369-378.

Wider de Xifra, E.A., Mendiara, S. and Batlle, A.M., FEBS Lett., 27 (1972) 275-278.

Wilchek, M., FEBS Lett., 7 (1970) 161-163.

Wilchek, M., Salomon, Y., Lowe, M. and Selinger, Z., Biochem. Biophys. Res. Commun., 45 (1971) 1177-1184.

Wiman, B. and Wallén, P., Eur. J. Biochem., 50 (1975) 489-494.

Chapter 12

Solid-phase immunoassay and enzyme-linked lectin assay

Immunoassays are analytical techniques based on the specific and high bioaffinity binding characteristics of antibodies with antigens. An extremely sensitive and selective immunoassay is used for the determination of the concentration of molecules against which specific antibodies can be prepared. Over the past three decades considerable progress has been made in devising new approaches to the immunochemical techniques in order to make determinations easier and the assay systems more reliable and precise. Various forms of immunoassay have been used extensively and effectively in human clinical diagnostics, e.g. for the analysis of such diverse analytes as drugs, hormones, microorganisms, specific antibodies, and tumour markers in samples such as blood, urine, faeces and tissue. A rapid expansion of these techniques has occurred into forensic, veterinary, food and agricultural analyses. In the analysis of foods and beverages these techniques are used to detect and quantitate materials such as additives, adulterants, fungal and bacterial contaminants, constituents, antinutritional factors, toxins or infectious agents (Allen and Smith, 1987). The potential for the rapid measurement of a minute quantity of a specific analyte from within a complex sample matrix, often with little or no sample clean-up, is one of the attractive features that has led to the widespread application of immunoassay (Rittenburg, 1990b). Many clinical, research or other laboratories are actively engaged in such work, every day.

The highly developed marker techniques are analytic tools which are also of general interest for research work in the field of bioaffinity chromatography because they provide excellent possibilities for fundamental investigations on a molecular level (Mohr and Pommerening, 1985). For example Pesce and Michael (1988), in their chapter on "The use of ELISA in the characterization of protein antigen structure and immune response", showed that enzyme-linked immunosorbent assay (ELISA) can be used to: (1) accurately measure polyclonal and monoclonal antibodies to the same antigen; (2) measure antibodies to both the whole protein molecule and to its components; (3) distinguish between strongly and weakly reacting determinants of the same

antigen; (4) discriminate between determinants on native and denatured structures; (5) detect the effects of charge and non-specific binding in general on antigen-antibody reactions; and (6) rapidly evaluate idiotypic/anti-idiotypic interactions. Thus, the ELISA technique is useful in studying immunochemical properties of antigens and antibodies.

A major difficulty throughout the history of immunoassays has been the separation of the unreacted (excess) antibody or antigen from the antibody-antigen complex. The problem of separating the free phase from the bound phase is greatly reduced by introducing a solid phase to which one reactant is bound (Smith, 1990). The use of a solid phase permits easy washing. Theoretical and practical aspects of enzyme-linked immunosorbent assay have been described by Clark and Engvall (1983). Solid phases and chemistries in complementary immunoassays have been summarized by Rasmussen (1988). Antibodies and antigens have been immobilized on a variety of solid surfaces. Immobilization is accomplished either passively, through hydrophobic interaction of the molecule to be immobilized and the solid support, or actively by use of covalent attachment to suitable sites on the solid phase surface. Commonly used solid supports are polystyrene, polyvinyl, Nylon, glass, nitrocellulose, silica, polyacrylamide and agarose (Rittenburg, 1990).

The separation of free reactants from immobilized reactants can be achieved through centrifugation or filtration from particulate solid phases such as agarose, polystyrene, or polyacrylamide beads. Magnetic fields can also be used for separating particles which have an iron oxide core (Guesdom and Avrameas, 1981). It is more common to use larger, disposable forms of the solid phase such as tubes, cuvettes, multiwell plates, balls, dipsticks, and adsorbent devices, which allow efficient separation of the bound from the unbound fractions through simple rinse steps. A large variety of automated and semi-automated equipment has been developed to simplify reagent transfer, rinsing steps, and interpretation of results. The 96-well plastic plate is one of the more commonly used forms of polystyrene and polyvinyl solid phases and is well suited to automated and high throughput uses. The 96 wells of a microtitre plate can be washed and read automatically in about a minute. However, not only does the protein binding capacity of microtitre

plates from different manufactures vary, there can also be differences in the ability of individual proteins to bind to them. Kemeny and Challacombe (1988b), in their chapter about microtitre plates and other solid phase supports, gave a comparison of the binding of different proteins to two commercial microtitre plates. Urbanek et al. (1985) bound four ^{125}I-radiolabelled proteins: bee venom phospholipase A_2(PLA_2), bovine serum albumin (BSA), bovine γ-globulin (BGG), and β-lactoglobulin (β-LACT) to two commonly used microtitre plates, one of which showed a high (Nunc immuno-1) and the other a low (Dynatech M129A) capacity for PLA_2. Fig. 12.1 shows that the irradiated Nunc plates bound all four proteins equally well, but there was reduced binding of PLA_2, BSA and β-LACT to the Dynatech M129A plates.

Kemp et al. (1985) recommended that enzyme immunoassay plates should be shaken before absorbance is measured. For reproducible results a consistent coating technique is essential. It is customary to try to reduce subsequent non-specific binding by using blocking agent such as Tween or bovine serum albumin in the reaction mixture (Blake and Gould, 1984).

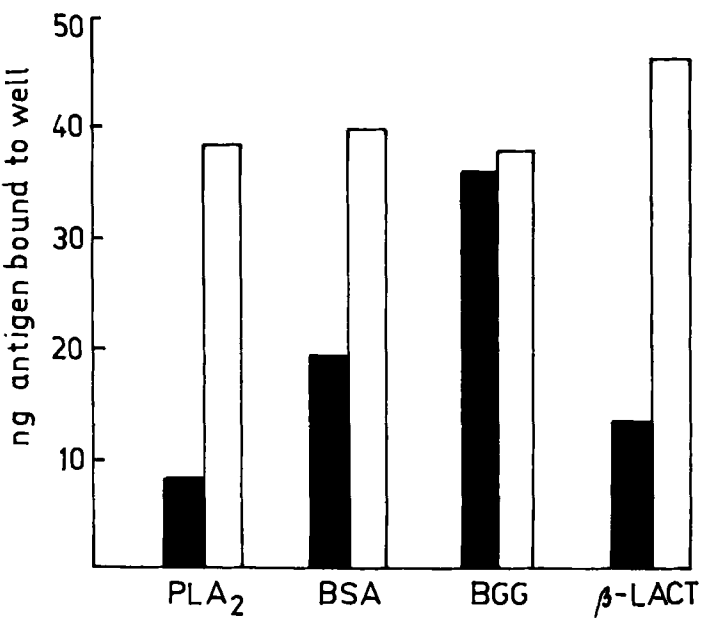


Fig. 12.1. Comparison of the binding of different proteins to Dynatech M129A (■) and NUNC immuno-1 (❑) microtitre plates. Reproduced with permission from R. Urbanek et al., J. Immunol. Methods, 79 (1985) 123-136.

Many reviews on various types of solid-phase immunoassays have been published in many chapters and books (e.g. Abraham, 1977; Langone and Van Vunakis, 1981 and 1983; Maggio, 1983; Collins, 1988; Kemeny and Challacombe, 1988a; Ngo, 1988; Rittenburg, 1990a; Zola, 1990). Review abouth microtitre plates was published by Kemeny and Challacombe (1988b). Modern immunoassays have arisen from the desire to detect and quantify complex biological molecules under conditions where chemical and physical analytical techniques are either unsuitable or unavailable. Several examples will be briefly described in this chapter.

12.1 SOLID-PHASE RADIOIMMUNOASSAY (RIA)

The classical immunoassay, described by Yalow and Berson (1960), is based on the ability of the unlabelled antigen to compete with the antigen, labelled with a radioactive isotope, in binding to a limited number of binding sites of the antibody. Hence, the process consists in the inhibition of the binding of pure labelled antigen onto the antibody under the effect of the unlabelled antigen that is to be determined. When the system is in equilibrium, the radioactivity bound to the antibodies is separated from the unbound radioactivity, and one or the other is then determined. On the basis of this measurement, the amount of the unlabelled antigen in the unknown sample can be determined. The sensitivity of the radioimmunoassay is within the concentration range 10^{-11}-10^{-17}mole and therefore it permits the quantitative determination of most of the various biologically active compounds that occur in biological systems in nanogram and picogram amounts. The method was substantially simplified by the utilization of the binding of antibodies onto solid supports (Catt et al., 1966; Wide and Porath, 1966). An example of a competitive radioimmunoassay technique is shown in Fig. 12.2 (Smith, 1990).

The use of antisera attached covalently to agarose, cellulose and Sephadex in radioimmunoassay systems for proteins and haptens was investigated by Bolton and Hunter (1973). They demonstrated that the covalent binding on the insoluble matrix did not affect unfavourably the antiserum. If a decreased sensitivity of the assay was observed, then it was caused simply by steric hindrance of high molecular weight

antigens. Guesdon and Avrameas (1981) described the immobilization of antibodies and antigens on magnetically responsive polyacrylamide-agarose beads and their use for the quantitation of total immunoglobulin E (IgE) and specific IgE in human sera. Their paper contains a table of magnetic suports for the immobilization of proteins in immunological techniques.

The magnetic solid phases used in radioimmunoassays are shown in Table 12.1. Gee and Langone (1983) studied immunoassays using antigen-coated plastic tubes and radiolabelled or enzyme-labelled Protein A. The enzyme and radioimmunoassays for

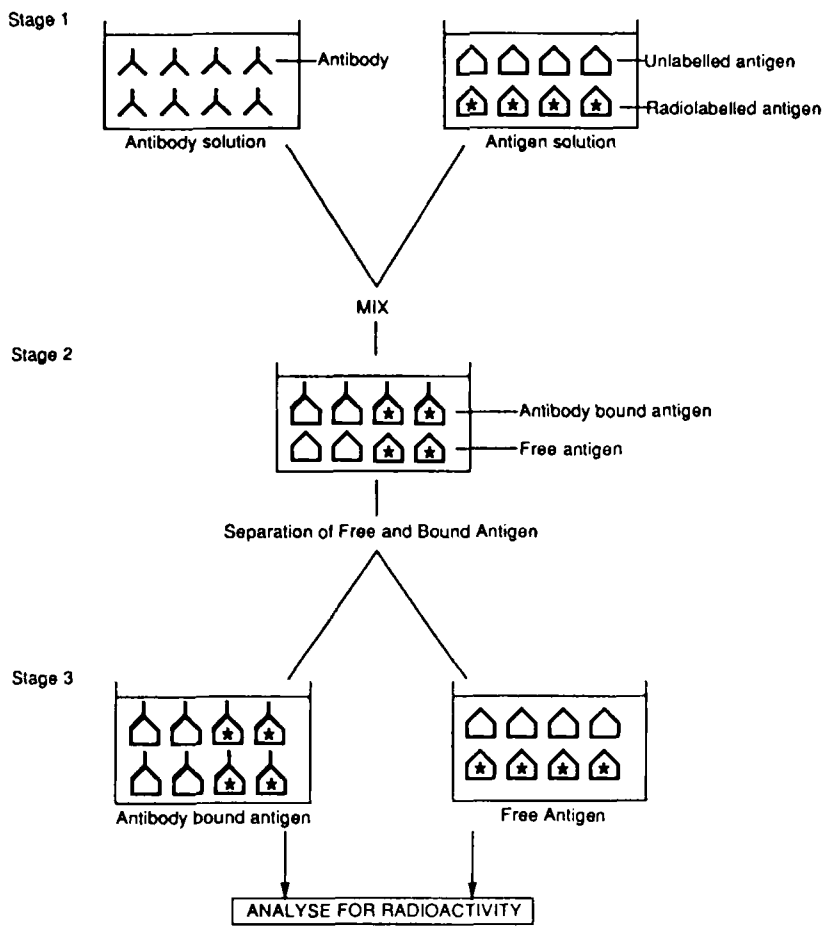


Fig. 12.2. Example of a competitive radioimmunoassay technique. Ratio of free to bound antigen gives original ratio of mixture. Reproduced with permission from C. J. Smith, in J. H. Rittenburg (Editor), Development and Application of Immunoassay for Food Analysis, Elsevier Appl. Sci., London and New York, 1990, pp. 3-27.

immunoglobulin were of similar sensitivity, whereas enzyme assays for haptens tended to be less sensitive. Bogdanove and Strash (1975) reported possible errors in radioimmunoassay, caused by the escape of radioiodine. Radioimmunoassay has become increasingly widely used, primarily in the field of drugs, hormones, chemotherapeutic agents, antibiotics, immunoglobulin, etc., as is evident from the reviews by, for example, Landon and Moffat (1976), Broughton and Strong (1976) and Cleeland et al.

Table 12.1.

Magnetic supports for the immobilization of proteins in immunological techniques

Solid phase	*Treatment of the solid phase*	*Coupling method*	*Use*
Iron oxide powder (magnetite, Fe_3O_4)	Silanization with p-aminobenzene trimethoxysilane	Activation by diazotation; azo bond formation mainly with tyrosine residues of protein	Radioimmunoassay of digoxin
Iron oxide powder (magnetite, Fe_3O_4)	Coated with diazoted m-diaminobenzene (Bismarck Brown)	Azo bond formation	Radioimmunoassay of thyroxine, human placental lactogen, and digoxin
Albumin magnetic microparticles: bovine serum albumin polymerized in presence of barium ferrite ($BaFe_{12}O_{19}$)			Thyroxine radioassays
Polyacrylamide-agarose containing iron oxide (Magnogel)		Activation with glutaraldehyde	Enzyme immunoassays of various antigens and antibodies
		Activation with glutaraldehyde	Affinity chromatography (immunoadsorbents)
		Activation with glutaraldehyde	Cell fractionation
Iron containing polymeric microspheres	Derivatized with diaminoheptane	Glutaraldehyde activation	Labeling and separation of cells

(1976). The book edited by Gupta (1975) is about the radioimmunoassay of steroid hormones.

An example of the application of radioimmunoassay in the solid phase is the determination of ornithine transcarbamylase synthesized in a coupled transcription-translation system, directed by the arg F gene carried on the specialized transducing bacteriophage (Eshenbaugh et al., 1974).

Anti-ornithine transcarbamylase antibody, bound covalently to Sepharose 4B (0.1 ml), was added to the solution to be assayed in a total volume of 0.5 ml, adjusted to 0.050 M phosphate, 0.15 M sodium chloride, pH 7.2. The assay mixture was mixed with continuous gentle rotation on a New Brunswick rotor drum at 5 rpm in an incubator at 33°C for 60 min, diluted with 5 ml of assay buffer, centrifuged at 500 g for 3 min and the supernatant was then discarded. The Sepharose was transferred to a Whatman CF/C 25 mm filter and washed with 50 ml of assay buffer. The radioactivity was determined using Beckman Ready-Solv VI scintillation fluid in a Beckman LS 230 liquid scintillation counter. The method is rapid, sensitive and specific for ornithine transcarbamylase and can be used in studies aimed at the elucidation of the control mechanism of the arginine synthetic pathway, carried out *in vitro*.

Since the initial studies by Yalow and Berson (1960) radioimmunoassays have developed into an extremely versatile analytical technique. The specificity is dependent on the antibodies and the sensitivity on both the antibodies and the radiolabel. However, radioisotopes have their drawbacks (Gould and Marks, 1988). The preparation of the radiolabelled antigen involves real risks, which are cumulative. Even when these are prepared commercially the product shows batch-to-batch variation and generally has a half-life limited to two months. Their toxic nature necessitates the application of strict regulatory control and their measurement requires the use of specialized, sophisticated and hence expensive equipment. The necessity for a separation step has prevented the development of simple automation.

12.2 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The disadvantages of radiolabels encouraged the search for alternatives. Engvall et al. (1971) and Van Weemen and Schuurs (1971) independently described the use of enzyme-labelled reagents. These are now well established and have allowed the development of a diverse range of assay protocols. The most general advantage of enzyme labels, compared to radiolabels, is their improved shelf-life. Enzyme labels may often be stored under sterile conditions for more than a year at 4°C or at room temperature when freeze-dried. Radiation hazards are obviously absent (Gould and Marks, 1988).

Engvall et al. (1971) used the principle of the radioimmunoassay described for the determination of rabbit immunoglobulin G (IgG), with the difference that instead of the labelling of antigens with radioactive isotope they conjugated it with the enzyme, using alkaline phosphatase. The basis of the competitive immunoassay is the separation of the free and antibody-bound antigen, so that the antigen can be determined quantitatively. This was facilitated by the fixation of the antibody onto the solid phase by physical adsorption on polystyrene tubes. The immunoassay was carried out in the following manner. Polystyrene tubes (80 x 11 mm) were each coated with 1 ml of a solution of antibody. The antibody solutions were diluted with 0.1 M sodium carbonate buffer of pH 9.8 or with phosphate-buffered saline. The tubes with the coating solution were allowed to stand at 37°C for 3 h and then stored in the same solution in the cold. Before determination, a suitable number of tubes were washed with 0.9% sodium chloride solution containing 0.05% of Tween 20. A 0.5-ml volume of one of the following was placed in each tube: (1) the standard solution of IgG; (2) the unknown sample; or (3) the buffer alone. This was followed by the addition of 0.1 ml of a dilute conjugate of akaline phosphatase and IgG (prepared by coupling with glutaraldehyde). All dilutions were carried out with a phosphate-buffered saline containing 1% human serum albumin and 0.02% sodium azide. The tubes were incubated at room temperature overnight (16 h) in a roller drum, so that the surface coated with the antibody was covered with the 0.6-ml volume present in the tubes. The tubes were then washed three times with a sodium chloride-Tween solution. The amount of the enzyme-linked IgG bound to the

tube coated with the antibody was determined by adding 1 ml of 0.05 M sodium carbonate buffer (pH 9.8) containing 1 mg/ml of p-nitrophenyl phosphate and 1 mM of magnesium chloride. After 30 min the reaction was stopped by the addition of 0.1 ml of 1 M sodium hydroxide solution. The absorbance measured at 400 nm was used to construct a standard graph by plotting the enzyme activity (increase in absorbance per unit time) of individual samples against their content of standard IgG.

Fig. 12.3, taken from the book of Mohr and Pommerening (1985), may serve as an example. It shows the ELISA technique for the determination of thyroxin in human serum (Kleinhammer et al., 1979). In the first step (immunosorption) the sample solution and a known quantity of thyroxine labelled with the covalently bound enzyme peroxidase are pipetted in an antiobody-coated plastic tube (e.g. polypropylene or polystyrene). The sample antigen and the labelled antigen compete with the solid-phase-bound antibodies, forming antigen-antibody complexes. After removing the

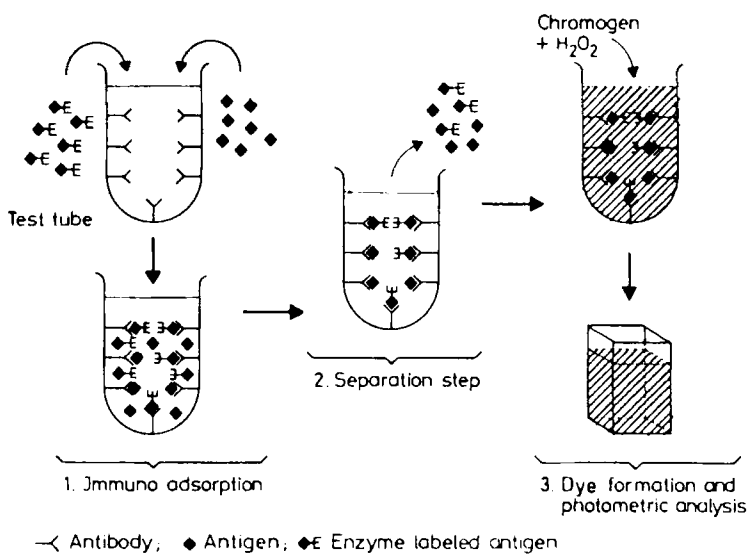


Fig. 12.3. Schematic representation of the determination of thyroxin by the ELISA technique. Reproduced with permission from P. Mohr and K. Pommerening, Affinity Chromatography, Practical and Theoretical Aspects, Marcel Dekker, New York and Basel, 1985, pp. 135-141.

unbound antigen molecules by washing (separation step), a chromogen [2,2-di-(ethylbenzthiozoline-6-sulphonate)] and H_2O_2 are added. The peroxidase label of the bound antigen then catalyzes dye formation, which can be followed spectrophotometrically at 405 nm in the case of this chromogen. The extinction determined after a given time correlates inversely with thyroxine concentration in the sample solution. The exact value is found by means of a standard curve that must be ascertained before the beginning of measurements using a set of solutions containing the antigen and labelled antigen in known concentrations.

The schematic representation of the ELISA technique allows us to characterize its essential features as follows.

ELISA is a solid phase assay using an enzyme label to measure the primary antibody-antigen interaction with the capability of being used to determine either antibody or antigen concentration. Briefly, one of the reactants is attached to a solid phase and the other reactant, antibody or antigen as appropriate, is allowed to react with it. This produces an antibody-antigen complex linked to the solid phase and any nonreacting material, e. g. antigen, can be removed by washing. This achieves the separation step, which can prove problematical in other assay formats. The extent of the reaction can then be determined by addition of the enzyme labelled fraction, if this was not added as a competitor in the original reaction mixture, and after further washing the enzyme substrate is added and the reaction product measured. There are a number of different variations on the methodology used for monitoring the antibody or antigen, either directly or indirectly (Smith, 1990). Fig. 12.4 shows examples of direct competitive and non-competitive ELISAs, and of an indirectly competitive ELISA. More details of these variants have been published by Rittenburg (1990b).

To minimize error in the interpretation of ELISA, a number of steps can be taken to facilitate standardization. Plasticware (96-well plates) varies from manufacturer to manufacturer, from batch to batch and with the manufacturer's post-moulding treatment. Generally speaking, more added antigen increases sensitivity up to a limit. Wright and Nielsen (1988) developed the essential features of a protocol for ELISA in 96-well plates for serodiagnosis of bovine brucellosis.

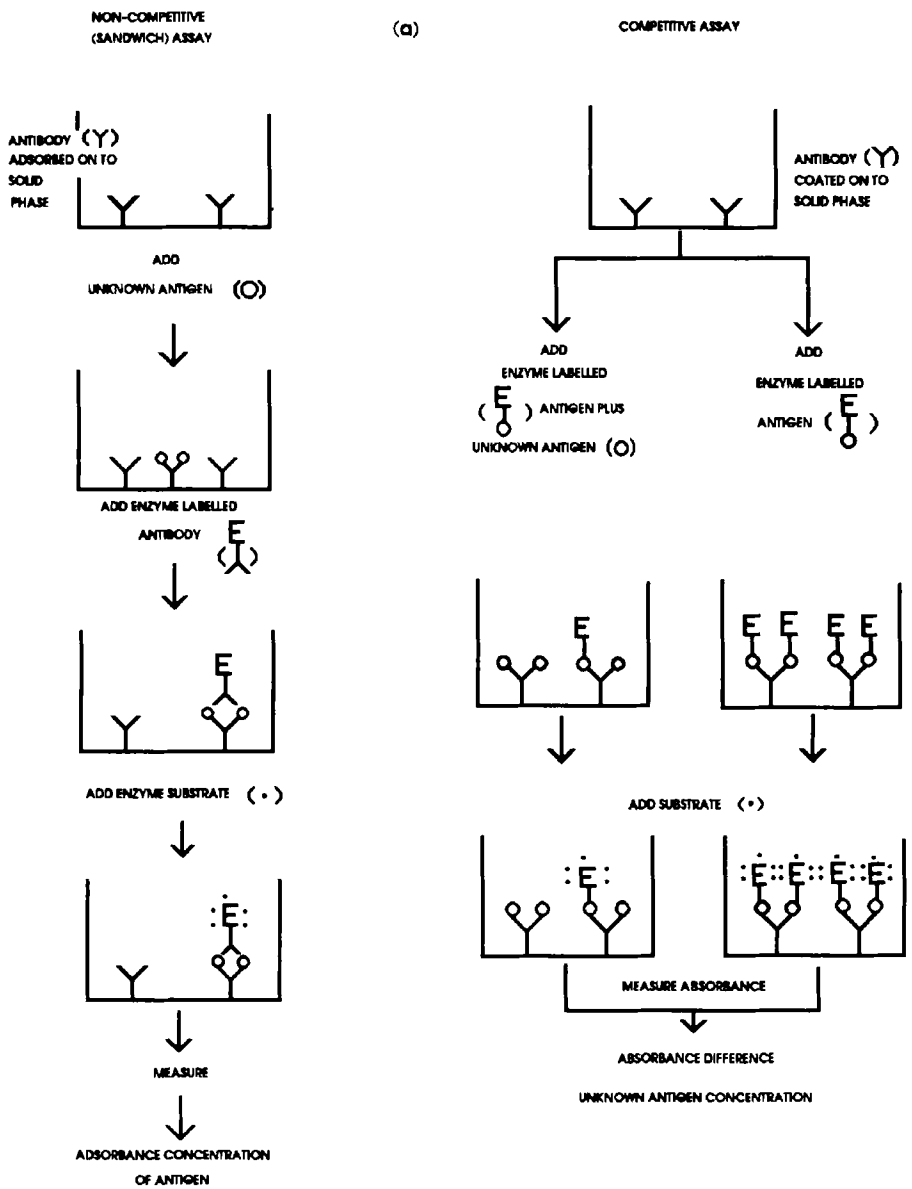


Fig. 12.4. (a) Examples of direct competitive and non-competitive enzyme-linked immunosorbent assays. Reproduced with permission from C. J. Smith, in J. H. Rittenburg (Editor), Development and Application of Immunoassay for Food Analysis, Elsevier Appl. Sci., London and New York, 1990, pp. 3-27.

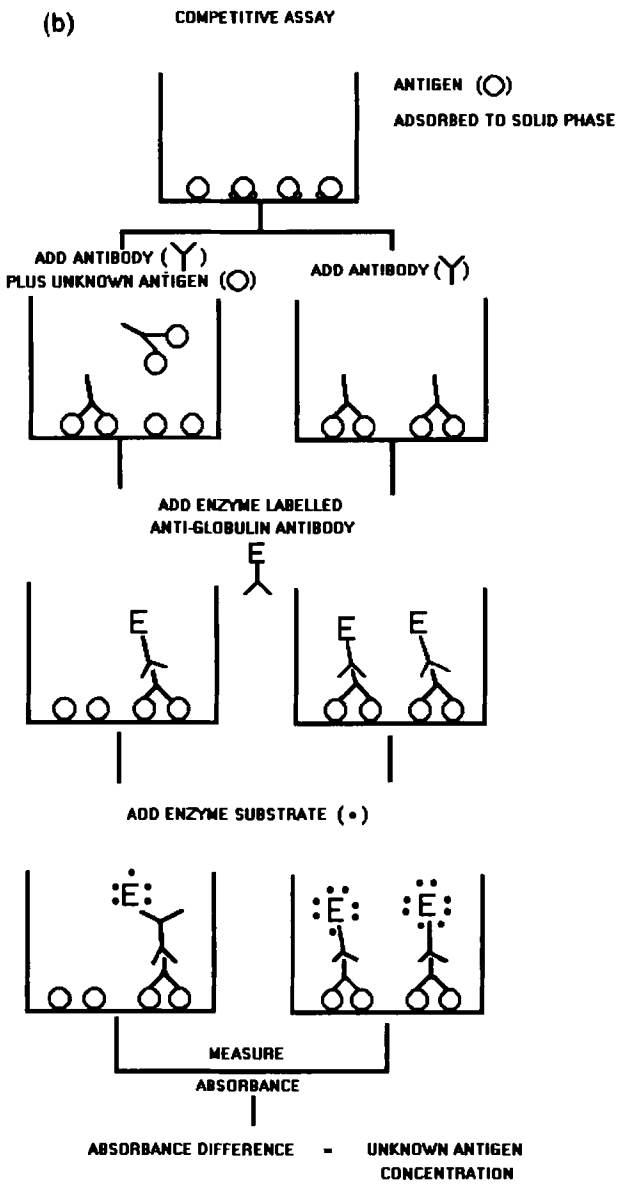


Fig. 12.4. (b) Example of an indirect competitive enzyme-linked immunosorbent assay. Reproduced with permission from C. J. Smith, in J. H. Rittenburg (Editor), Development and Application of Immunoassay for Food Analysis, Elsevier Appl. Sci., London and New York, 1990, pp. 3-27.

1. 96-well polystyrene plates are rinsed in distilled water immediately prior to use. Excess water is removed by sharply striking the plate, upside down, onto absorbent, lint-free material.

2. The plate wells are coated with 200 μl of antigen solution per well. The antigen solution contains 1.0 μg/ml of smooth lipopolysaccharide (S-LPS) prepared from a *B. abortus* strain in 0.06 M carbonate buffer pH 9.6. The plates are sealed with an adhesive plastic sheet and incubated at room temperature for approximately 18 h.

3. Serum samples to be tested are diluted 1:100 in 0.01 M phosphate, pH 7.2, containing 0.15 M NaCl and 0.05 % Tween 20 (PBS/T).

4. Immediately prior to use the antigen coated plates are washed four times with PBS/T and excess wash buffer is removed as in Step 1. The samples and controls are dispensed in 200 μl volumes in a quadrant pattern (Fig. 12.5). The plates are resealed and incubated for 3 h at room temperature.

5. Unbound serum proteins are removed by washing as above and 200 μl of appropriately diluted detecting reagent is added to each well. The detection reagent used initially was a commercially prepared horseradish peroxidase (HRP)-conjugated rabbit anti-bovine IgG (H + L). An HRP-conjugated mouse monoclonal anti-bovine L chain (prepared from ascites fluid and conjugated by the periodate method) is likely to replace the polyclonal conjugate. The plates are resealed, incubated at room temperature for 1 hour followed by washing in PBS/T.

6. Substrate (4 mM hydrogen peroxide) and chromogen (1 mM 2,2'-azino-bis(3-ethylbenzthiazolinesulphonic acid) in solution in 0.05 M citrate, pH 5.0, is dispensed in 200 μl amounts into each well. The plate is maintained at room temperature. Timing of the reaction begins immediately and the plate is placed on a plate shaker to assure uniform enzyme-substrate contact and formation of a uniform meniscus.

7. After exactly 4 min of development the plate is assessed for development in a photometric 96-well plate scanner pre-blanked on 200 μl of undeveloped substrate/chromogen solution in a separate plate. The average optical density (OD) of a target reference serum placed in 4 wells (Fig. 12.5) is used to extrapolate the time required for the target serum to achieve an OD of 1.0 using the equation:

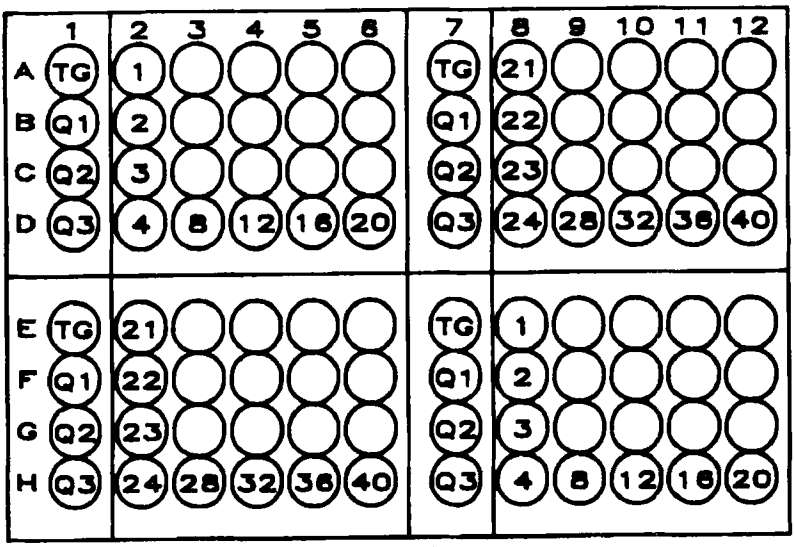


Fig. 12.5. Sample placement configuration used in the indirect ELISA; TG, target serum; Q1, positive quality control serum; Q2, negative quality control serum and Q3, buffer control (no serum). Reproduced with permission from P.F. Wright and K. H. Nielsen, in T. T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 129-146.

$$\text{Final development time} = \frac{OD\ at\ 4\text{min}}{4.56939} + 0.1393$$

Given an average development of about 10 min, OD units derived in this manner will not require between plate or day-to-day variability corrections.

8. The results are interpreted for diagnostic relevance using an O.D. threshold.

The enzyme immunoassay, involving antigen, hapten or antibody labelled with an enzyme, combines the specific recognition properties of antibodies with the high sensitivity characteristic of enzyme-based analytical techniques. The enzymes most commonly used in ELISA are horse-radish peroxidase (HRP), calf intestinal alkaline phosphatase (AP), and β-galactosidase from *Escherichia coli* (β-Gal.) HRP is currently the cheapest and has 10 - 15 % of its mass as carbohydrate, which is useful in some conjugation reactions. A review on the enzymes mentioned above and others most commonly used as labels in immunoassay systems has been published by Gould and Marks (1988), describing some of their properties.

Enzyme reactions may be made quantitative by a variety of measuring techniques, determined by the respective substrate and the features of the product resulting from this, such as luminescence technology, fluorimetry, potentiometry, amperometry or colorimetry. The most frequently applied technique, however, is the measurement of a coloured reaction product which is formed from a colourless substrate due to the catalytic activity of the marker enzyme. In this case the chromogen can be a direct product of the reaction of the marker enzyme, or can be formed as a consequence of a reaction coupled with an indicator enzyme or an auxiliary/indicator enzyme system.

Such combined reactions as recycling or amplifying systems result in a multiplication of the chromogen formation and, by this means, in a particularly sensitive enzyme detection. The optimum composition of substrate and stopping solutions for measuring activity of various marker enzymes (e. g. HRP, AP, β-Gal, urease etc.) have been described by Porstmann and Porstmann (1988). Colorimetry offers many advantages: great sensitivity, easy handling, simple and cheap measuring equipment and a visual way of evaluating the material. Haptens and antigens can be detected and quantified with a comparable sensitivity to radioimmunoassay (picomol to femtomol range). Chromogen concentrations can be differentiated both by simple photometers within the visible range (mostly 400 - 600 nm) and semiquantitatively or qualitatively by the naked eye. Hence ELISA makes visual yes - no decisions possible, which has opened up a new era: highly sensitive, specific and practicable screening tests under field conditions.

As an example we may mention ELISA using urease as the enzyme marker described by Lee and Lambros (1988). A visual, enzyme linked immunosorbent assay using urease (ELISA-U) as the enzyme marker was adapted for the rapid detection of antibody against *Plasmodium falciparum*. Flat-bottomed, 96-well microtitre plates were coated with *P. falciparum* soluble antigen obtained by saponin and NP-40 treatment of parasite cultures. Antibody was detected by successive incubations with test sera, urease-conjugated rabbit-human antibody, and urease substrate. Reactive sera developed a definite and easily observed purple colour. Sera from patients with single infections of *P. vivax* or *P. ovale* were unreactive. No cross-reactivity was noted with sera from patients with rheumatoid arthritis, filariasis, amoebiasis, schistosomiasis, dengue, scrub typhus,

leptospirosis, or toxoplasmosis. The procedure can be performed at room temperature and completed within 1 h. The sensitivity of the assay is comparable to that of the indirect fluorescent antibody test at all but the lowest dilutions tested.

Enzyme-linked immunosorbent assay has been used by Lucas et al. (1988) for the determination of contaminants resulting from the immunoaffinity purification of recombinant proteins. Contamination levels of less than 56 ppm of antibody were determined in the purified recombinant proteins.

Methods for the enzyme labelling of antigens, antibodies and their fragments have been described by Ishikawa et al. (1988). Their chapter characterizes conjugates of antibody-enzyme prepared by conjugation methods with maleimide, pyridyl disulphide, glutaraldehyde and periodate.

12.3 AMPLIFICATION SYSTEMS FOR ENZYME IMMUNOASSAY

The basic principles of all heterogeneous enzyme immunoassays are the same: an antibody (or antigen) linked to an enzyme is allowed to react with the corresponding immobilized antigen (or antibody) and the enzyme activity is then revealed by specific measurement. The most important elements in these procedures are the effectiveness of the antibody- (or antigen)-enzyme conjugate employed and the sensitivity of the enzyme detection method. Generally, the enzyme-antibody conjugate is prepared by covalently coupling the enzyme to the antibody. It was observed that partial denaturation of the enzyme often occurs during the coupling procedure. For this reason, enzyme immunoassays based on a biospecific interaction between the antibody and the enzyme have been proposed. As shown by Guesdon (1988), biospecific interactions, such as those between avidin-biotin, lectin-saccharide or antibody-antigen, could lead to a signal amplification which cannot be obtained with chemical binding. This is probably due primarily to the conditions used to link the enzyme molecules to the antigen-specific antibody; indeed in this case, the enzyme marker is used after mild modification (biotinylation) or even without any chemical modification (procedures based on the enzyme/anti-enzyme antibody or enzyme/lectin interactions), and secondarily to the increased number of enzyme molecules linked to each antibody

molecule. Use of microwell plates carrying hydrazide groups to enhance antibody immobilization in ELISA was described by Brillhard and Ngo (1991). Oriented immobilization of IgG after periodate oxidation resulted in greatly increased sensitivity. Furthermore, the hydrazide functionalized plates exhibited lower non-specific binding.

Biotin can be covalently bound to antibody or enzymes under relatively mild conditions at a high specific activity without affecting the antigen-binding capacity of the antibody or the activity of the enzyme. The use of biotinylated antibodies instead of antibody-enzyme conjugates minimizes the problem of steric hindrance. Biotin-avidin interaction is a versatile and specific method when an immunochemical detection method cannot be used (antigen-antibody cross-reactions). Many biotinyl derivatives are commercially available to bind biotin to the functional groups of proteins.

In contrast to hybrid antibody molecules with dual specificity, which must be prepared by reduction and subsequent reoxidation of a solution containing a mixture of antigen specific and enzyme-specific antibodies, the chimera antibodies are easily prepared with high yield. This technique offers several other advantages: in contrast to the classical enzyme immunoassay using enzyme-antibody conjugate, the chimera antibody technique may be performed with crude enzyme preparations, which could be of particular interest when the enzyme used as marker is difficult to obtain in pure form. Moreover the enzyme is used in its native form, i.e. without chemical modification, and thus its turnover rate is unchanged.

A certain background is always seen in the enzyme immunoassay technique. This background, which may be due to non-specific ionic interactions between the conjugate and the protein-coated plastic, lowers the detection capacity of this kind of assay. In contrast to the techniques using covalently prepared enzyme-antibody conjugate, the assays which employ biospecific interactions to link an enzyme to an antibody make use of homogeneous, unmodified proteins. Thus, non-specific binding, observed during the amplified immunoassay, may be eliminated by simply changing the pH of the incubation medium so as to neutralize the net electric charge of the protein causing the background. It is not possible to proceed in this way with classical enzyme-antibody conjugates because these preparations bind noncovalently to link an enzyme to an antibody, in

contrast to a covalent linkage. For example, it was possible to completely eliminate the non-specific binding of avidin to the solid phase by using solutions of alkaline pH and high ionic strength (Guesdon et al., 1979).

Numerous studies of the enzyme immunoassay have demonstrated the potential of this method for ultrasensitive immunoassays. Recent advances in biological technology have enabled substantial improvements to be made, and by intensifying the specific signal, assays have been designed which are even more sensitive than the radioimmunoassay technique. As examples we may mention a biotin-avidin sandwich ELISA for quantation of intact complement component C9 (Takata et al., 1989), quantitative determination of staphylococcal enterotoxin A by ELISA using a combination of polyclonal and monoclonal antibodies, and biotin-streptavidin interaction (Edwin, 1989) or serodiagnosis of tuberculosis tested against the immunoadsorbent antigen by ELISA with biotin-conjugated anti-human globulin and avidin-peroxidase reagents (Thongkrajai et al., 1989).

Kenney et al. (1990) developed quantitative two-site ELISA as a specific, quantitative and convenient method for measuring soluble proteins. The ELISA which they developed uses two populations of antibodies (Abs) to an antigen. Fig. 12.6 shows a schematic representation of quantitative two-site ELISA. An antibody to one site of the Ag is adsorbed onto an assay plate well. The second antibody to another site on the Ag contains attached biotin. This Ab-biotin conjugate is added to the well along with either known concentration of Ag or test samples. Ag is bound by both the adsorbed and biotinylated Abs. Streptavidin-peroxidase is added which binds to biotin on the second Ab *via* its streptavidin moiety. Following the addition of the appropriate substrate the peroxidase enzyme generates a product which absorbs visible light. Ag in test samples is quantitated by comparing the absorbance of the samples with the absorbances of known concentration of Ag.

A possible alternative to the biotin-avidin system is the fluorescein isothiocyanate (FITC)-anti-FITC system in which an FITC label is detected with monoclonal anti-FITC antibodies labelled with either an enzyme or a radioisotope. Harmer and Samuel (1989) employed alkaline phosphatase (AP) adsorbed onto microtitre wells as the target

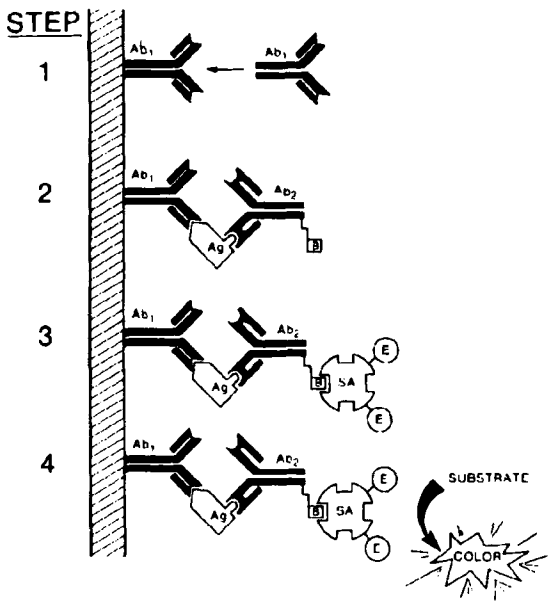


Fig. 12.6. The two-site ELISA. In step 1, Ab to one site (Ab_1) is adsorbed to a microplate well. In step 2, Ag is bound by both Ab_1 and Ab directed to a different site on the Ag (Ab_2). Ab_2 has a molecule of biotin (B) covalently attached to it. In step 3, streptavidin (SA) conjugated to an enzyme (E) binds biotin. In step 4, the complex is made visible by the enzymatic conversion of a substrate into a coloured product. Reproduced with permission from J. S. Kenney et al., in H. Zola (Editor), Laboratory Methods in Immunology, Vol. 1, CRC Press, Inc., Boca Raton, Florida, 1990, pp. 231-240.

antigen (Ag) in a model ELISA system. The Ag was then reacted with a monoclonal antibody to AP either unlabelled or labelled with (a) FITC and (b) biotin. The bound anti-AP was then detected with horse-radish peroxidase (HRP) conjugates of polyvalent anti-mouse IgG, and the FITC and biotin anti-AP conjugates with HRP conjugates of a monoclonal anti-FITC system proved to be of similar sensitivity as the biotin-streptavidin system detecting 140 a mol compared to 350 a mol of Ag. Both these methods of Ag detection were superior to the anti-mouse IgG reagent (2100 a mol). In contrast to biotinylated antibodies, FITC-labelled antibodies are highly coloured and fluorescent. These features aid the preparation, purification and characterization of conjugates. In addition, very low non-specific binding is encountered with enzyme conjugates of anti-FITC and this may confer an advantage over enzyme conjugates of avidin/streptavidin reagents.

12.4 ELISA-PLAQUE (ELISPOT) ASSAY

The technique, called the enzyme-linked immunosorbent assay (ELISA)-plaque or ELISPOT can be used to detect and enumerate a wide variety of secreting cell types. Sedgwick et al. (1990) described the basic elements of ELISA-plaque assays for the detection and quantitation of antibody-secreting lymphocytes specific for viral antigens or for the enumeration of cytokine-secreting cells. The general principles of the ELISA-plaque technique for the detection of cells secreting antibody or cytokine are shown in Fig. 12.7. The ELISA-plaque assay is based on the standard ELISA and differs only in the addition of cells rather than a solution (Step 3) and the use of a substrate that yields an insoluble, rather than a soluble end product (Step 6). Enumeration of antibody secreting cells with specificity for viral antigens is a useful tool in the characterization of virus-specific humoral immunity during the course of a virus infection. In contrast to the determination of virus-specific antibody titers in body fluids, this technique allows the quantitation of inflammatory virus-specific B-cells secreting virus-specific antibodies in individual organs or compartments of the body. Thus, development of local humoral immunity to viruses can be characterized with respect to kinetics, specificity for viral antigens, and quantity. As an example, quantitation of virus-specific antibody secreting cells isolated from rats has been described: a 96-well microdot blot chamber (e.g. BioRad Model "Bio-Dot" or Bethesda Research Laboratory Model "Hybri-Dot") with nitrocellulose or 25 square-well polystyrene replica dishes for bacteriology (Sterilin, Hounslow, U. K. or Greiner, Nürtigen, Germany) were used for antigen immobilization.

As a second example we may mention the ELISA-plaque assay used to estimate the number of cytokine-secreting cells. The principle is also shown in Fig. 12.7. The assay can be used to examine the cytokine production by cells present in any inflammatory infiltrate and may prove useful in the diagnosis and treatment of disease as well as in elucidating the underlying mechanisms of tissue pathology. The assay vessels used in this method were 24 round well or 96-well, flat bottomed polystyrene microtitre plates

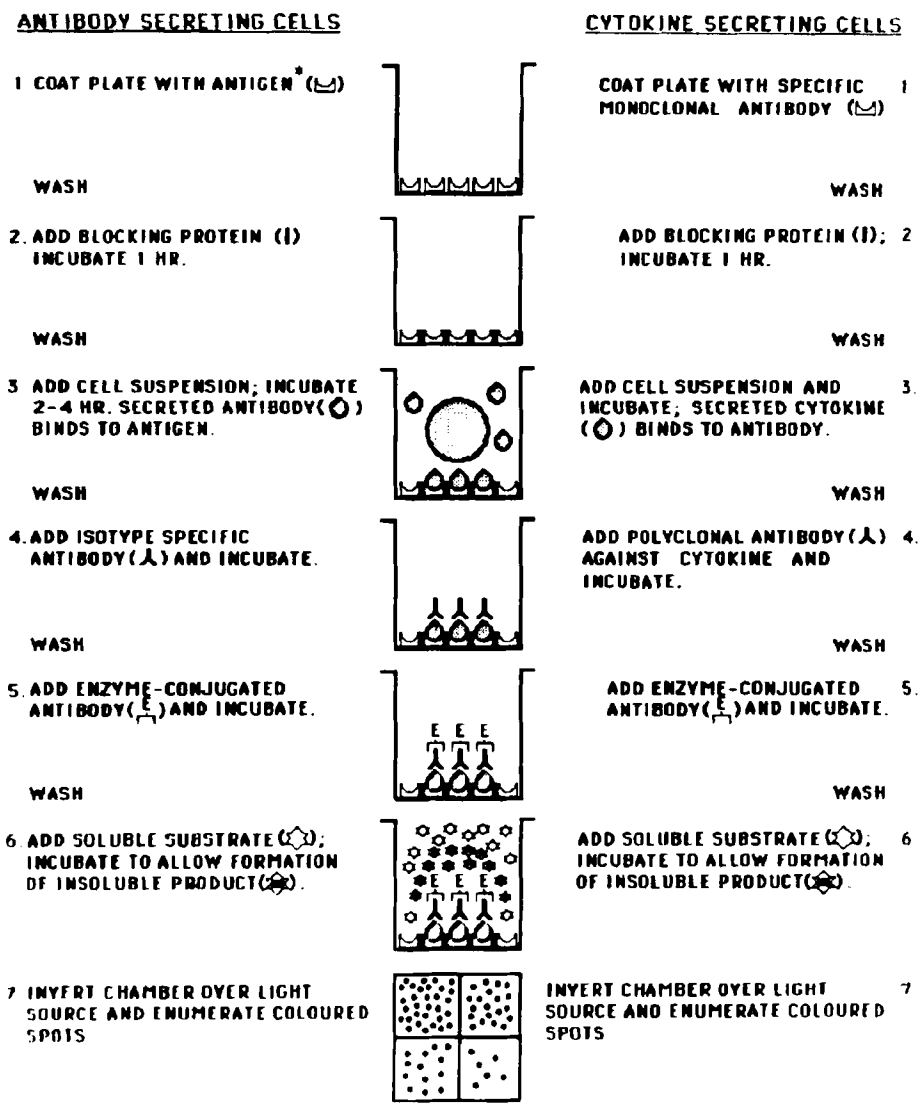


Fig. 12.7. General principles of the ELISA-plaque (ELISPOT) technique for the detection of cells secreting antibody or cytokine. Specific antibody may be substituted for measurement of total antibody secreting cells. Reproduced with permission from J. Sedgwick et al., in H. Zola (Editor), Laboratory Methods in Immunology, Vol. 1, CRC Press, Inc., Boca Raton, Florida, 1990, pp. 103-115.

(Nunc, Denmark). Cytokine-specific monoclonal antibodies for coating were purified by bioaffinity chromatography, because a purified product should be obtained.

12.5 CELLULAR ENZYME-LINKED IMMUNOSPECIFIC ASSAY (CELISA) AND ENZYME-LINKED LECTIN ASSAY (ELLA)

A micromethod that detects antibodies to cell-surface antigens was developed by Morris et al. (1982) under the name cellular enzyme-linked immunospecific assay (CELISA). The authors described an indirect immunoassay capable of detecting human anti-human leucocyte antigens (HLA) antibodies bound to lymphocytes. This CELISA utilizes an antiglobulin covalently linked to alkaline phosphatase to quantitate the amount of anti-HLA antibody bound to cell-surface human leucocyte antigens. During the CELISA V-bottom wells of polyvinyl chloride 96-well microplates (Dynatech, Inc., Alexandria, VA, USA) served as the recepticle in which as little as 5 μl of sera and as few as 25, 000 lymphocytes per well were incubated. The authors devised a rapid and simple technique to transfer the cells from the original V-bottom plate to a flat-bottom plate before adding the enzyme substrate. This strategy eliminated background noise due to the non-specific adsorption of the different protein immunoreactants to plastic. A schematic diagram of this method is shown in Fig. 12.8.

The detection of antibody bound to cell-surface antigens is of fundamental importance to basic and applied immunology. The work described in this article, as well as unpublished work of these authors, indicates that CELISA might be used to:

1. screen hybridoma culture fluids for the production of monoclonal antibodies to cell-surface antigens;
2. aid in the phenotyping of cellular antigens associated with subpopulations of cells;
3. detect and define antibodies to tumour-associated cell-surface antigens;
4. evaluate autoimmune disorders;
5. analyze antibodies directed towards platelet, granulocyte, and monocyte-endothelial cell antigens.

Since this assay is capable of sensitively and objectively quantitating antibody bound to cell-surface antigens, it may be of value in the areas of transplantation, blood banking,

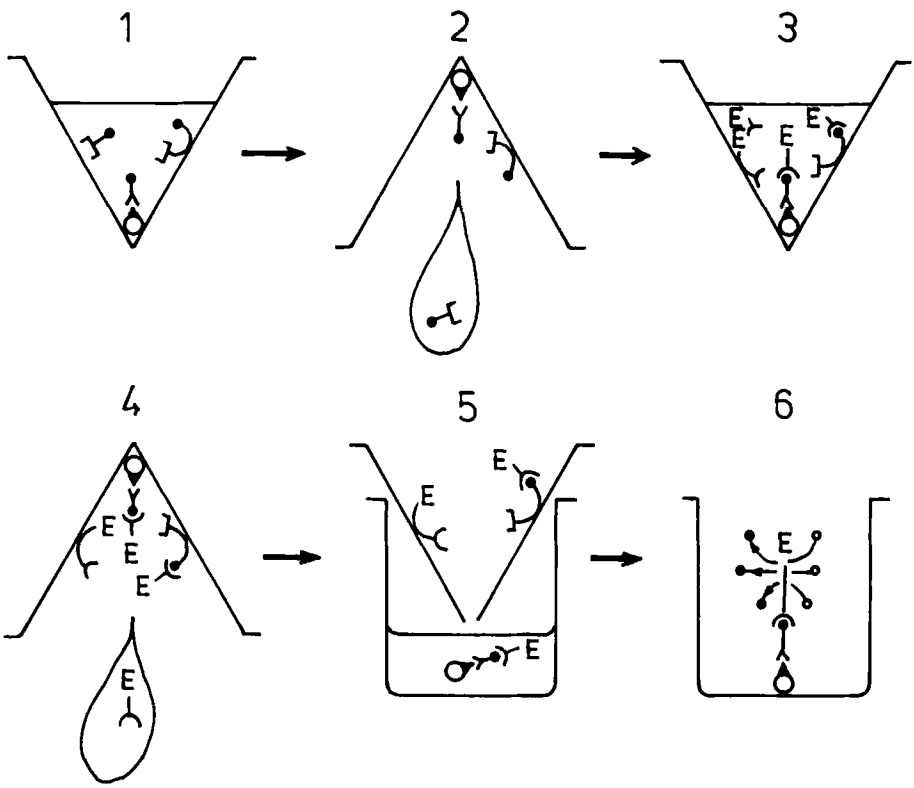


Fig. 12.8. A schematic diagram of the method used to perform the suspension-phase CELISA technique. Step 1: "First" antibody binds to its cell-surface antigen while some nonimmune immunoglobulin (Ig) adsorbs nonspecifically to the well surface. Step 2: Decantation of the unbound Ig after the target cells have been pelleted. Step 3: The enzyme-antiglobulin conjugate adsorbs nonspecifically to the well surface and binds to the "first" antibody on the cell and to adsorbed Ig on the well surface. Step 4: Decantation of unbound conjugate. Step 5: Transfer of target cells but not nonspecifically adsorbed immunoreactants from original V-bottom well to flat-bottom well. Step 6: Enzymatic conversion of clear substrate solution to a solution of coloured product. Reproduced with permission from R. E. Morris et al., Human Immunology, 5 (1982) 1-19.

autoimmune disease, tumour immunology, and the study of cell-surface differentiation and viral antigens.

The inhibition of endogenous cellular alkaline phosphatase activity in CELISA was studied by Morris and Horowitz (1984). They found that the endogenous alkaline phosphatase in human peripheral blood mononuclear cells was incompletely inhibited by EDTA or L-cysteine. Levamisole, however, completely inhibited endogenous cellular alkaline phosphatase without impairing the sensitivity of the CELISA. The use of levamisole is recommended for an assay that uses alkaline phosphatase conjugates to

detect molecules on the surfaces of cells that also contain endogenous alkaline phosphatase.

Another type of enzyme-linked immunoassay for the determination of antigens of blood cells by the use of membrane-associated immunoglobulins was described by Kiefel and Mueller-Eckhardt (1990). The principle of their method is a competitive enzyme-linked immunoassay (CELIA). This assay has been developed for the quantitation of platelet-associated immunoglobulins. However, it can be easily adopted to the determination of these immune proteins on other blood cells.

McCoy et al. (1983) described an enzyme-linked lectin assay (ELLA) as a simple microassay which may be used for the detection of specific carbohydrate units, either directly on immobilized glycoproteins or indirectly by inhibition of soluble sugars. The technique may be performed in a semiautomated and quantitative manner using commercially available ELISA plate readers. It is apparent that different lectins may be conjugated in a similar fashion to alkaline-phosphatase and employed for the detection and quantitation of carbohydrate units. ELLA was used by McCoy et al. (1984) to detect specific carbohydrate units on the surface of unfixed cells. The assay may be read by standard ELISA plate readers, since the cell-bound enzyme-lectin conjugate is specifically eluted from the cells prior to development of the conjugate. ELLA, when read using an ELISA plate reader, allows better detection and relative quantitation of specific surface carbohydrate units than is possible by standard immunofluorescence with fluorescein-conjugated lectins. The ELLA methodology may find use in blood banking and experimental cell research to detect cells bearing specific carbohydrate groups. Additionally it should be readily adaptable to microbiology for the detection of carbohydrate groups on fungal and bacterial cells.

12.6 MICROFLUORIMETRIC IMMUNOASSAY

In order to circumvent some of the disadvantages of radioimmunoassay, such as the instability of the radioactive labelling or the denaturation of proteins during the labelling, Haaijman and Bloemmen (1975) employed a fluorescent rather than a radioactive antiserum or antigen. They studied two types of fluorescent immunoassay systems: fluorescence inhibition immunoassay and fluorescence immuno-competition assay. The former system was studied with the use of human immunoglobulin G and mouse immunoglobulin M and the latter with ovalbumin. They used fluorescein or tetramethylrhodamine isothiocyanate for labelling. These methods were ten times less sensitive than the radioimmunoassay.

For the study of the distribution of tetanus antibodies in various classes of immunoglobulin, Hernandez et al. (1973) developed a simple immunofluorescent method. A 0.02-ml volume of a suspension of antigen, containing 10 mg of hydrated tetanus-toxicoid-agarose, was mixed with 0.2 ml of diluted (usually 1 : 10) serum to be tested and incubated at room temperature for 1 h. After washing the beads three times with a barbital buffer of pH 7.2, containig 1 M sodium chloride, 0.2 ml of rabbit anti-human immunoglobulin M, A or G was added, and both the incubation and the washing were then repeated. Finally, 0.2 ml of fluorescein-labelled goat anti-rabbit immunoglobulin was added and the incubation and washing were repeated. After the last wash the sediment was placed on a slide and observed in blue light under a fluorescence microscope. The presence of specific antibodies was revealed by brilliant green beads with fluorescent borders.

Good results of solid-phase radioimmunoassay for immunoglobulins were described by Daugharty (1981). However, fluorescence assay is potentially capable of spectacular sensitivity, but such sensitivity can only be achieved if the background luminescence is extremely low. Blood plasma, irradiated at around 350 nm, has a broad fluorescence spectrum with a maximum around 400 nm, and a tail extending to very long wavelengths. Many other materials, notably the plastics, often used for solid-phase supports or for disposable cuvettes, also have appreciable fluorescence. This background fluorescence

is not particularly intense, but nevertheless leads to a very poor signal-to-noise ratio if very high sensitivity is sought. This is a particular problem in homogeneous fluorescence immunoassay, where fluorescence measurement is required on a solution which may contain a high proportion of plasma.

These difficulties have ensured that, until recently, fluorescence immunoassay has not achieved the sensitivity of which it is theoretically capable. In connection with these difficulties Bailey et al. (1988) showed that terbium forms strongly fluorescent chelates with a variety of ligands containing aromatic groups. These chelates absorb at wavelengths characteristic of the aromatic group, and have emission spectra characteristic of terbium, consisting of a few narrow lines. These terbium chelates are suitable for the direct labelling of immunoassay reagents. Kronick (1988) described the use of phycoerythrin labels which results in significant increases in sensitivity of anywhere from two to ten times greater than that obtained with the use of conventional labels such as fluorescein. The summary of this chapter is that phycobiliproteins have become an important tool in the use of fluorescence in immunoassay. More details about fluorescence immunoassays are given in the book edited by Ngo (1988).

Fluorescence activated cell sorting (FACS) using fluorescent labelled antibodies to cell surface antigens to sort sub-populations of cells automatically has proved to be successful. The system depending on fluorescence resulting from the stimulation of the fluorochrome label by laser has been already developed commercially (Smith, 1990).

REFERENCES

Abraham, G.E. (Editor), Handbook of Radioimmunoassay, Marcel Dekker, New York, 1977, pp. 1-822.

Allen, J.C. and Smith, C.J., Trends Biotechnol., 5 (1987) 193-199.

Bailey, M.P., Rocks, B.F. and Riley, C., in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 187-197.

Blake, D.A. and Gould, B.J., Analyst, 109 (1984) 533-547.

Bogdanove, E.M. and Strash, A.M., Nature, 257 (1975) 426-427.

Bolton, A.E. and Hunter, W.M., Biochim. Biophys. Acta, 329 (1973) 318-330.

Brillhart, K.L. and Ngo, T.T., J. Immunol. Methods, 144 (1991) 19 - 25.

Broughton, A. and Strong, J.E., Clin. Chem., 22 (1976) 725-729.

Catt, K., Niall, H.D. and Tregear, G.W., Biochem. J., 100 (1966) 31c-33c.

Clark, B.R. and Engvall, E., in E.T. Maggio (Editor), Enzyme-Immunoassay, CRC Press, Inc., Boca Raton, Florida, 1983, pp. 167-179.

Cleeland, R., Christenson, J., Usategui-Gomez, M., Heveran, J., Davis, R. and Grunberg, E., Clin. Chem., 22 (1976) 813-816.

Collins, W.P. (Editor), Complementary Immunoassays, John Wiley and Sons, Chichester, New York, Brisbane, Toronto, Singapore, 1988, pp. 1-280.

Daugharty, H., Methods Enzymol., 73 (1981) 597-608.

Edwin, C., J. Clin. Microbiol., 27 (1989) 1496-1501.

Engvall, E., Jonsson, K. and Perlmann, P., Biochim. Biophys. Acta, 251 (1971) 427-434.

Eshenbaugh, D.L., Sens, D. and James, E., Advan. Exp. Med. Biol., 42 (1974) 61-73.

Gee, A.P. and Langone, J.J., Methods. Enzymol., 92 (1983) 403-413.

Gould, B.J. and Marks, V., in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 3-26.

Guesdon, J.L., in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 85-106.

Guesdon, J.L., Ternynck, T. and Avrameas, S., J. Histochem. Cytochem., 27 (1979) 1131-1139.

Guesdom, J.L. and Avrameas, S., Methods. Enzymol., 73 (1981) 471-482.

Gupta, D. (Editor), Radioimmunoassay of Steroid Hormones, Verlag Chemie, Weinheim, 1975, pp. 1-224.

Haaijman, J.J. and Bloemmen, F.J., Ann. N. Y. Acad. Sci., 254 (1975) 137-150.

Harmer, I.J. and Samuel, D., J. Immunol. Methods, 122 (1989) 115-121.

Hernandez, R., Just, M. and Bürgin-Wolff, A., Z. Immun. Forsch., 145 (1973) 376-384.

Ishikawa, E., Imagawa, M., Hashida, S., Yoshitake, S., Hamaguchi, Y. and Ueno, T., J. Immunoassay, 4 (1983) 209-327.

Ishikawa, E., Hashida, S., Kohno, T. and Tanaka, K., in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 27-55.

Kemeny, D.M. and Challacombe, S.J. (Editors), ELISA and Other Solid Phase Immunoassays, John Wiley and Sons, Chichester, 1988a, pp. 1-367.

Kemeny, D.M. and Challacombe, S.J., in D.M. Kemeny and S.J. Challacombe (Editors), ELISA and Other Solid Phase Immunoassays, Theoretical and Practical Aspects, John Wiley and Sons Ltd., Chichester, New York, Brisbane, Toronto, Singapore, 1988b, pp. 31-55.

Kemp, M., Husky, S. and Jensenius, J.C., Clin. Chem., 31 (1985) 1090-1091.

Kenney, J.S., Masada, M.P. and Allison, A.C., in H. Zola (Editor), Laboratory Methods in Immunology, Vol.1, CRC Press, Inc., Boca Raton, Florida, 1990, pp. 231-240.

Kiefel, V. and Mueller-Eckhardt, C., in H. Zola (Editor), Laboratory Methods in Immunology, Vol.1, CRC Press, Inc., Boca Raton, Florida, 1990, pp. 241-248.

Kleinhammer, G., Deutsch, G., Linke, R. and Staehler, F., in W. Vogt (Editor), Georg Thieme Verlag, Stuttgart, 1979, pp. 43-49.

Kronick, M.N., in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 163-185.

Landon, J. and Moffat, A.C., Analyst, 101 (1976) 225-243.

Langone, J.J. and Van Vunakis, H., Methods Enzymol., 73 (1981) 79-739.

Langone, J.J. and Van Vunakis, H., Methods Enzymol., 92 (1983) 147-647.

Lee, M. and Lambros, C., Amer. J. Trop. Med. Hyg., 39 (1988) 421-426.

Lucas, C., Nelson, C., Peterson, M.L., Frie, D., Vetterlein, D., Gregory, T. and Chen, A.B., J. Immunol. Methods, 113 (1988) 113-122.

Maggio, E.T. (Editor), Enzyme-Immunoassay, CRC Press, Inc., Boca Raton, Florida, 1983, pp. 1-295.

Mc Coy, Jr., J.P., Varani, J. and Goldstein, I.J., Anal. Biochem., 130 (1983) 437-444.

Mc Coy, Jr., J.P., Varani, J. and Goldstein, I.J., Exp. Cell Res., 151 (1984) 96-103.

Mohr, P. and Pommerening, K., Affinity Chromatography, Practical and Theoretical Aspects, Marcel Dekker, New York and Basel, 1985, pp. 135-141.

Morris, R.E., Thomas, P.T. and Hong, R., Human Immunol., 5 (1982) 1 - 19.

Ngo, T.T. (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 3-495.

Pesce, A.J. and Michael, J.G., in D.M. Kemeny and S.J. Challacombe (Editors), ELISA and Other Solid Phase Immunoassays, Theoretical and Practical Aspects, John Wiley and Sons Ltd., Chichester, New York, Brisbane, Toronto, Singapore, 1988, pp. 181-195.

Porstmann, B. and Porstmann, T., in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 57-84.

Rasmussen, S.E., in W.P. Collins (Editor), Complementary Immunoassays, John Wiley and Sons Ltd., Chichester, New York, Brisbane, Toronto, Singapore, 1988, pp. 43-55.

Rittenburg, J.H. (Editor), Development and Application of Immunoassays for Food Analysis, Elsevier Appl. Sci., London and New York, 1990a, pp. 3-259.

Rittenburg, J.H., in J.H. Rittenburg (Editor), Development and Application of Immunoassays for Food Analysis, Elsevier Appl. Sci., London and New York, 1990b, pp. 29-57.

Sedgwick, J., Cooke, A., Dörries, R., Hutchings, P. and Schwender, S., in H. Zola (Editor), Laboratory Methods in Immunology, Vol.1, CRC Press, Inc., Boca Raton, Florida, 1990, pp. 103-115.

Smith, C.J., in J.H. Rittenburg (Editor), Development and Application of Immunoassays for Food Analysis, Elsevier Appl. Sci., London and New York, 1990, pp. 3-27.

Takata, Y., Moriyama, T., Fukumori, Y., Yoden, A., Shima, M. and Inai, S., J. Immunol. Methods, 117 (1989) 107-113.

Thongkrajai, P., Lulitanon, V. and Chamnanvanakit, C., J. Med. Microbiol., 30 (1989) 101-104.

Urbanek, R., Kemeny, D.M. and Samuel, D., J. Immunol. Methods, 79 (1985) 123-131.

Van Weemen, B.K. and Schuurs, A.H.W.M., FEBS Lett., 15 (1971) 232-236.

Wide, L. and Porath, J., Biochim. Biophys. Acta, 130 (1966) 257-260.

Wright, P.F. and Nielsen, K.H., in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York and London, 1988, pp. 129-146.

Yalow, R.S. and Berson, S.A., J. Clin. Invest., 39 (1960) 1157-1175.

Zola, H. (Editor), Laboratory Methods in Immunology, Vol.1, CRC Press, Inc., Boca Raton, Florida, 1990, pp. 81-265.

This Page Intentionally Left Blank

Chapter 13

Several examples of the application of biospecific adsorption in medicine

Bioaffinity chromatography can be used for the isolation, determination or removal of many biologically active compounds from the human body. Many examples have been given in Chapter 9. Bioaffinity chromatographic techniques have been also used many times to study molecular interactions, to reduce unwanted material by extracorporeal removal, or to use antibody-targeted drugs. Several examples will be shown but they are by no means exhaustive.

13.1 STUDIES OF VARIOUS COMPONENTS IN THE HUMAN BODY

The application of bioaffinity chromatographic techniques for separations and studies on molecular interactions of the components of the blood coagulation system has been reviewed by Andersson (1981).

The plasma coagulation system, which is responsible for the formation of fibrin, consists of fourteen components, nearly all of them being proteins. Fig. 13.1 shows the coagulation-fibrinolysis system, the coagulation being shown on the left-hand side. The coagulation system is often called the coagulation cascade or "waterfall", because it begins with a small triggering effect in a sequence of reactions involving one protease activating another protease, and is then amplified to a large final effect, the formation of the clot. Most of the reactions occur on surfaces such as in the contact activation phase, which is believed to take place on the subendothelium of the damaged vessel, or in the activation of prothrombin and Factor X, which occurs on the surface of the aggregated platelets. In the later stages of the process the thrombin formed cleaves off the two fibrino-peptides from fibrinogen, exposing the polymerization sites, resulting in the formation of fibrin polymers and a fibrin gel.

As the coagulation cascade begins with a small triggering effect which is strongly amplified, it would be a rather labile system if there were no other controlling factors

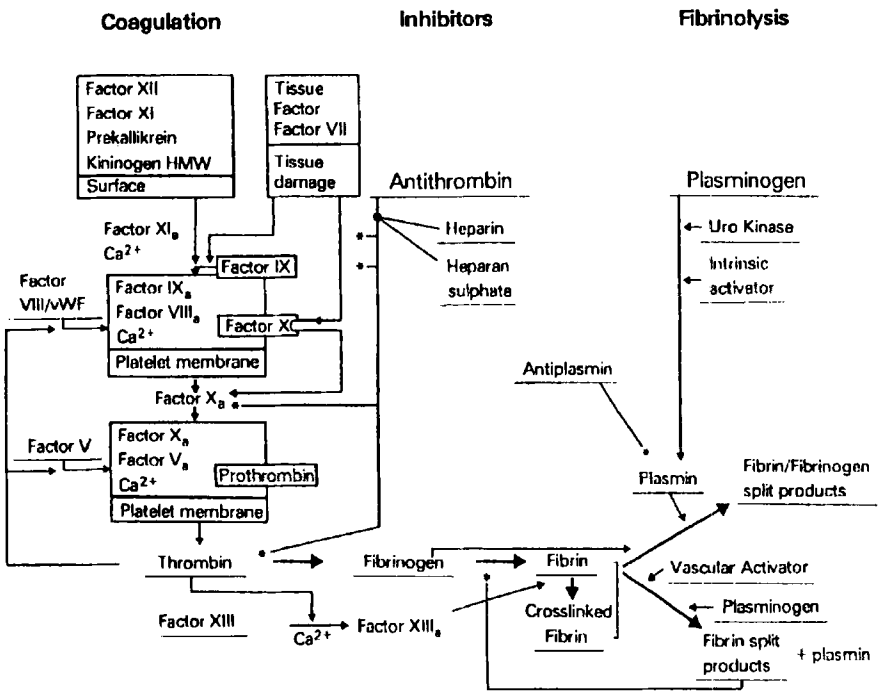


Fig. 13.1. Coagulation-fibrinolysis system. * , Inhibition; ● , rate enhancement.

present. Such factors are present, however, one of the most important being the coagulation inhibitors.

Antithrombin III is the main coagulation inhibitor and it is also identical with heparin cofactor, i.e. the factor in plasma, which is necessary for the anticoagulant activity of heparin. Antithrombin inhibits coagulation enzymes, both early and late in the coagulation cascade.

Another important defence system which protects against thrombosis is the fibrinolytic system. In this system the proenzyme plasminogen can be activated to the protease plasmin, which then degrades the fibrin clot or the thrombus.

Much work has been devoted to purifying the various coagulation factors and other components of the haemostatic system. One reason for this has been the need to gain

knowledge regarding the structure and properties of the separate components, which is necessary in order to understand how the whole system functions. Another reason has been that, for the clinical treatment of an hereditary deficiency of certain coagulation factors, there is a need for the development and production of concentrated preparations of certain coagulation factors. A good example of this is Factor VIII, which is needed for the treatment of classical haemophilia (haemophilia A).

The work has been fairly successful, as most of the components have been purified and more or less well characterized. Bioaffinity chromatographic methods have been used fairly often in the various procedures devised for purification. There are also several reasons why bioaffinity chromatography should be of particular value in this field. First, as is evident from Fig. 13.1, there is a number of interactions between the various components in the haemostatic system that could potentially be used for bioaffinity chromatographic purposes. Second, the concentrations in plasma of most of the factors are very low, which requires that the separation procedures used are very efficient and specific. This is also supported by the fact that many of the coagulation factors have very similar structures and properties and thus are difficult to separate from each other. Finally, several of the coagulation components are labile and it is advantageous to use gentle purification procedures, a requirement which is often fulfilled by the bioaffinity chromatographic methods.

Bioaffinity chromatographic techniques have also been used successfully to clarify which parts of fibrinogen and fibrin interact during fibrin polymerization, which parts of the plasminogen molecule interact with lysine, and the nature of the Factor VIII/Von Willebrand Factor complex. The discovery of the heterogeneity of heparin with respect to anticoagulant activity and binding to antithrombin III is also important.

It can be expected that bioaffinity chromatographic techniques will be of great value in further studies of the interactions and mechanisms involved in the haemostatic system. In particular, surface-dependent reactions will probably be studied using this type of technique. Various models of the different steps in the coagulation cascade, for instance, could be constructed by various gel-bound systems.

Finally, as regards practical applications, there is one example where bioaffinity chromatography has been the essential step for the purification of a component of clinical importance, viz., antithrombin III. Without applying bioaffinity chromatography it would not have been possible to prepare it on a large scale at reasonable cost. Antithrombin III is now used for the treatment of hereditary deficiency cases in connection with surgery, childbirth and thrombosis. Direct depletion and recovery of human coagulation factor IX from plasma using immobilized monoclonal antibody was described by Bessos et al. (1988). Development of an immunoaffinity process for factor IX purification was published by Tharakan et al. (1990). Bioaffinity chromatography of fibroblast growth factors on substituted polystyrene was described by Jacquot Dourges et al. (1990).

Snake venoms represent a very complex mixture, including about 80 protein components of various biological activities: procoagulant, fibrinogenolytic, prothrombin-converting and many others. Multi-step procedures are usually necessary to obtain an enzyme of desirable purity using common separation methods. Fořtová et al. (1990) isolated fibrinogen-converting enzyme ficozyme from the venom of *Bothrops asper* by one-step bioaffinity chromatography on Blue Sepharose.

As early as 1978 Torchilin and coworkers described the preparation and some of the properties of heparin-bound α-chymotrypsin. The conclusion of their paper was that heparin-bound enzymes could have a wide range of medical applications. Immobilized enzymes for thrombolytic therapy were summarized by Torchilin et al. (1988). Beginning in 1980 the neutral proteinase streptokinase (EC 3.4.24.4) from *Staphylococcus aureus* immobilized on oxidized dextran was produced under the name Streptodekaza in the USSR on an industrial scale. It has been used for the treatment of acute myocardial infarction, acute pulmonary artery thromboembolism, peripheral arterial and deep vein thrombosis, and haemophthalmia. Table 13.1 shows a comparison of the clinical efficacy of streptokinase/heparin and Streptodekaza/heparin administration in the treatment of patients with acute myocardial infarction.

However, Poznansky (1988) described several serious drawbacks which have limited the common use of enzymes in medicine: (1) availability of the enzyme in a sufficiently

Table 13.1.

Comparison of a clinical efficacy of streptokinase/heparin and streptodekaza/heparin administration in the treatment of patients with acute myocardial infarction

Clinical criterion	*% of cases*	
	Streptokinase/ heparin (n = 37)	*Streptodekaza/ heparin (n = 30)*
Relief of pain syndrome	63.9	83.8
Fast ECG dynamics (first 24 h)	45.7	67.6
Prolonged course and secondary infarction	13.3	5.4
Cardiac tamponade	13.3	2.7
Circulatory insufficiency (%)		
On admission	42.8	59.4
Disappearance on 14th day	20.0	51.3
Specific complications		
Hemmorrhagia	71.4	-
Thromboembolism	14.3	5.4
Allergic reactions	26.6	-

pure and nontoxic form; (2) biodegradation of administered enzyme due to proteolysis and heat inactivation; (3) immunological reactivity of the administered enzyme, usually a foreign protein, often bacterial; (4) delivery of enzyme to appropriate and specific sites of action tissues, cells, and often intracellular organeles. His approach to the development of possibilities for enzyme replacement therapy was to use albumin, a natural plasma protein, to immobilize enzymes in a soluble conjugated form in an effort to produce a stable, nonimmunogenic and potentially targetable enzyme product.

The influence of various agents on the fibrinolytic activity of soluble and immobilized urokinase was studied by Ohshiro et al. (1988). They immobilized urokinase on the surface of an ethylene-vinyl acetate copolymer (Evatate) which was used for intravenous catheters. Fig. 13.2 shows the influence of a combination of soluble and immobilized urokinase with inhibitor, antibody, cephalothin sodium (CET, cephem antibiotics) and their mixtures on their fibrinolytic activity. The experiment was performed at 37°C after 1 h incubation. The storage stability of immobilized urokinase may be explained in such

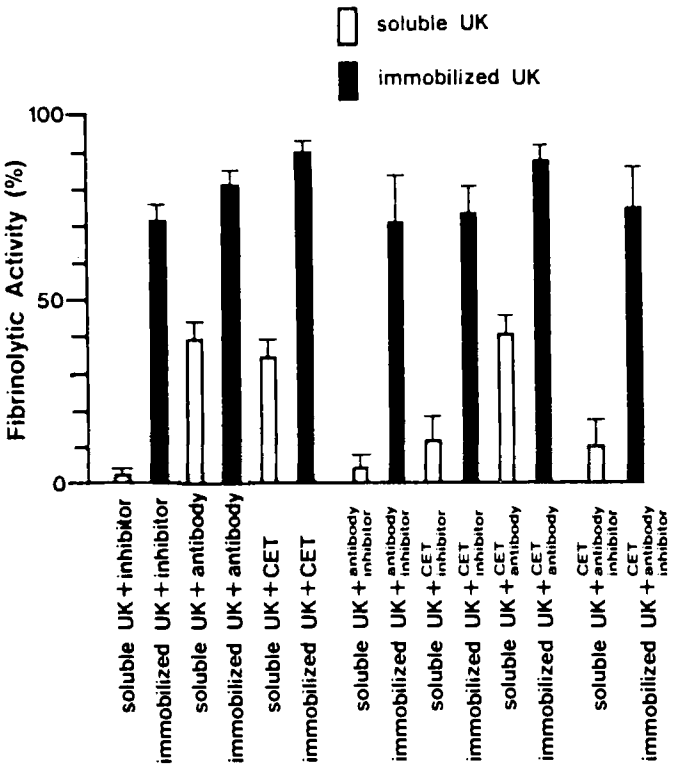


Fig. 13.2. Influence of various agents on fibrinolytic activity of soluble and immobilized urokinase. Reproduced with permission from T. Ohshiro et al., Methods Enzymol., 137 (1988) 529-545.

a way that the amino group in the lysine residue of a urokinase molecule is protected by immobilization, resulting in protection against proteolysis by lysine-specific protease and against autolysis by urokinase itself.

Urokinase-treated Evatate tubes for intravenous catheters have been studied in clinical applications. Fig. 13.3 shows the fibrinolytic activity of urokinase-treated Evatate tubes after catheterization. Seventy catheters were utilized for total parenteral nutrition in 50 patients after surgery and in 20 terminally ill patients. Fever due to catheterization was noted only in five patients, but there was no correlation between thrombus formation and pyrexia. On the basis of the results acquired, the thrombus formation seems to correlate not with the catheterization period, but with the fibrinolytic

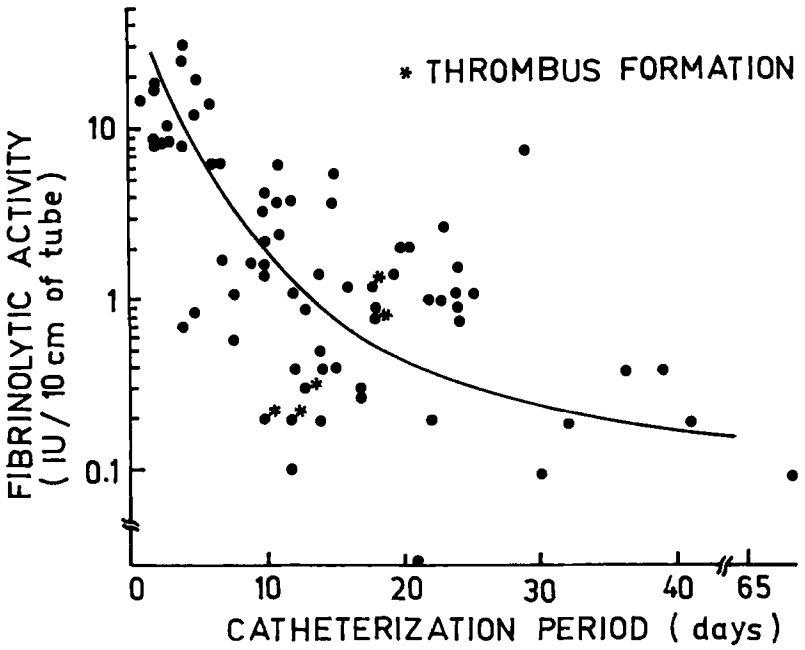


Fig. 13.3 Fibrinolytic activity of urokinase-treated Evatate tube after catheterization. Reproduced with permission from T. Ohshiro et al., Methods Enzymol., 137 (1988) 529-545.

activity. The fibrinolytic ability of urokinase-treated tubes decreased abruptly after clinical application in contrast to the stability *in vitro*.

The application of immobilized proteinase in medicine has been described by Turková (1990). Immobilization *via* reductive alkylation for the coupling of chymotrypsin to periodate-oxidized cellulose beads was used for the preparation of a proteolytic wound cleansing agent. In clinical practice chymotrypsin immobilized on bead cellulose not only possessed proteolytic activity with respect to the necrotic tissues, pus and fibrin, but also appeared harmless towards healthy tissue. In agreement with the hydrophilicity and porous structure of the cellulose matrix, it possessed a high absorption capacity. The exudate containing degraded tissue necroses and bacteria was sucked from the wound. The attachment of enzyme to the carrier prevented the autolysis of the former and thus any losses of activity during its action. Because of the immobilization proteolytic activity is localized on the surface of the wound only, the extended

proteolytic activity of chymotrypsin led to the release of necroses from the wound to a great extent and to its gradual cleansing. The early infected secretion was actively observed between powder particles. A combination of the two principles of the wound powder action - namely the proteolytic and the absorption one - helped to achieve rapid purification of infected necrotic defects, formation of pure granulation and quick healing of the wound (Turková et al., 1983; Štamberg et al., 1988).

Another reported use of bioaffinity chromatography in medicine is the separation of human pepsins from fresh human gastric juice or from an extract of human gastric mucosa obtained from resected parts of the stomachs of patients suffering from gastric carcinoma or ulcer diseases (Kučerová, 1986 and 1989). It may be suggested that the amount of enzymes may correlate with genetic type and gastric disease.

Many ligands have been described that interact with specific receptors on cells, with subsequent effects on cellular physiology. Advances in immunology and molecular biology have greatly enhanced our understanding of how ligands and receptors interact. The development of both polyclonal and monoclonal antireceptor antibodies has allowed the isolation and subsequent characterization of a wide variety of receptor structures. Molecular cloning techniques allowed the elucidation of the primary amino acid sequences of many receptors and their cognate ligands. A review on the development and use of receptor binding peptides derived from antireceptor antibodies has been published by Wiliams et al. (1989).

13.2 EXTRACORPOREAL REMOVAL OF SUBSTANCES IN VIVO

It has been proved that antibodies and immune complexes may cause some diseases. The therapy of many of these immunologically mediated illnesses was dependent on the use of immunosuppressive drugs which suppress host immunity non-specifically. Specific elimination from the circulation of immune substances that are pathological in the disease might represent another therapeutic approach. Schenkein et al. (1971) eliminated antibodies against bovine serum albumin (BSA) from the plasma of positively immunized rabbits by letting their blood circulate through an immunoadsorbent prepa-

red by binding BSA to bromoacetylcellulose. Using a similar system in a similar manner, DNA antibodies were eliminated specifically from the blood of actively immunized rabbits (Terman et al., 1974, 1975). Immunoadsorbents for the elimination of serum hepatitis antigen from blood, blood plasma and plasma products were prepared by Charm and Wong (1974) by coupling goat serum with the antiserum hepatitis antigen-antibody on Sepharose 2B after activation with cyanogen bromide. The elimination of substances from the blood by bioaffinity chromatography was studied further by Plotz et al. (1974) and Scharschmidt et al. (1974), who employed albumin attached to Bio-Gel A5m for the elimination of bilirubin and other albumin-bound substances.

Terman et al. (1976) used BSA immobilized on a microcapsule for the elimination of antibodies against BSA from canine blood. The microcapsule chambers were composed of a 8 x 2.5 cm I.D. glass cylinder with a 40-mesh stainless steel screen at its ends. This chamber was filled with approximately 400 microcapsules of diameter 1.0-1.1 mm and then connected with a Travenol roller pump using polyethylene tubes. The capsules were washed with 2 l of water at 30°C for 60 min, hydrolysed with 500 ml of 3 M hydrochloric acid by circulating it for 60 min at 30°C and a flowrate of 30 ml/min, and then washed with water. A 12.5% solution of glutaraldehyde in 0.1 M borate buffer of pH 8.3 was circulated at 30°C for 15 min at a flow rate of 30 ml/min, and the capsules were then washed with 0.1 M borate buffer of pH 8.3 at 30°C. After the exchange of the tubes, a solution of 200 mg of BSA with 0.2 μg of [^{125}I]BSA added as a marker in 0.9% sodium chloride solution was circulated through the capsules for 30 - 60 min. Finally, 200 ml of 10 μM $Na_2S_2O_5$ solution were circulated through the capsules at a flowrate of 200 ml/min. After washing with 0.9% sodium chloride solution, when the wash water did not contain any ^{125}I (approximately 500 ml), the capsules were transferred in solution into a siliconized chamber for *in vivo* use. In this manner, 32.1 - 34.5 mg of BSA was bound to microcapsules.

A scheme of the extracorporeal system *in vivo* is shown in Fig. 13.4. The dogs were anaesthetized with sodium pentobarbital and the femoral artery and vein were canulated with wide-bore polyethylene tubing. Sodium heparin (3 mg/kg) was injected intravenously and the femoral artery and venous catheters were connected to a Travenol

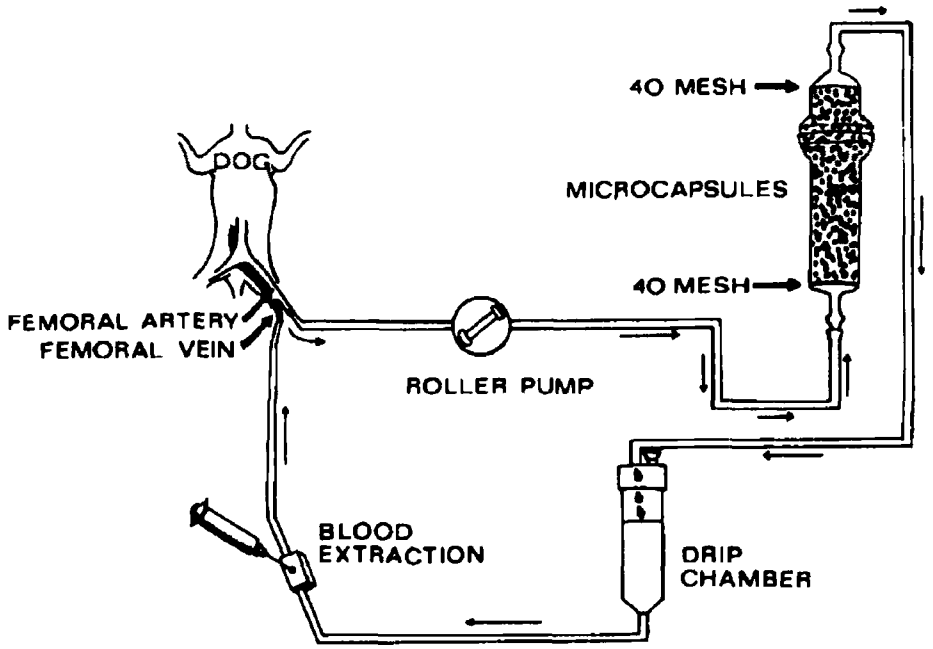


Fig. 13.4. Schematic representation of the in vivo extracorporeal circulation system. Reproduced with permission from D.S. Terman et al., J. Immunol., 116 (1976) 1337-1341.

roller and then to the microcapsule chamber. The latter was coated with silicone before use. The insertion of a three-way stopcock permitted the interruption of the blood flow through the chamber at 7 - 12 min intervals, so as to prevent the microcapsules from impeding the flow by clogging the screens. The chamber was connected to a bubble trap and then to the femoral vein. The heparinized blood was circulated through the extracorporeal system for 20 min before the injection of antibodies. The flowrate was kept at 200 ml/min during the whole procedure. Blood samples were withdrawn from the venous line and sampled for antibodies at various intervals.

The rabbit antiserum against BSA and human serum albumin (HSA) was infused simultaneously into the anaesthetized dogs, using a dilution at which the antiserum binds 95% of its corresponding antigens. For the anti-BSA this was a 1:520 dilution and for the anti-HSA a 1:270 dilution. After a 12 min equilibration the BSA microspheres were introduced into the extracorporeal circulation. The specific reduction of BSA-binding

antibodies in the four dogs investigated was fastest during the first 15 min after the introduction of BSA microcapsules, while the drop was much slower after the next 60 min. The anti-HSA binding remained constant in the same time interval. In order to prove that the observed reduction in the BSA binding is not caused by the release of BSA from microcapsules during the experiments, the vital organs and the serum of the dogs were analysed for the presence of ^{125}I at the end of the experiments. No significant radioactivity was found in the organs or in the serum of the dogs. A haematocrit and a leucocyte count in the dogs were carried out before and after the extracorporeal circulation through BSA microcapsules and had almost identical values. Only one of the four dogs displayed a reduction in leucocyte count. After the termination of the experiments, the microcapsules were analysed for thrombotic materials or cellular debris, which, however, could not be demonstrated. The specific removal of circulating antibodies against BSA in a canine host with a BSA collodion charcoal extracorporeal immunoadsorbent was described by Terman et al. (1978). A cartridge system containing Protein A immobilized over a charcoal collodion matrix was used in clinical application by Terman et al. (1981), who treated five patients with recurrent breast cancer.

Extracorporeal removal of cholesterol from blood with heparin-agarose conjugates was studied by Lupien et al. (1980) as a means of reducing the plasma cholesterol concentration in two patients with homozygous familial hypercholesterolemia. The decrease in plasma cholesterol was mainly due to the removal of low density lipoproteins. The circulating level of high density lipoproteins was unaffected by this treatment. Lupien et al. (1982) used the same extracorporeal technique with batch bioaffinity chromatography in two familial hypercholesterolemic heterozygote patients for a period of two years. In both cases low density lipoproteins (LDL)-cholesterol levels were drastically lowered and high density lipoproteins (HDL)-cholesterol levels were normalized. No undesirable side effects were noted during the two year experimental period. These results confirmed the safety and simplicity of this technique for considerably lowering LDL-cholesterol levels in whole blood of familial hypercholesterolemic patients who do not adequately respond to conventional medical treatment.

Selective immunoadsorption (SI) systems were defined by Buffaloe (1983) as extracorporeal plasma treatment systems that employ immunologic reagents for the selective removal or modification of specific plasma components. The immunologic reagents mentioned in his paper include not only antigens and antibodies, but also other molecules with similar binding properties, such as Protein A, which binds certain classes of immunoglobulins (cf. Table 3.3 in Section 3.3), or bovine conglutinin, which binds components of the complement system. The SI system's contribution to disease treatment is revealed by its safety, efficacy, and economics. Table 13.2 shows these three areas, which provide the framework for establishing system design approaches.

Extracorporeal blood treatment systems must conform to general criteria of biological nonreactivity and physiological compatibility. The use of biological materials in SI

Table 13.2.

SI system design

Safety (biocompatibility)	
Biological nonreactivity	*Physiological alignment*
Sterility	Electrolytes
Pyrogen-free	Proteins
Nontoxicity	Temperature
Stability	pH
Thrombogenicity	Volume
Side reactions	
Purity	
Efficacy	
Disease	*Treatment*
Pathogenic plasma component identified	Quantity
Location	Frequency
Quantity	Duration
Generation rate	
Economics	
Cost	*Value*
Immunoadsorbent	Patient population
Immobilization technology	Reimbursement
	Cost/benefit
Complete therapy system	Alternative treatments

systems presents a particular challenge in that this material may be immunogenic if released into the patient, and the process of binding of immune reactants may initiate undesirable biological reactions, such as complement activation. Additional challenges, such as rendering biological material sterile and pyrogen-free during the processing of SI systems, are of particular importance because of the fragile nature of many of these immunological reagents.

Efficacy considerations constitute the single most challenging area for the development of immunoadsorbent therapy systems. Identification of the specific pathogenic plasma components leads to delineation of their distribution and activity in the body and thus can indicate treatment regimens. Without such an understanding, treatment efficacy must be seriously questioned. In general, the more selective a system design may be, the more information that is necessary concerning the disease pathogenesis. Maintenance of the desired biological activity of immune reactants during processing presents unique challenges. Technology developed from laboratory preparations of biological material may be helpful in this regard.

Economic criteria can be divided into cost and value considerations. At various points in the development of SI systems, economic factors dominate either the technical progress or the practical aspects of a project. The overall development costs of SI systems will likely be significant, thus the critical evaluation of economic factors may become a dominant consideration when compared with alternative approaches.

An appropriate overlap between laboratory, animal, and clinical studies at various stages of development can result in timely progress toward the realization of useful SI therapy systems.

Laboratory studies are helpful in a number of ways. The identification and quantitation of the particular circulating pathogenic species is a high priority for the establishment of the basis of the SI system therapy protocols. Sizing experiments can yield valuable information concerning physical SI system design, cost, and compatibility with extracorporeal technology. Safety and biocompatibility testing will reflect the suitability of the system for animal and human exposure.

Animal models offer opportunities to study interrelationships between the SI system and living organisms that do not exist under laboratory conditions. Experiments using normal animals are useful in studying toxicity and biocompatibility, and the effectiveness of an experimental SI system may be examined in association with passive infusion of target material. If models involving active immunization disease-like states are adduced, studies might aid in projections of the effectiveness of the test SI system in the treatment of human disorders. Spontaneous animal disease models could be quite helpful in developing therapy strategies for clinical studies.

Experimental human studies involving SI systems present the same considerations as any new approach to disease treatment. Analysis of risk/benefit versus current therapy alternatives is the primary consideration. In many cases, therapeutic plasma exchange treatment regimens can serve as the "control" therapy for the evaluation of efficacy. The monitoring of long-term patient exposure to biological material, particularly immunologic parameters, should be a part of such studies.

A number of creative research groups which have contributed to the development of SI systems were described in this very lucid paper of Buffaloe (1983). Several examples of SI system research activities in animal and clinical studies are included.

Bensinger et al. (1985) adapted immunoadsorption columns capable of removing anti-A or anti-B red cell antibody from plasma to a whole blood perfusion system by coating the silica with a thin layer of collodion and incubating with albumin (1%). Patients were given heparin, 25 units/kg intravenously, followed by a continuous infusion of 3% citrate and heparin, 10 units/ml, to achieve a ratio of 1:16 - 1:20 (anticoagulant to whole blood) before delivery through the columns. Effective and specific removal of anti-A or anti-B antibody was achieved with this technique. The effects on coagulation parameters and the complement system were acceptably small.

A critical review on Protein A immunoadsorption techniques in clinical trials was published by Fer and Oldham (1985). They summarized that the role of Protein A immunoadsorption/immunoactivation as a form of cancer therapy requires a lot of further clarification before such procedures can be considered effective.

Freiburghaus et al. (1988) described extracorporeal systems for the adsorption of antibodies against Factor IX in patients with haemophilia. Their chapter describes the *in vivo* elimination of inhibitors by two different extracorporeal bioaffinity chromatography systems. The first was a group-specific system in which immunoglobulins were adsorbed to matrix-bound Protein A in a continuous two-column plasma system. In the second system the antibodies against Factor IX were specifically adsorbed to purified Factor IX (FIX) covalently linked to macrobeads of agarose in an extracorporeal whole-blood system. The clinical set up for the adsorption of specific antibodies in a continuous whole-blood system using FIX-Sepharose is shown in Fig. 13.5.

The whole-blood system is controlled by an immunotherapy monitor, ITM 10 (Gambro AB, Lund, Sweden). The apparatus consists of two pumps, one for blood and one for the addition of sodium citrate (ACD). The monitor is also equipped with pressure gauges and air detectors for the protection of the patient as well as of the columns.

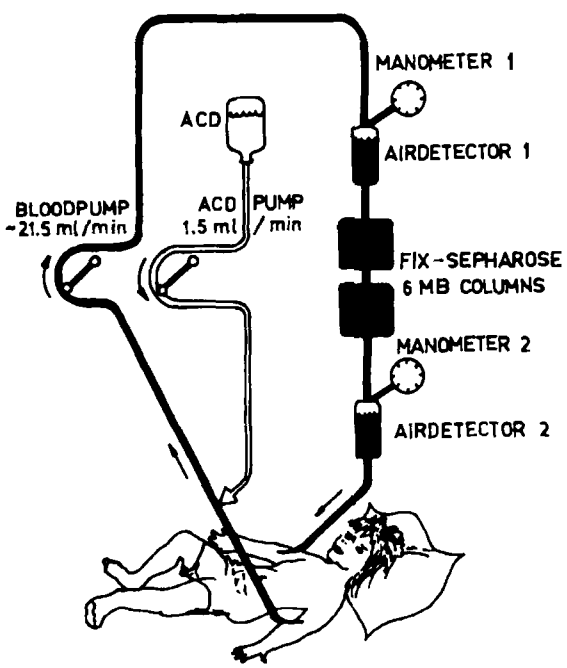


Fig. 13.5 Clinical set up for adsorption of specific antibodies in a continuous whole-blood system. Reproduced with permission from C. Freiburghaus et al., Methods Enzymol., 137 (1988) 458-466.

Venous blood is drawn at a rate of approximately 20 ml/ min and citrated directly. While the blood passes over the column, it is depleted of its antibodies against Factor IX. After adsorption the blood is retransfused to the patients. The extracorporeal volume of the system is only 200 ml.

The first patient treated was a 10-year-old boy with severe haemophilia B and a high inhibitor titre. The conclusion of the case report is that these adsorption techniques are both safe and rapid. With citrate added to the blood there are no signs of activation of the coagulation, of the fibrinolytic system or the complement one, nor is there any sign of haemolysis.

Circulating immune complexes (CIC) are already known to be present in cancer patients and are responsible for much of the cancer-associated immunosuppression. Removal or modulation of these "blocking factors" can reverse the immunosuppression. Protein A from *Staphylococcus aureus* has the unusual property of binding to CIC with high avidity. Use of Protein A as an immunoadsorbent in extracorporeal immunotherapy bioaffinity columns has resulted in antitumour and antiviral responses in animals.

Messerschmidt et al. (1988) developed a multicentre trial to assess toxicity and antitumour response with this biologic response modifier alone. Overall, 24% (21 of 87 patients) had objective tumour regressions, including both partial responses (PR) and less than PR. No complete responses (CR) were observed. Responses were observed in acquired immune deficiency syndrome (AIDS)-related Kaposi 's sarcoma (six of 17 PR; two of 17 < PR; overall, 47%), breast adenocarcinoma (five of 22 PR; three of 22 < PR; overall response, 36%), colon adenocarcinoma, (one PR, one < PR; overall response, 11%), and non-oat cell lung carcinoma (two of seven < PR). The procedure was well tolerated and could be performed on an outpatient basis. No adverse reaction was observed in 735 of 1113 treatments (66%). The most common adverse effect was an "influenza-like" syndrome consisting of fever and chills. Pain was present in 12% of the patients. There were no study-related deaths. Serum IgG and CIC levels did not change statistically due to therapy in responding or nonresponding patients. Complement levels remained within the normal range. Liver and renal tests remained stable

throughout the study. In summary, Protein A immunoadsorption of plasma is well tolerated in the outpatient clinic, has demonstrated antitumour activity in resistant solid tumours, and functions as a biological response modifier.

The equipment containing magnetic beads developed by Ugelstad et al. (1988) has been used successfully in clinical application for the depletion of tumour cells. The scheme is shown in Fig. 8.25 in Section 8.3.6. The efficiency of the same immunomagnetic beads in purging human myeloma cells from bone marrow *ex vivo* was evaluated by Shimazaki et al. (1988). Bone marrow purging for multiple myeloma by avidin-biotin immunoadsorption was described by Lemoli et al. (1989). Avidin-biotin immunoadsorption was used to remove neoplastic plasma cells from the bone marrow of patients with multiple myeloma.

13.3 SELECTIVE TARGETING OF DRUGS

Most pharmacologically active agents are low molecular weight compounds which rapidly penetrate into almost all tissues and body fluids and are efficiently and rapidly removed from the body by natural excretion mechanisms. In order to maintain the therapeutic effect, large and repeated doses must be applied. Attachment of drugs to macromolecular carriers alters their rate of elimination from the body and limits their uptake by cells to the process of endocytosis. They accumulate in the lysosomal compartment of the cell where they are released from the macromolecular carrier by the action of lysosomal enzymes and subsequently exhibit their pharmacological effects (Říhová and Říha, 1985).

There are many serious diseases caused by extensive proliferation or hyperactivity of some cell subpopulations. Among them certainly the most common are different types of cancer and autoimmune diseases. The cytotoxic and immunosuppressive drugs which are used for the treatment of these diseases, and in transplanted patients, are not specific enough i.e. not only is the pathological process affected by the therapy, but the suppressive activity affects other normal cellular systems, especially the hematopoiesis, gonads, normal functions of mucous membranes, and urinary tract. These side effects,

especially after intensive treatment, very often lead to unwanted complications, such as a dramatic increase of sensitivity to different bacterial or viral infections, and are therefore the limiting factors for the use of the most effective and cytotoxic substances. For this reason, at the beginning of this century Ehrlich suggested attachment of the drugs to carriers capable of targeting them specifically to the affected tissue.

In recent times the development of targetable drugs has become the topic of acute interest in many laboratories. Some serum protein nitrogen mustard complexes with high chemotherapeutic selectivity were described by Wade et al. (1967). Another example is chemotherapy which uses nucleic acid as carrier. Trouet et al. (1972) published a paper on chemotherapy using lysosomes with a DNA-daunorubicin complex. The carrier for cytostatic drug daunomycin used by Kitao and Hattor (1977) was lectin concanavalin A. Varga et al. (1977) stated that the carrier can be also a hormone; a melanotropic-daunomycin conjugate shows receptor-mediated cytotoxicity in cultured murine melanoma cells. Recently, attention has been focused on the possibility of treating malignant melanoma with polymeric N-(2-hydroxypropyl)methacrylamide (HPMA) adriamycin (ADR) conjugates with covalently bound α-melanocyte stimulating peptide hormone (MSH). It was found that polymer bound MSH *in vivo* promotes the interaction of HPMA copolymers with melanoma cells in culture. An HPMA-ADR conjugate targeted with MSH demonstrated antitumour activity against intraperitoneal and subcutaneous melanoma, increasing the lifespan of mice (Ulbrich, 1991). Table 13.3 is adopted from the review of drug targeting published by Gregoriadis (1977).

Wilchek (1978) described two different approaches to the preferential concentration of designer drugs in the target tissue, both based on the extraordinary specificity of an immunological reaction. The first approach, termed immunotherapy, concerns the production of antibodies to specific marker antigens on cancer cells. With the help of complement such antibodies should kill the cancerous cells, leaving the normal cells untouched. The second approach, called affinity or bioaffinity therapy, utilizes antibodies as carriers for cytotoxic drugs in order to destroy tumour cells chemically. In this

Tab 13.3.

Drug carriers in medicine and biology

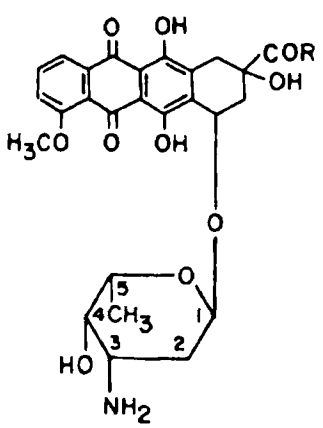
Carrier	*Associated drugs*
Macromolecules	
Immunoglobulins	Methotrexate, daunomycin, adriamycin, chlorambucil, radioactive iodine , diphtheria toxin, glucose oxidase
Desialylated glycoproteins	Lysozyme, albumin
Albumin	Amanitin, phalloidin, deoxycholic acid
Fibrinogen	Dichloroethylphosphoramide
Deoxyribonucleic acid	Daunomycin, adriamycin
Dextran	Insulin, noradrenaline, amphetamine, novocaine, coenzyme B_{12}
Cells	
Erythrocytes	β-glucosidase, β-galactosidase, glucuronidase, asparaginase, glucose oxidase, methotrexate, adriamycin
Hepatocytes	Uridine diphosphate glucuronyltransferase
Synthetic systems-non-biodegradable	
Nylon semi-permeable microcapsules	Carbonic anhydrase, uricase, asparaginase, urease, catalase
Polyacrylamide gel	Asparaginase
Liquid surfactant membranes	Urease
Glass beads	Uricase
Synthetic systems-biodegradable	
Albumin microspheres	6-mercaptopurine, actinomycin D
Multiple oil emulsions	Naltrexone
Lactic acid polymers	Cyclazocine
Liposomes	Lysozyme, amyloglucosidase, neuraminidase, α-mannosidase, glucocerebroside, β-glucosidase, hexosaminidase A, asparaginase, peroxidase, glucose oxidase, lectins,immunoglobulins, insulin, diphtheria toxoid, Poly(I):Poly(C), actinomycin D, 5-fluorouracil, methotrexate, bleomycin, cytosine arabinoside, 8-azaguanine, mechlorethamine, bichloroethyl nitrosourea, colchicine, penicillin G, steroids, cyclic AMP, pepstatin, glutamate, nitroblue tetrazolium

Association of drugs with carriers can be effected through covalent, hydrophohic, hydrogen or other types of bonding. In synthetic systems passive entrapment of drugs is also possible

approach potential carriers can be various molecules possessing an affinity to certain cells.

If bioaffinity therapy is to succeed, both the carrier and the drug must retain their activity after they are chemically linked. In particular the activity and specificity of the carrier must remain intact, since the conjugated drug may regain its activity following its release into the target cell. Fig. 13.6 shows the structure of drugs which Wilchek (1978) coupled to antibodies specific to several experimental murine tumour cells. Three different methods of covalent binding of the drug to antibody were checked. In all cases the amino group of the sugar moiety of the drug was used.

Water-soluble carbodiimide was employed to bind the drug through its amino group to the carboxyl groups of the antibody. About four drug residues per antibody were bound. Unfortunately, this method gave conjugates which were almost devoid of drug activity. Dialdehydes, especially glutaraldehyde, were also used to crosslink the amino groups of the protein with that of the drug. This method gave higher ratios of drug to antibody, about 7 - 10 moles per mole; but most of the drug and the protein was aggregated due to protein cross-linking. In the third method periodate cleavage of the C_3-C_4 bond of the amino sugar residues of the drug was also tested. This cleavage produced aldehyde groups capable of reacting with amino groups on the protein.



R = CH_2OH, adriamycin

R = CH_3, daunomycin

Fig. 13.6. Structure of drug.

Subsequent reduction of the resultant Schiff base with sodium borohydride gave a stable conjugate. An average of 2 - 5 moles of drug per antibody was obtained in different runs. The bond was stable over several weeks of storage at neutral pH and the drug was not dissociated from the antibody. No binding occurred when the drug was mixed with the protein without periodate oxidation.

In order for this approach to succeed, the drug and antibody should retain their potency when bound together. The drug activity of the conjugated daunomycin antibody was tested *in vitro* on tumour and normal cells, by measuring the inhibition of cellular RNA synthesis expressed by [^{3}H]-uridine incorporation into the cells. In these studies it was found that the conjugates retained substantial amounts of both drug and antibody activity. The drug activity of these conjugates was less than that of free daunomycin at low concentrations; but conjugated and free drug produced the same inhibition of RNA synthesis at higher concentrations. In addition, the free drug was more active after a short incubation time, whereas prolonged incubation produced equivalent maximum effects with both the free and bound drugs. In a drug-antibody conjugate comprising two moles of drug per mole of antibody, 64% antibody activity was retained. At six moles per mole only 25% activity was left.

To determine whether daunomycin covalently bound to antitumour antibodies exhibited selective cytotoxicity against the tumour cells, daunomycin was conjugated to immunoglobulins containing antibodies against two different mouse lymphoid tumours. The drug was shown to preferentially affect cells against which the antibodies were prepared, as measured by inhibition of uridine incorporation.

The periodate oxidation method yielded conjugates which contained about 2 - 5 moles of drug per mole of antibody. This amount seemed to be too low for *in vivo* studies, since large quantities of antibodies would be required. Therefore alternative methods of binding daunomycin to andibodies were developed, which resulted in conjugates comprising higher degrees of substitution with concomitant retention of antibody and drug activity.

In order to increase the amount of drug coupled to the antibody, dextran was used as a bridge between the antibody and daunomycin. The dextran was oxidized with

sodium periodate to give the corresponding polyaldehyde, and daunomycin was coupled to part of the aldehydes *via* its amino sugar. This complex was further conjugated to the lysines of the antibody. This conjugate could be used directly or could be stabilized by further reduction with sodium borohydride. Separation between the free drug, daunomycin-dextran, and the daunomycin-dextran antibody was achieved by gel filtration on Sephadex G-100. Dextrans of different molecular weights were used, ranging from 10000 up to 500000. The drug-dextran-antibody preparations were tested both for their drug and antibody activity. The drug activity was found to decrease to about 20 - 50% of the free drug, but a prolonged incubation time resulted in the same maximum activity as that of the free drug. The antibody activity was also about half that of the original antibody. By this method, however, about 20 moles of drug per mole of antibody were bound.

Another approach was to couple the periodate oxidized drug to linear polyacrylhydrazide (PAH). The polyacrylhydrazide daunomycin was linked to the antibody using glutaraldehyde. This was performed in several steps: glutaraldehyde was linked to the drug-PAH complex, the excess reagent was removed by gel filtration on Sephadex G-25, and the activated complex was then allowed to react with the antibody. Analysis of the drug and antibody activities showed that both functions were almost entirely lost.

The use of t-RNA as a bridge between the drug and antibody was based on the idea that since the drug could bind to the t-RNA noncovalently to form a complex, the drug would be slowly released at the target cells. Commercial *E. coli* t-RNA was oxidized by $NaIO_4$. The antibodies were coupled to the aldehyde group of the periodate oxidized t-RNA. The conjugate was further stabilized by reduction with sodium borohydride. The drug was then allowed to react with the t-RNA antibody conjugate. The resultant complex was separated from free drug by gel filtration. The complex retained most of its drug activity and about 50% of its antibody activity.

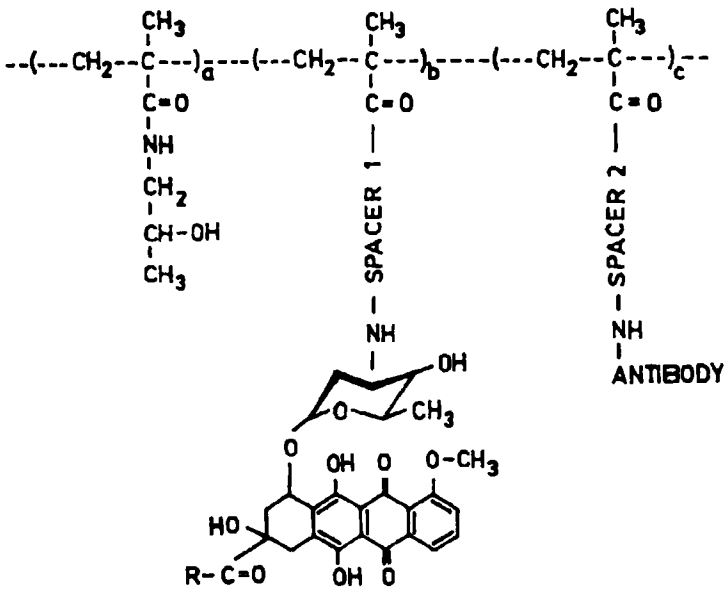
Immunological problems of polymer-bound drugs were reviewed by Říhová and Říha (1985). T lymphocytes, important for all types of immune reaction, were used as target cells by Říhová et al. (1986). Antibodies against their surface Thy 1.2 alloantigen

(anti-Thy 1.2) served as the targeting structure. They were bound by aminolysis either to soluble synthetic copolymers based on HPMA containing different oligopeptide side sequences or to pharmacologically active HPMA copolymer with quaternary ammonium group. Summary of their paper about bioaffinity therapy with antibodies and drugs bound to soluble synthetic polymers may be given as follows: (1) HPMA copolymers containing targeting anti-Thy 1.2 antibodies were 70 times more cytotoxic against T lymphocytes than HPMA copolymers with non-specific immunoglobulin. (2) Daunomycin conjugated to a biodegradable side-sequence (Gly-Phe-Leu-Gly) was effective in a concentration 100 times lower than daunomycin conjugated to a non-cleavable sequence (Gly-Gly). (3) HPMA copolymers containing drug and targeting antibodies were effective both *in vitro* and *in vivo*.

The *in vivo* effectiveness and limited toxicity of daunomycin conjugated to HPMA copolymers and targeting antibody was described by Říhová et al. (1988). Application of free daunomycin led to a significant irritation of Kupffer cells in liver while none of the daunomycin-antibody-copolymer conjugate had such an effect. Targeted polymeric prodrugs based on HPMA were tested by Říhová et al. (1990) on human peripheral blood lymphocytes or mouse splenocytes triggered *in vitro* to proliferation by T cell specific (concanavalin A) or B cell specific (*S. aureus* Cowan I) mitogens. Fig. 13.7 shows the structure of copolymer HPMA studied and Table 13.4 characterizes used HPMA copolymers. Selective inhibition of ^{3}H-thymidine incorporation by T lymphocytes was observed only after *in vitro* incubation with prodrugs prepared by covalent attachment of daunomycin or adriamycin and antibody (anti-CD3; anti-Thy1.2) to biodegradable oligopeptide side chains of HPMA. The *in vitro* results were confirmed *in vivo*.

The advantages of carbohydrates used as a tool for the oriented immobilization of glycoproteins have been discussed in Section 4.3.4. In accordance with these facts, Krinick et al. (1990) increased the final effect of the polymeric conjugate by the binding of the antibody to the polymeric carrier through the oxidized carbohydrate Fc part of the antibody molecule.

The results of the project "Water-soluble Polymeric Drug Carriers" carried out in cooperation between teams of Dr. B. Říhová from the Institute of Microbiology, Czech.



a,b,c = content of oligopeptide side chains in mol %

Sample	Spacer 1	Spacer 2	Antibody	R
I	-GlyGly-	-GlyGly-	anti-CD_3	$-CH_2.OH$
II	-GlyPheLeuGly-	-GlyPheLeuGly-	anti-CD_3	$-CH_2.OH$
III	-GlyPheLeuGly-	---	---	$-CH_2.OH$
IV	---	-GlyPheLeuGly-	anti-Thy 1.2	---
V	-GlyGly-	-GlyGly-	anti-Thy 1.2	$-CH_3$
VI	-GlyPheLeuGly-	---	---	$-CH_3$
VII	-GlyPheLeuGly-	-GlyPheLeuGly-	anti-Thy 1.2	$-CH_3$
VIII	-GlyPheLeuGly-	-GlyGly-	anti-Thy 1.2	$-CH_3$

Fig. 13.7. Structure of copolymers of N-(2-hydroxypropyl)methacrylamide studied.

Acad.Sci. of the Czech Republic (AS CR) and Dr. K. Ulrich, Institute of Macromolecular Chemistry, AS CR in Prague, Professor R. Duncan, University of Keele, U.K., and Professor J. Kopeček, University of Utah, USA, have been published in many papers. The results of this cooperation are also the targeting of drugs by means of amino sugars. Targeting of HPMA copolymers to liver by the incorporation of galactose residues was described by Duncan et al. (1983). It was shown that poly(HPMA) conjugates containing the same quantities of bound amino sugars (glucosamine, galacto-

Table 13.4.

Characterization of N-(2-hydroaxypropyl)methacrylamide copolymers.

No.	*Structure of copolymers*	*Content in wt %*		M_w
		Drug	*Antibody*	
I	GlyGly-ADR | P | GlyGly-ATG	8.0	25	18,000
II	GlyPheLeuGly-ADR | P | GlyPheLeuGly-ATG	7.5	25	17,500
III	P-GlyPheLeuGly-ADR	8.5	–	24,000
IV	P-GlyPheLeuGly-ATS	–	25	22,500
V	GlyGly-DNM | P | GlyGly-ATS	5.7	25	17,500
VI	P-GlyPheLeuGly-DNM	6.8	–	22,500
VII	GlyPheLeuGly-DNM | P | GlyPheLeuGly-ATS	6.1	25	18,000
VIII	GlyPheLeuGly-DNM | P | GlyGly-ATS	7.0	25	58,500

P – polymer backbone; DNM – daunomycin; ADR – adriamycin; ATS – anti-Thy 1.2 antibody; ATG – anti-CD3 antibody; Mw – molecular weight of polymeric precursor

samine, mannosamine) are differently distributed in the organism. The clearance of intravenously injected HPMA conjugates bearing D-galactosamine from the bloodstream is the fastest process.

In the *in vivo* testing of poly(HPMA) conjugates bearing adriamycin and galactose it was found that such conjugates may be targeted to the hepatocyte galactose receptor. This may prove useful for the organ-specific chemotherapy of primary and metastatic liver cancer. The dependence of the rate of blood clearance of the conjugates on the content of bound galactosamine and on the dose of the intravenously administered conjugate was investigated, as well as the effect of the repeatedly administered conjugate on the rate of pinocytic uptake by liver cells was assessed. The results give some

indication as to the optimum way of administering the drug. The accumulation of adriamycin conjugates by human hepatoma cell line (HepG2) was also studied. It was found that HepG2 retains the galactose receptor, thus accumulating the conjugate twice as fast as mouse liver. Probably due to the present lysosomal enzyme, pinocytosis by the HepG2 cell lines is followed by a comparatively fast release of activated adriamycin. The clinical testing of this poly(HPMA) conjugates is being carried out in Britain (Ulbrich, 1991).

The preparation and application of magnetic polymers for the targeting of drugs were described by Mosbach and Schröder (1979) and by Torchilin et al. (1985). Ferromagnetic fluids have been proposed to enable drug targeting by the effects of strong magnetic fields on those substances. Magnetic microspheres carrying antineoplastic drugs have already beeen successfully employed for selective cancer chemotherapy. The studies of Sprandel et al. (1987) were concentrated on magnetically responsive erythrocyte ghosts. This chapter is part of Volume 149 of Methods in Enzymology which deals with drug and enzyme targeting. Special attention should be paid to the chapter on the use of platelets as drug carriers for the treatment of haematologic diseases (Ahn et al., 1987) and the chapter on the use of avidin-biotin technology for liposome targeting (Rivnay et al., 1987).

REFERENCES

Ahn, Y.S., Harrington, W.J. and Mylvaganam, R., Methods Enzymol., 149 (1987) 312-325.

Andersson, L.O., J. Chromatogr., 215 (1981) 153-164.

Bensinger, W.I., Buckner, C.D. and Clift, R.A., Vox Sanguinis, 48 (1985) 357-361.

Bessos, H., Prowse, C.V. and James, K., Med. Lab. Sci., 45 (1988) 261-265.

Buffaloe, G.W., in Plasmaphoresis : Therapeutic Applications and New Techniques, Y.Nose, J.W.Smith, P.S.Malchesky and R.S.Krakauer, Editors, Raven Press, New York, 1983, pp. 211-216.

Charm, S.E. and Wong, B.L., Biotechnol. Bioeng., 16 (1974) 593-607.

Duncan, R., Kopeček, J., Rejmanová, P. and Lloyd, J.B., Biochim. Biophys. Acta, 775 (1983) 518-521.

Fer, M.F. and Oldham, R.K., in Immune Complexes and Human Cancer Contemporary Topics in Immunobiology, F.A. Salinas and M.G. Hanna, Editors, Vol. 15, Plenum Publ. Corp., New York, 1985, pp. 257-276.

Fořtová H., Dyr, J.E. and Suttnar, J., J. Chromatogr., 523 (1990) 312-316.

Freiburghaus, C., Ohlson, S. and Nilsson, I.M., Methods Enzymol., 137 (1988) 458-466.

Gregoriadis, G., Nature, 265 (1977) 407-411.

Jacquot Dourges, M.A., Gulino, D., Courty, J., Badet, J., Barritault, D. and Jozefonvicz, J., J. Chromatogr., 526 (1990) 35-45.

Kitao, T. and Hattori, K., Nature, 265 (1977) 81-82.

Krinick, N.L., Říhová, B., Ulbrich, K., Strohalm, J. and Kopeček, J., Makromol. Chem., 191 (1990) 839-856.

Kučerová, Z., J. Chromatogr., 376 (1986) 409-412.

Kučerová, Z., J. Chromatogr., 489 (1989) 390-393.

Lemoli, R.M., Gobbi, M., Tazzari, P.L., Tassi, C., Dinota, A., Visani, G., Grassi, G., Mazza, P., Cavo, M. and Tura, S., Transplantation, 47 (1989) 385-387.

Lupien, P.J., Moorjani, S., Gagne, C., Brun, L.D., Lou, M. and Dagenais, G., Artery, 10 (1982) 286-300.

Lupien, P.J., Moorjani, S., Lou, M., Brun, D. and Gagne, C., Pediatric Res., 14 (1980) 113-117.

Messerschmidt, G.L., Henry, D.H., Snyder, H.W., Bertram, J., Mittelman, A., Ainsworth, S., Fiore, J., Viola, M.V., Louie, J., Ambinder, E., Mac Kintosh, R., Higby, D.J., O'Brien, P., Kiprov, D., Hamburger, M., Balint, J.P., Fisher, L.D., Perkins, W., Pinsky, C.M. and Jones, F.R., J. Clin. Oncol., 6 (1988) 203-212.

Mosbach, K. and Schröder, U., FEBS Lett., 102 (1979) 112-116.

Ohshiro, T., Chung Liu, M., Kambayashi, J. and Mori, T., Methods Enzymol., 137 (1988) 529-545.

Plotz, P.H., Berk, P., Scharschmidt, B.F., Gordon, J.K. and Vergalla, J., J. Clin. Invest., 53 (1974) 778-785.

Poznansky, M.J., Methods Enzymol., 137 (1988) 566-574.

Říhová, B., Kopeček, J., Kopečková-Rejmanová, P., Strohalm, J., Plocová, D. and Semorádová, H., J. Chromatogr., 376 (1986) 221-233.

Říhová, B., Kopečková, P., Strohalm, J., Rossman, P., Větvička, V. and Kopeček, J., Clin. Immunol. Immunopath., 46 (1988) 100-114.

Říhová, B. and Říha, I., CRC Critical Reviews in Therapeutic Drug Carrier Systems, 4 (1985) 311-374.

Říhová, B., Strohalm, J., Plocová, D. and Ulbrich, K., J. Bioact. Compat. Polym., 5 (1990) 249-266.

Rivnay, B., Bayer, E.A. and Wilchek, M., Methods Enzymol., 149 (1987) 119-123.

Scharschmidt, B.F., Plotz, P.H., Berk, P.D., Waggoner, J.G. and Vergalla, J., J. Clin. Invest., 53 (1974) 786-795.

Schenkein, I., Bystryn, J.C. and Uhr, J.W., J. Clin. Invest., 50 (1971) 1864-1868.

Shimazaki, C., Wisniewski, D., Scheinberg, D.A., Atzpodien, J., Strife, A., Gulati, S., Fried, J., Wisniewolski, R., Wang, C.Y. and Clarkson, B.D., Blood, 72 (1988) 1248-1254.

Sprandel, U., Lanz, D.J. and von Hörsten, W., Methods Enzymol., 149 (1987) 301-312.

Štamberg, J., Turková, J., Hubková, M. and Pitrák, V., Chemically Modified Surfaces, Vol.2, Gordon and Breach, 1988, pp. 79-90.

Terman, D.S., Garcia-Rinaldi, R., Dannemann, D., Moore, D., Crumb, C. and Tavel, A., Clin. Exp. Immunol., 34 (1978) 32-41.

Terman, D., Harbeck, R., Hoffman, A., Stewart, I., Robinette, J. and Carr, R., Fed. Proc. Fed. Amer. Soc. Exp. Biol., 34 (1975) 976.

Terman, D.S., Stewart, I., Hoffmann, A., Carr, R. and Harbeck, R., Experientia, 30 (1974) 1493-1494.

Terman, D.S., Tavel, T., Petty, D., Tavel, A., Harbeck, R., Buffaloe, G. and Carr, R., J. Immunol., 116 (1976) 1337-1341.

Terman, D.S., Young, J.B., Shearer, W.T., Ayus, C., Lehane, D., Mattioli, C., Espada, R., Howell, J.F., Yamamoto, T., Zaleski, H.I., Miller, L., Frommer, P., Feldman, L., Henry, J.F., Tillquist, R., Cook, G. and Daskal, Y., N. Engl. J. Med., 305 (1981) 1195-1200.

Tharakan, J., Strickland, D., Burgess, W., Drohan, W.N. and Clark, D.B., Vox Sang., 58 (1990) 21-29.

Torchilin, V.P., Il'ina, E.V., Streltsova, Z.A., Smirnov, V.N. and Chazov, E.I., J. Biomed. Mater. Res., 12 (1978) 585-590.

Torchilin, V.P., Maksimenko, A.V. and Mazaev, A.V., Methods Enzymol., 137 (1988) 552-566.

Torchilin, V.P., Papisov, M.I. and Smirnov, V.N., J. Biomed. Mater. Res., 19 (1985) 461.

Trouet, A., Deprez-de Campeneere, D. and de Duve, C., Nature New Biology, 239 (1972) 110-112.

Turková, J., in Interbiotech '89, Mathematical Modelling in Biotechnology, A. Blažej and A. Ottova, Editors, Elsevier, Amsterdam, 1990, pp. 377-385.

Turková, J., Štamberg, J., Pitrák, V., Hostomská, Z., Jelínková, L., Šebesta, K., and Šebestík, M., Czech. Patent Appl., AO 2493 11 (1983).

Ugelstad, J., Berge, A., Ellingsen, T., Aune, O., Kilaas, L., Nilsen, T.N., Schmid, R., Stenstad, P., Funderud, S., Kvalheim, G., Nustad, K., Lea, T., Vartdal, F. and Danielsen, H., Makromol. Chem., Macromol. Symp., 17 (1988) 177-211.

Ulbrich, K., J. Bioact. Compat. Polym., 4 (1991) 348-357.

Varga, J.M., Asato, N., Lande, S. and Lerner, A.B., Nature, 267 (1977) 56-58.

Wade, R., Whisson, M.E. and Szekerke, M., Nature, 215 (1967) 1303-1304.

Wilchek., M., Enzyme Engineering, 4 (1978) 435-440.

Williams, W.V., Weiner, D.B., Cohen, J.C. and Greene, M.I., Bio/Technology, 7 (1989) 471-475.

Chapter 14

Application of bioaffinity chromatography to the quantitative evaluation of specific complexes

Association processes undoubtedly play one of the most important roles in biochemical processes. The initial degree of bonding to a substrate has an important effect on the catalytic properties of an enzyme. Control processes in biology depend on the association of the repressor with the operon, hormones with specific receptors, etc. The formation of specific complexes of nucleic acid chains or of antibodies with antigens plays an important role. From this point of view the overall goals may be characterized as the evaluation of the role of recognition in increasingly complex systems, the detection and quantitation of macromolecular and cellular interactions and methods by which these may be achieved. These aims remain central to the study of recognition in biology and its application in biotechnology and medicine. Methods in which the ligand - protein interaction is used for direct analysis, such as equilibrium dialysis, ultrafiltration, gel filtration, spectroscopic methods and steady-state kinetic analysis, have been supplemented by a technique for the determination of dissociation constants based on bioaffinity chromatography. Reviews of the development in quantitative bioaffinity chromatography have been published by Chaiken (1986), Kasai et al. (1986) and Winzor (1992).

For the separation of isoenzymes of lactate dehydrogenase, Brodelius and Mosbach (1973) used Sepharose with N^6-(6-aminohexyl)-AMP. Five peaks due to separated isoenzymes could be eluted by increasing the NADH concentration, as shown in Fig. 14.1. The separation has been interpreted as a result of the differences in dissociation constants (K_{diss}) for the binary enzyme - NADH complex. Brodelius and Mosbach (1974), using the same support and in an analogous manner, subsequently chromatographed a series of lactate dehydrogenases from a variety of sources. The dissociation constants of the materials were known. Fig. 14.2 shows a direct proportionality between these K_{diss} values and the elution concentration of NADH. The linearity indicates that, in this case, the dissociation constants for the enzyme - NADH complex

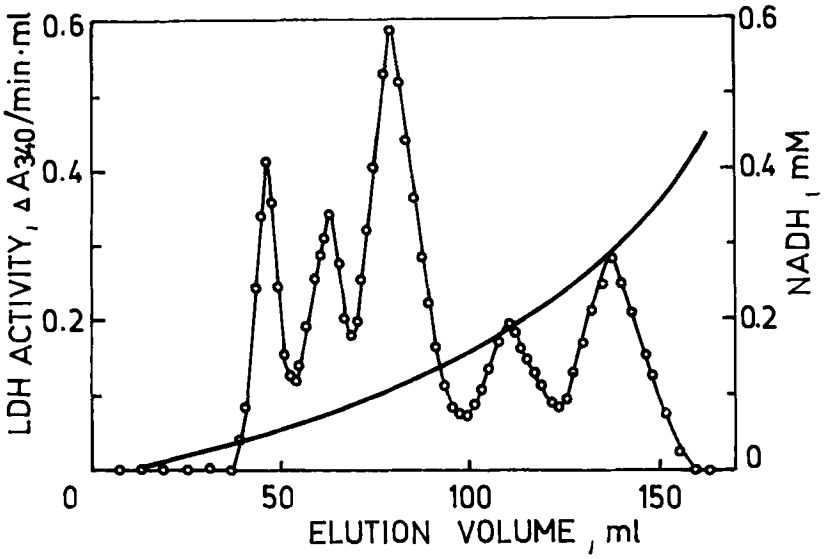


Fig. 14.1. Elution of lactate dehydrogenase isoenzymes with a concave gradient of NADH. Protein (0.2 mg) in 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.0), 1 mM β-mercaptoethanol and 1 M sodium chloride were applied to an AMP-analogue - Sepharose column (140 x 6 mm, containing 2.5 g of wet gel) equilibrated with 0.1 M sodium phosphate buffer (pH 7.5). The column was washed with 10 ml of the latter buffer, and the isoenzymes were then eluted with a concave gradient of 0.0-0.5 mM NADH in the same buffer, containing 1mM β-mercaptoethanol. Fractions of 1 ml were collected at the rate of 3.4 ml/h. Reproduced with permission from P. Brodelius and K. Mosbach, FEBS Lett., 35 (1973) 223-226.

play a greater role than those for the complex between the enzyme and the immobilized affinity ligand (AMP). Hence, it is possible to determine the dissociation constants for binary complexes between the enzyme and NADH on the basis of the determination of elution concentrations of NADH. No differences in K_{diss} values were observed if either a crystalline or crude preparation was used. Other proteins present in crude preparations, even when bound in the column, do not affect the elution pattern. This is a great advantage of this determination in comparison with the conventional methods for the determination of dissociation constants, which require not only pure enzymes but also homogeneous isoenzymes. Moreover, the use of bioaffinity chromatography is very rapid and requires only a very small amount of enzyme for each determination.

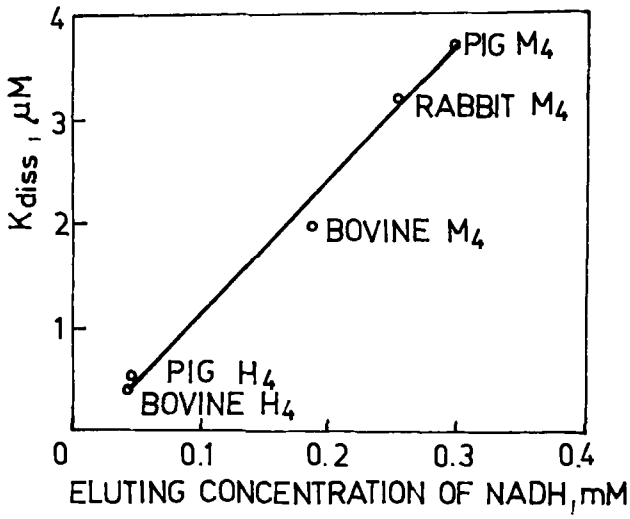


Fig. 14.2. Dissociation constants for the binary complex between enzyme and NADH as a function of eluting concentration of NADH. Reproduced with permission from P. Brodelius and K. Mosbach, Biochem. Soc. Trans., 2 (1974) 1308-1310.

4.1 DETERMINATION OF DISSOCIATION CONSTANTS BY ELUTION ANALYSIS

The most commonly used method of quantitative bioaffinity chromatography is based on the elution of a biological macromolecular substance from an affinity matrix with soluble affinant solutions of various concentrations (Dunn and Chaiken, 1975). The elution volume of a macromolecular substance depends directly on the concentration of the affinity ligand bound to a solid support if the concentration of the soluble affinity ligand is constant. Further, it is inversely proportional to the concentration of the soluble affinant if the concentration of the immobilized affinant is constant. These dependences can be expressed by Eqn. (14.1)

$$V = V_0 + \frac{(V_0 - V_m)\frac{[L]}{K_L}}{\left(1 + \frac{[I]}{K_I}\right)} \qquad (14.1)$$

where V = elution volume; V_o = volume at which the macromolecular substance is eluted if no interaction with the immobilized ligand takes place (for example, with an enzyme in the presence of a strong inhibitor); V_m = void volume, determined, for example, by means of blue dextran elution; [L] = concentration of the immobilized ligand, determined on the basis of the operating capacity of the sorbent; K_L = dissociation constant for the interaction of the macromolecular substance (solute) with the immobilized ligand; [I] = concentration of the soluble affinity ligand; and K_I = dissociation constant for the soluble binary complex.

The validity of this equation was checked by Dunn and Chaiken (1975) by examining the chromatography of staphylococcal nuclease on thymidine 3'-(p-Sepharose-aminophenyl phosphate)5'-phosphate (abbreviation: pdTpAP-Sepharose) as a function of the concentration of both the immobilized nucleotide and the soluble nucleotide used for the elution of nuclease.

Fig. 14.3 shows the effect of the dilution of the affinity matrix with unsubstituted Sepharose. The same amount of nuclease is always eluted with a solution of the inhibitor thymidine 3', 5'-bisphosphate (pdTp) from columns of pdTpAP-Sepharoses differing only in the concentration of the immobilized thymidine 3'-(p-aminophenyl phosphate) 5'-phosphate (pdTpAP). The elution volume of nuclease is directly proportional to the concentration of the immobilized nucleotide (Fig. 14.3B). The dependence found agrees with Eqn. 14.1.

Extrapolation of the plot of V versus [L] to [L] = 0 gives the value of V_o. The value of V_o obtained from Fig. 14.3 B was in excellent agreement with the value obtained experimentally by the elution of phenol red from the same column, or by the elution of nuclease from the same volume of unmodified Sepharose. On the basis of Eqn. 14.1, the slope of the curve in Fig. 14.3 B is given by the relationship (14.2)

$$\text{Slope} = \frac{(V_0 - V_m)/K_L}{\left(1 + \frac{[I]}{K_I}\right)} \qquad (14.2)$$

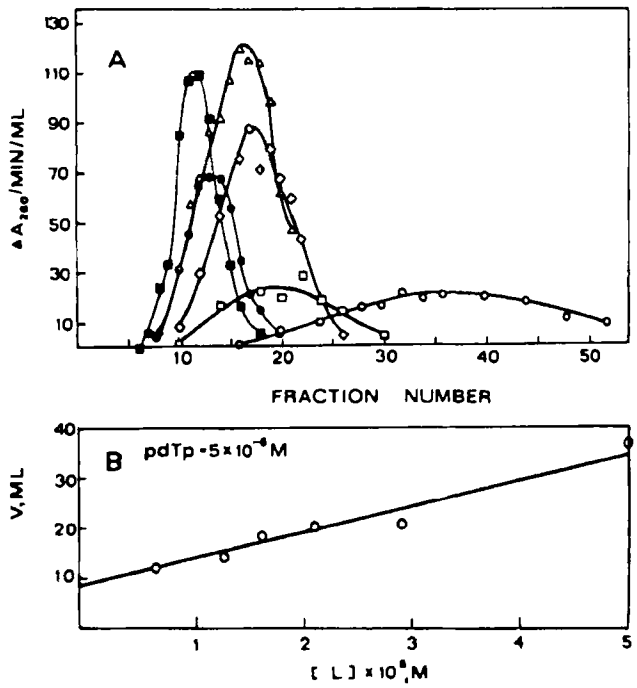


Fig. 14.3. (A) Composite plot of chromatography of nuclease on thymidine 3'-(p-Sepharose-aminophenyl phosphate) 5'-phosphate (pdTpAP-Sepharose) with various bound ligand concentrations ([L]). The affinity matrix was mixed with underivatized Sepharose 4B in the following proportions (milliliters of pdTpAP-Sepharose to millilitres of Sepharose 4B): 5.0 (o), 2.5 (□), 2:4 (◇), 1.5:4.5 (Δ), 1:4 (●), 0.5:4.5 (■). The mixtures were then used to prepare 5-ml columns of packed gel equilibrated with $5x10^{-6}$M thymidine 3',5'-bisphosphate (pdTp); 200 μg samples of nuclease were applied and eluted with 0.1 M ammonium acetate buffer containing $5x10^{-6}$M pdTp.
Δ A_{260}/min/ml is the activity in the hydrolysis of salmon sperm DNA. (B) Plot of elution volume versus concentration of immobilized affinity ligand. Reproduced with permission from B.M. Dunn and I.M. Chaiken, Biochemistry, 14(1975) 2343-2349.

Hence, when one of the dissociation constants (K_L or K_I) and the slope are known, the other constant can be calculated.

Fig. 14.4 illustrates the dependence of the elution volume of nuclease on the concentration of the soluble nucleotide. The same amount of nuclease was always introduced into columns of pdTpAP-Sepharose equilibrated with pdTpAP solutions of various concentrations, and eluted with a solution of pdTpAP of adequate concentration (Fig. 14.4B). With decreasing concentration of the inhibitor, [I], the protein peak becomes broader as the retention time increases.

Eqn. 14.1 can be transformed into (14.3)

$$\frac{1}{V - V_o} = \frac{1}{(V_o - V_m)\frac{[L]}{K_L}} + \frac{[I]}{K_I(V_o - V_m)\frac{[L]}{K_L}} \qquad (14.3)$$

In Fig. 14.4B, $1/(V - V_0)$ is plotted against [I], the latter being obtained on the basis of elution volumes from Fig. 14.4A:

$$\text{Slope} = 1/K_I A \qquad (14.4)$$

$$\text{Intercept} = 1/A \qquad (14.5)$$

where $A = (V_o - V_m)([L]/K_L)$.

From Eqns. 14.4 and 14.5 it follows that the intercept/slope ratio is equal to K_I. The expression for the intercept on the $1/(V - V_0)$ axis (Fig. 14.4B) contains K_L and, if [L], V_o and V_m are known, K_L can be calculated from that expression. Table 14.1 gives the values for K_I and K_L obtained by the method described, in comparison with the values of K_I obtained from kinetic measurements or from equilibrium dialysis. On the basis of the agreement between the given constants, it can be concluded that in this system the binding of nuclease on the affinity matrix is completely reversible and that the immobilization of the ligand does not limit the complex formation.

On the basis of the determination of phosphate in the acid hydrolysate of pdTpAP-Sepharose, the amount of the bound pdTpAP-Sepharose was determined as 2.1×10^{-4}M. However, when dissociation constants were calculated, the concentration of pdTpAP which was determined from the operating capacity was used, which gave 5×10^{-5}M. From a comparison of these values it follows that only 24% of the insolubilized ligand molecules are capable of binding nuclease.

K_L values were obtained independently by elution with six different affinity ligands and their values varied within the range $0.6 - 2.6 \times 10^{-6}$M. The scatter in K_L seems to be independent of the nature of the substituents bound to thymidine. The agreement of the

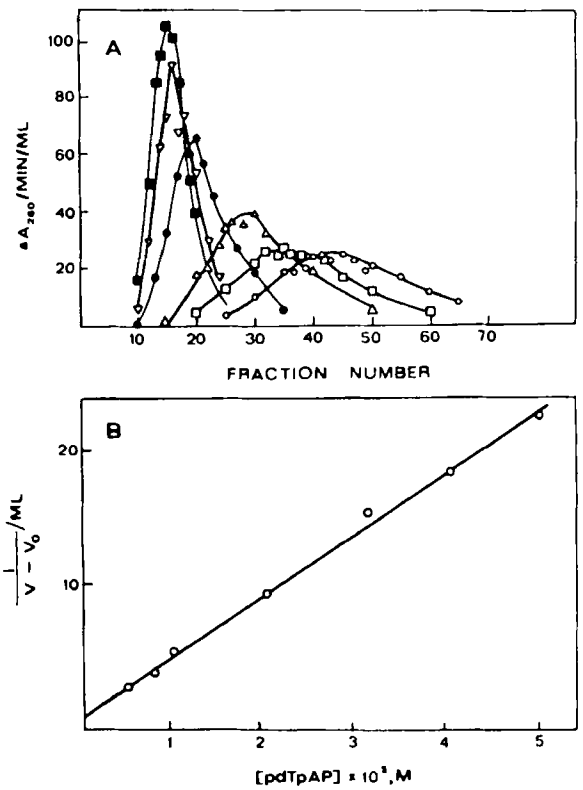


Fig. 14.4. (A) Composite plot of chromatography of nuclease on thymidine 3'-(p-Sepharose-aminophenyl phosphate) 5'-phosphate(pdTpAP-Sepharose) with various concentrations of soluble thymidine 3'-(p-aminophenyl phosphate)5'-phosphate (pdTpAP) in the eluting buffer; 200 μg-samples of nuclease were applied to columns equilibrated with 0.5×10^{-5}M (o), 0.75×10^{-5}M (□), 1.0×10^{-5}M (Δ), 2×10^{-5}M (●), 3×10^{-5}M (V) and 4×10^{-5}M (■) pdTpAP each in 0.1 M ammonium acetate solution, and eluted with these buffers. (B) Plot of $1/(V - V_o)$ versus [pdTpAP] according to Eqn. 14.3, correlation coefficient r = 0.997. For the sake of clarity, the result obtained for [pdTpAP] = 5×10^{-4}M is included in Fig. 14.4 (B) but the elution profile is omitted from Fig. 14.4 A. Reproduced with permission from B.M. Dunn and I.M. Chaiken, Biochemistry, 14 (1975) 2343-2349.

K_L values (within experimental error) shows that the nuclease-affinity matrix interaction is independent of the presence of another ligand. In contrast to the K_L values, the values of K_I depend strongly on the substitution of the ligand. Virtually identical K_I values for pdTp and pdTpAP indicate that the aminophenyl group of the spacer arm has no effect on the interaction of nuclease with the immobilized nucleotide. Similarly, a comparison of K_I (2.3×10^{-6}M) for pdTpAP shows that the binding of the nucleotide on a solid

Table 14.1.

Dissociation constants for the interaction of affinity ligand with solute staphylococcal nuclease (pH 7.5, in the presence of 10 mM $CaCl_2$)

Affinity ligand	$K_I(M)$		$K_L(M)$
	Chromatography	*Kinetics*	
Thymidine 3'-(*p*-aminophenyl phosphate) 5'-monophosphate	$2.3 . 10^{-6}$	$2.5 . 10^{-6}$	$1.1 . 10^{-6}$
Thymidine 3',5'-bisphosphate	$2.5 . 10^{-6}$	$5.9 . 10^{-6}$	$1.0 . 10^{-6}$
Thymidine 5'-monophosphate	$1.6 . 10^{-5}$	$2.8 . 10^{-5}$	$0.9 . 10^{-6}$
Thymidine 3'-phosphate 5'-(*p*-nitrophenyl phosphate)	$1.1 . 10^{-5}$	$6.3 . 10^{-6}$	$0.6 . 10^{-6}$
Thymidine 3'-(*p*-nitrophenyl phosphate) 5'-phosphate	$4.1 . 10^{-6}$	$3.5 . 10^{-6}$	$2.6 . 10^{-6}$
Thymidine 3'-(*p*-nitrophenyl phosphate)	$4.3 . 10^{-3}$		$1.5 . 10^{-6}$
None			$1.2 . 10^{-6}$

support does not affect the interaction of nuclease with pdTpAP. Hence, the same forces are operative in the binding, both in solution and after the binding of the nucleotide on a solid support. Hence, this method not only serves for the determination of dissociation constants but also for the evaluation of the effect of immobilization on complex formation.

Objections have been expressed by Nichol et al. (1974) to the equations derived by Dunn and Chaiken (1974). Dunn and Chaiken (1975) restricted their validity to cases when $[E]/K_L$ is small, in which event a very low concentration of enzyme has to be used. A further restriction concerns the interactions with K_L values which are too low. If the binding constant is 10^{-9}M, for example, then the calculated rate constant for the dissociation of the complex EL will be very low, viz. about 0.042/sec (Dunn and Chaiken, 1975). The elution of the protein will then be affected on the basis of the kinetic effect, and it will be unattainable in an experimentally feasible time. The addition of the dissolved ligand to the eluting solvent will not affect the elution of the protein because the dissociation is unimolecular. These kinetic effects explain the failure, which is sometimes observed, of some enzymes to be eluted from affinity sorbents with buffers

that contain strong inhibitors. In some instances the protein peak is so broad that it is undetectable. The method is mainly suitable for cases when sorbents are available that contain ligands with a medium bonding strength. In such cases the system is completely reversible within the time period of the chromatographic experiment.

Experimental aspects of quantitative bioaffinity chromatography have been published by Winzor (1985). From the viewpoint that the total concentration of accessible matrix sites, ($\overline{\overline{m}}_x$) is a parameter whose magnitude requires evaluation from the experimental results, column chromatography is the simplest procedure to employ for quantitative bioaffinity chromatography, since the same value of $\overline{\overline{m}}_x$ then relates to all results obtained with the same solute on the same column. However, in some instances, particularly those involving biological affinity matrices such as muscle myofibrils (Kuter et al., 1983), the column technique is not feasible because of flow-rate problems. Accordingly partition equilibrium experiments also find application in quantitative bioaffinity chromatography.

With the possible exception of a requirement that experiments be conducted on a relatively small scale, the procedures for setting up quantitative bioaffinity chromatography do not differ substantially from those for any other column experiment. However, the following points should be taken into consideration. (1) In view of the general dependence of chemical equilibria upon temperature, a jacketed column should be used, thermostatically maintained at the temperature of interest.

(2) Since the evaluation of equilibrium constants requires the accurate assessment of elution volumes, precautions are necessary to ensure sufficient precision in the measured values of elution volume. If the column effluent is being divided into fractions for assay, the size of each fraction is most accurately determined from its weight: collection of fractions in previously weighed tubes is therefore recommended. Alternatively, an accurately controlled and measured flow-rate may be used in conjunction with continuous monitoring of the column effluent.

(3) The quantitative expressions contain the total concentration of partitioning solute in the mobile phase. Frontal chromatography should therefore be used in preference to the usual zonal procedure.

14.2 DETERMINATION OF DISSOCIATION CONSTANTS BY FRONTAL ANALYSIS

The version of quantitative bioaffinity chromatography based on frontal analysis (Kasai and Ishii, 1975) is based on the assumption that if the total length of the agarose column is l and the total amount of the immobilized ligand in the adsorbent is L_t, then $L_o = L_t/l$, where $[L_o]$ is the density of affinity ligands per unit length of the agarose column. When an affinity adsorbent with a weak affinity is used, an enzyme solution of concentration $[E_o]$ is continuously applied to the column, and the enzyme will be eluted from the column with a volume V. Then the following equation can be written:

$$(V - V_o)\,[E_o] = l[EL] \tag{14.6}$$

where V_o is the elution volume if no interaction takes place between the active site of the enzyme and the immobilized affinity ligand (for example, in the presence of a soluble, strongly competitive inhibitor) and [EL] is the density of the enzyme-immobilized ligand complex per unit length of the agarose column. Equilibrium is attained during the complex formation:

$$E + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EL$$

If the concentration of free enzyme [E] = $[E_o]$, the following equation can be written:

$$K_L = \frac{k_{-1}}{k_{+1}} = \frac{[E][L]f_L}{[EL]f_{EL}} = \frac{[E_o]([L_o] - [EL])f_L}{[EL]f_{EL}}$$

$$= \frac{1}{(V - V_o)f_{EL}}[L_o]f_L - [E_o]\frac{f_L}{f_{EL}} = \frac{L_t f_L}{(V - V_o)f_L}[E_o]\frac{f_L}{f_{EL}} \tag{14.7}$$

where K_L is the dissociation constant of the enzyme-immobilized ligand complex, [L] is the density of the unoccupied ligand per unit length and f is the activity coefficient of

the reactant, the mobility of which is restricted. If chromatography takes place under the conditions when $[E_o] << K_L$, Eqn. 14.7 can be rewritten as

$$K_L = L_t f_L / (V - V_o) f_{EL} \tag{14.8}$$

If the enzyme is eluted with volumes V_1 and V_2 under two different conditions, we can write

$$\frac{K_{L(1)}}{K_{L(2)}} = \frac{(V_2 - V_o)}{(V_1 - V_o)} \tag{14.9}$$

where $K_{L(1)}$ and $K_{L(2)}$ are the corresponding dissociation constants. Hence, Eqn. 14.9 is also applicable to a comparison of different enzymes.

If the enzyme is eluted in the presence of a competitive inhibitor of concentration $[I_o]$ (where $[I_o] >> [E_o]$) by a volume V_i, then the following equation can be deduced:

$$K_{L(I)} / K_L = 1 + [I_o] / K_I = (V - V_o) / (V_i - V_o) \tag{14.10}$$

where K_I is the dissociation constant of the enzyme-soluble inhibitor complex, defined by the relationship

$$K_I = \frac{[E][I]}{[EI]} \tag{14.11}$$

$K_{L(I)}$ is defined as an apparent dissociation constant of the enzyme-immobilized ligand complex, in the presence of soluble inhibitor:

$$K_{L(I)} = ([E] + [EI])\ [L] f_L / [EL] f_{EL} \tag{14.12}$$

Eqn. 14.10 can be rewritten as

$$(V - V_i) / (V - V_o) = 1 / (1 + K_I / [I_o]) \quad (14.13)$$

It follows that $(V - V_i)/(V - V_o)$ is equal to the ratio of the enzyme-soluble inhibitor complex concentration to the total enzyme concentration. The concentration of the inhibitor at which $(V - V_i)/(V - V_o) = 0.5$ gives the values for K_I. Eqn. 14.13 can be written as

$$V_i = V_o + K_I (V - V_i) / [I_o] \quad (14.14)$$

If V_i is plotted against $(V - V_i)/[I_o]$, then K_I and V_o can be calculated from the slope and the intercept on the ordinate. If V_o and V are known, then the value of K_I for any competitive inhibitor can be obtained from a single chromatographic run in the presence of the corresponding inhibitor.

The chromatography of bovine trypsin on Sepharose with covalently bonded glycylglycyl-L-arginine (2.2 μmole of peptide per millilitre of Sepharose) is illustrated in Fig. 14.5A. A solution of trypsin was always introduced, together with an inhibitor of corresponding concentration onto the affinity sorbent, equilibrated with the given concentrations of inhibitor benzyloxycarbonylarginine. A straight line was obtained from the plot of V_i *versus* $(V - V_i)/[I_o]$ (Fig. 14.5B), confirming the validity of the derived equation. The values $K_I = 0.29$ mM and $V_o = 11.6$ ml were obtained from the slope and the intercept on the ordinate. The K_I value for benzyloxycarbonylarginine, determined under the same conditions in a solution obtained from the inhibition of the enzymatic hydrolysis of benzoyl-L-arginine p-nitroanilide with trypsin according to the method of Dixon, was 0.17 mM. The slight difference may be caused by using conditions that are optimal for sorption, but less suitable for kinetic analysis.

Using the method described, Kasai and Ishii (1975) determined K_I values for various inhibitors. For α- and β-trypsin and *Streptomyces griseus* trypsin they determined K_L using glycylglycyl-L-arginine bound to Sepharose. With β-trypsin they also determined

the effect of various concentrations of salts on the K_L values. Since nothing is known about the activity coefficient (f), all f values were taken as unity.

An advantage of the method of Kasai and Ishii is its sensitivity and simplicity: it requires only a small amount of the protein being investigated; in comparison with the method of Dunn and Chaiken (1975), the enzyme concentration is negligibly low in comparison with the K_L value; the use of frontal analysis simplifies the equation deduced; and the elution volumes can be determined more accurately because their

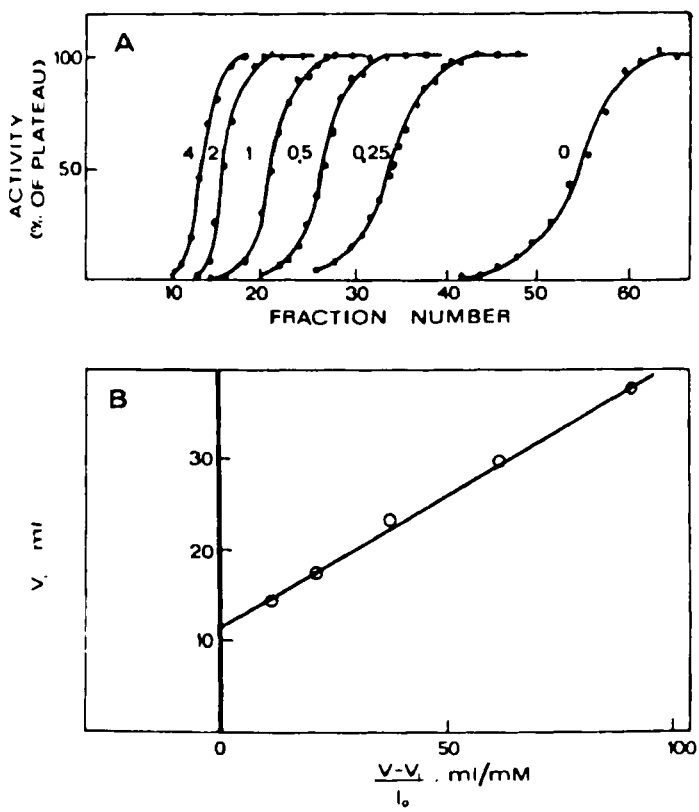


Fig. 14.5. (A) Chromatography of bovine trypsin on glycylglycyl-L-arginine-Sepharose (180 x 6 mm, 5.1 ml). A column was equilibrated with 0.1 M Tris-acid maleate-NaOH buffer, pH 6.2, containing the indicated concentration (mM) of benzyloxycarbonylarginine. Bovine β-trypsin solution in the same buffer (about 10 μg/ml, $5x10^{-7}$M) was applied continuously. The flow-rate was 3 ml/h and fractions of 1.1 ml were collected. Chromatography was carried out in a cold room (4°C). Elution of trypsin was monitored by measuring enzymatic activity using benzoyl-DL-arginine p-nitroanilide as substrate. The elution volume was determined as the volume in which the enzyme concentration reached half that of the plateau. (B) Plot of V_i against $(V - V_i)/[I_o]$. Reproduced with permission from K. Kasai and S. Ishii, J. Biochem. Tokyo,77 (1975) 261-264.

dependence on concentration is negligible. The theory and applications of frontal bioaffinity chromatography to studies of specific interactions of biomolecules in multivalent systems have been reviewed by Kasai et al. (1986).

14.3 DEVELOPMENTS IN QUANTITATIVE BIOAFFINITY CHROMATOGRAPHY

The zonal method of quantitative bioaffinity chromatography enjoys greater popularity at present than the frontal method, presumably due to the fact that the latter technique is considered to employ too much solute. However, the difference between the amounts of solute required is not as great as might be expected at the first sight. Although the volume of solution applied is much larger in a frontal than in a zonal experiment, the concentration can be considerably lower because of the absence of any dilution. This point is clearly evident from the results of Kyprianou and Yon (1982) shown in Fig. 14.6. They compared the effect of NADH concentration on the advancing elution profile obtained in frontal affinity chromatography of rat liver lactate dehydrogenase on a 0.1 ml column (0.08 x 5.0 cm) of 10-carboxydecylamino-Sepharose with the corresponding profiles obtained by zonal chromatography on a ten-times larger column (0.50 x 1.28 cm) of the same affinity matrix.

The amount of lactate dehydrogenase used to generate the frontal profiles of Fig.14.6a ranged between 9 and 54 pmol (1 - 6 ml of 9 nM enzyme), compared with 18 pmol of enzyme (0.1 ml, 180 nM) for each zonal experiment (Fig. 14.6 b). It should also be noted that, whereas the frontal patterns allow precise delineation of the median bisector of the boundary irrespective of the NADH concentration being studied, the location of the equivalent position in the zonal profiles (Fig. 14.6 b) becomes increasingly difficult with decreasing ligand concentration. Finally, even though the zonal method does prove to be more economical in terms of solute, the important point remains that results so obtained are uninterpretable unless certain simplifying approximations may be assumed (Winzor, 1985).

High-performance bioaffinity chromatography was used for the determination of equilibrium and rate constants of immobilized concanavalin A by Anderson and Walters

(1986). Values obtained by zonal and frontal analysis on columns of variable concanavalin A coverage were in close agreement and were approximately two-fold greater than literature values obtained from solution studies. Since diffusional band broadening was significant, even when small sugars were chromatographed on 5-μm supports, it is apparent that even smaller or non-porous particles are needed to accurately measure dissociation rate constants in the range of 5 s^{-1}. The peak decay method for the measurement of dissociation rate constants by high-performance bioaffinity chromatography was therefore described by Moore and Walters (1987). Theory and computer modeling were used to establish conditions such that each solute molecule would undergo only a single dissociation step during its passage through the column. As a result, the dissociation rate constant could easily be determined from the slope of the logarithm of the tailing portion of the peak. An experimental system, consisting of immobilized concanavalin A and a fluorescent sugar, was used to test the theory and to compare the peak decay method with the conventional isocratic method.

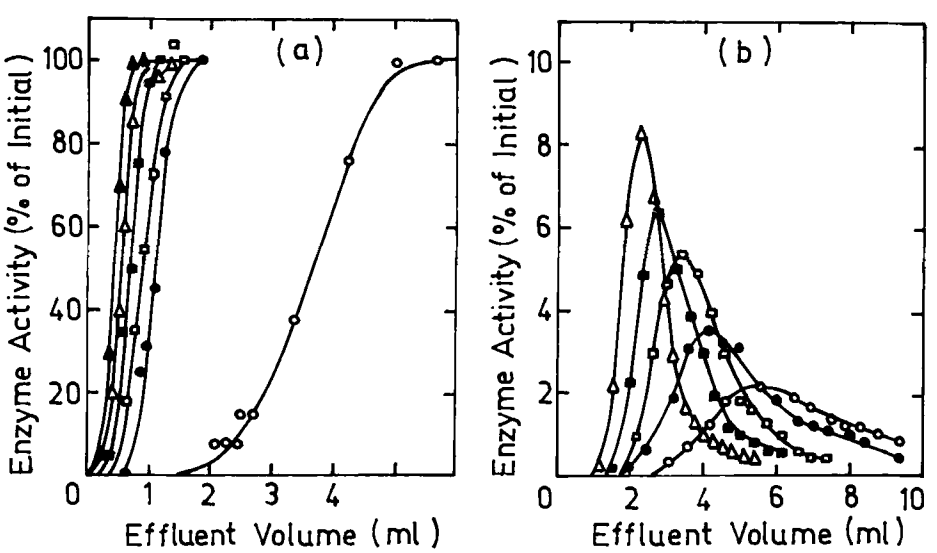


Fig. 14.6. Comparison of the effects of NADH concentration on the elution profiles obtained in frontal and zonal chromatography of rat liver lactate dehydrogenase on 10-carboxydecylamino-Sepharose. (a) Advancing elution profiles obtained in frontal chromatography of enzyme (9nM) on a 0.1 ml column in the absence of NADH (o) and in the presence of 2 μM (●), 3 μM (❒), 4 μM (■), 5 μM (Δ) and 6 μM (▲) coenzyme. (b) Elution profiles obtained in zonal chromatography of enzyme (0.1 ml, 0.18 μM) on a 1.0 ml column in the presence of 8 μM (o), 10 μM (●), 12 μM (❒), 15 μM (■) and 18 μM (Δ) NADH. Reproduced with permission from P. Kyprianou and R. J. Yon, Biochem. J., 207 (1982) 549-556.

Lee et al. (1990) made a systematic study of nonlinear effects on the determination of the binding constant in affinity chromatography. The criteria for the applicable range of linear theory are derived for both frontal and zonal analysis. The difference in the criteria for zonal and frontal analysis indicates that the latter may not be preferred in some instances. An investigation of experimental data from the literature showed that the effect of isotherm nonlinearity on elution volume is not small in affinity chromatography, where the binding constant is very large and the experimental design does not permit work in the linear region.

A close, quantitative relationship between equilibrium constants obtained by use of quantitative bioaffinity chromatography and analogous constants determined fully in solution for a large number of proteins was reviewed by Chaiken (1986). This consistently observed correlation has formed the basis for extending theoretical treatments in order to evaluate not only monovalent molecular systems of varying types but also multivalently interacting macromolecules, including those which function cooperatively. Characterization of the multiple interactions in the neurophysin-neurohypophysial hormone system was presented as an example.

Recently Winzor (1992) reviewed quantitative expressions derived specifically for each type of bioaffinity chromatography scheme: (1) ligand-facilitated elution, reflecting a competition between ligand and biospecific matrix sites for partitioning solute, or between solute and ligand for biospecific matrix sites; (2) ligand-retarded desorption, reflecting an interaction of solute-ligand complexes with matrix sites, or of solute with matrix-ligand complexes. However, because these expressions assume a rather daunting form in situations where the partitioning solute is multivalent in its interaction with matrix sites, a more general approach has been adopted in this review. Irrespective of the interplay of equilibria that gives rise to solute adsorption by biospecific matrix sites, the concentration of partitioning solute associated with the matrix is used to define an effective binding constant for the interaction of the solute constituent with biospecific matrix sites. This constitutive equilibrium constant is then rationalized in terms of equilibrium constants for the particular combination of interactions responsible for the affinity chromatographic behaviour. The logical starting point is the

interaction of partitioning solute with matrix in the absence of ligand, this being the situation in which the effective binding constant is also the equilibrium constant for the solute-matrix interaction.

In the initial bioaffinity chromatography studies (Andrews et al., 1973; Dunn and Chaiken, 1975; Kasai and Ishii, 1975) the partitioning solute, A, was considered to be univalent in its interaction with biospecific matrix sites, X. However, affinity chromatography of an enzyme such as lactate dehydrogenase on Blue Sepharose, 10-carboxydecylamino-Sepharose or trinitrophenyl-Sepharose involves a situation in which the nucleotide-binding site on each of the four subunits is a candidate for interaction with the matrix. Consequently, evaluation of the intrinsic affinity constant for the solute-matrix interaction, k_{AX}, relies upon account being taken of the tetravalency of the enzyme. For the interaction of f-valent partitioning solute, A, with univalent biospecific matrix sites, X, the binding function, r_f, is defined as

$$r_f = ([\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f}) / [\overline{\overline{X}}] \qquad (14.15)$$

where $[\overline{A}]$ and $[\overline{\overline{A}}]$ denote the liquid-phase and total solute concentrations, respectively, of partitioning solute for a system with an effective total concentration $[\overline{\overline{X}}]$ of matrix sites: in the absence of ligand $[\overline{A}]$ is also the free concentration of solute. Adaptation of Eqn. 14.15 to frontal column chromatographic data in an experiment with applied solute concentration $[\overline{A}]$ is effected by noting (Hogg and Winzor, 1984) that the ratio of the accessible volume (V_A^*) to the measured elution volume ($\bar{V}_A$) defines the proportion of solute in the liquid phase. On making this substitution of $V_A^*/\bar{V}_A$ for $[\overline{A}]/[\overline{\overline{A}}]$, Eqn. 14.15 may be rewritten as

$$r_f[\overline{\overline{X}}] = [\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f} = [\overline{A}]^{1/f}\{(V_A/V_A^*)^{1/f} - 1\} \qquad (14.16)$$

The decision to combine the denominator of Eqn. 14.15 with r_f reflects the fact that the effective total concentration of biospecific matrix sites is not a parameter of known

magnitude in either column chromatographic experiments or partition equilibrium studies.

Provided that a single intrinsic association constant, k_{AX}, governs all solute-matrix interactions, the general counterpart of the Scatchard analysis becomes (Hogg and Winzor, 1985)

$$r_f[\bar{\bar{X}}]/[\bar{A}]^{1/f} = k_{AX}[\bar{\bar{X}}] - fk_{AX}r_f[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f} \qquad (14.17)$$

which enables k_{AX} and $[\bar{\bar{X}}]$, the effective total concentration of matrix sites, to be calculated from a linear plot of $r_f[\bar{\bar{X}}]/[\bar{A}]^{1/f}$ *versus* $r_f[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f}$. In frontal affinity chromatography $[\bar{\bar{A}}]$ is replaced by $(\bar{\nu}_A/\nu_A^*)[\bar{A}]$, and $r_f[\bar{\bar{X}}]$ by $\{(\bar{\nu}_A/\nu_A^*)^{1/f} - 1\}\,[\bar{A}]^{1/f}$ (Eqn. 14.16), whereupon Eqn. 14.17 becomes

$$(\bar{\nu}_A/\nu_A^*)^{1/f} - 1 = k_{AX}[\bar{\bar{X}}] - fk_{AX}(\bar{\nu}_A/\nu_A^*)^{(f-1)/f}[\bar{A}]\,\{(\bar{\nu}_A/\nu_A^*)^{1/f} - 1\} \qquad (14.18)$$

Fig. 14.7a presents such an analysis of frontal chromatographic data for the interaction of p-nitrophenylmannoside with immobilized concanavalin A, a system for which f may be assigned a value of unity on the grounds of the small size of the partitioning carbohydrate. Values of 2.4 ($\pm$ 0.5) x 10^4 M^{-1} and 13.3 μM are obtained for the intrinsic binding constant (k_{AX}) and effective matrix-site concentration ($[\bar{\bar{X}}]$), respectively. In a corresponding study (Hogg et al., 1987) of the interaction of concanavalin A with the carbohydrate matrix of Sephadex G-50 (Fig. 14.7b), the partitioning protein was considered bivalent because of its two equivalent and independent sites for carbohydrates: an intrinsic binding constant of 1.3 ($\pm$ 0.3) x 10^4 M^{-1} and an effective matrix site concentration of 46.3 μM result from this analysis. Fig.14.7 serves to emphasize that an obvious prerequisite for the application of Eqns. 14.17 and 14.18 to affinity chromatographic data is the assignment of a magnitude to the solute valence, f; and in that regard, having recourse to a conventional Scatchard plot (f = 1 in Eqns. 14.17 and 14.18) merely means that unity has been selected as the most appropriate valence.

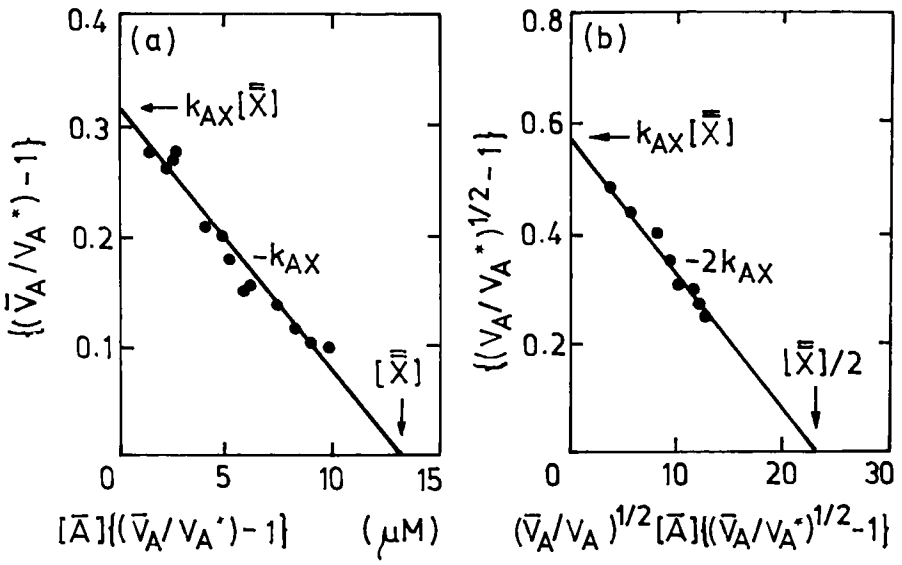


Fig. 14.7. Evaluation of the intrinsic association constant for the solute matrix interaction, k_{AX}, in frontal affinity chromatographic studies of the interactions of (a) p-nitrophenylmannoside with concanavalin A immobilized on glyceryl-CPG 170, and (b) concanavalin A with Sephadex G-50. Results are plotted according to Eqn. 14.18 with the valences (f) of p-nitrophenylmannoside and concanavalin A being taken as 1 and 2, respectively. Reproduced with permission from D. J. Winzor, J. Chromatogr., 597 (1992) 67-82.

Although the problem of making allowance for solute multivalency has been solved in the sense that Eqns. 14.17 and 14.18 provide the general counterpart of the Scatchard analysis, such a procedure is open to the criticism that the use of a linearly transformed version of the binding equation distorts the consequences of experimental uncertainty. The obvious answer to such criticism is to employ the untransformed binding equation, but for multivalent solutes it transpires that Eqn. 14.17 has been derived without recourse to the expression of which it is the linear transform: the latter therefore needs to be identified. By a relatively simple rearrangement and multiplication throughout by

$f[\bar{\bar{A}}]^{(f-1)/f}$, Eqn. 14.17 may be written as

$$fr_f[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f} = \frac{fk_{AX}[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f}[\bar{A}]^{1/f}}{1 + fk_{AX}[\bar{\bar{A}}]^{(f-1)/f}[\bar{A}]^{1/f}} \quad (14.19)$$

from which the dependence of $fr_f[\bar{\bar{X}}][\bar{\bar{A}}]^{(f-1)/f}$ upon $f[\bar{\bar{A}}]^{(f-1)/f}[\bar{A}]^{1/f}$ is seen to be a rectangular hyperbolic relationship. Standard nonlinear regression analysis of results in such terms may thus be used to yield k_{AX} and the total concentration of biospecific matrix sites, $[\bar{\bar{X}}]$.

14.4 RECYCLING PARTITION EQUILIBRIUM SYSTEM

An alternative to quantitative column bioaffinity chromatography is to conduct a series of partition equilibrium experiments in which the concentrations of solute in the liquid phase are determined directly for mixtures with known total concentration of partitioning solute. Kuter et al. (1983) investigated the interaction between aldolase and myofibrils derived from rabbit skeletal muscle by partition equilibrium studies at pH 6.8 and physiological ionic strength (I = 0.158M). The results are interpreted in terms of an intrinsic association constant of 410,000 M^{-1} for the interaction of four sites on aldolase with myofibrillar sites, there being one such site for every 10-12 heptameric repeat units of the F-actin-tropomyosin-troponin thin filament. Involvement of the active site of the enzyme in the adsorption process was indicated by the fact that competitive inhibition of the phenomenon by phosphate may be accounted for by an intrinsic association constant of 400 M^{-1} for the aldolase-phosphate interaction, a value in good agreement with that describing phosphate inhibition of the enzymatic hydrolysis of fructose-1,6-bisphosphate under similar conditions. On the basis of these equilibrium constants plus the aldolase and thin filament contents of muscle, resting muscle was indicated as containing a significant proportion (25-30%) of aldolase in the bound form, with changes in the subcellular distribution of the enzyme being likely during exercise

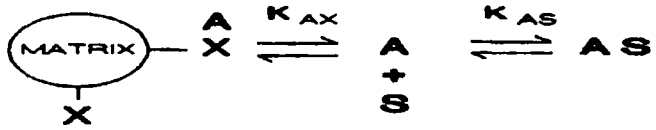
due to the increased concentrations of Ca^{2+} and fructose-1,6-bisphosphate that then prevail.

As well as reinforcing the concept that reversible adsorption of aldolase is a physiologically significant phenomenon in skeletal muscle, the results of this investigation also signify the involvement of the enzyme active site in the interaction with muscle matrix.

The effect of calcium ion on the interaction of aldolase with rabbit muscle myofibrils was studied by Harris and Winzor (1989). A partition equilibrium study has shown calcium ion to be a noncompetitive inhibitor of the adsorption of aldolase by myofibrils. This inhibition was interpreted quantitatively in terms of a tenfold decrease in the intrinsic association constant for the aldolase-myofibril interaction upon Ca^{2+} binding to either or both of the low-affinity troponin sites associated with regulation of muscle contraction.

The versatility of quantitative bioaffinity chromatography (QBAC) in the evaluation of the binding of macromolecular ligands to macromolecular acceptors has been increased substantially as a result of the derivation of the equations which describe the partitioning of acceptor between biospecific matrix-bound and soluble forms in terms of total, rather than free, ligand concentrations. A summary of the parameters used by Hogg et al. (1991) in the study of the interaction between heparin and human antithrombin III by recycling partition quantitative bioaffinity chromatography on heparin-Sepharose is shown in Fig. 14.8. In addition to simplifying the performance of the binding experiments, this development makes it possible to apply the technique to systems characterized by affinities higher than those previously amenable to investigation by QBAC.

Fig. 14.9 shows a schematic representation of the recycling partition system used in studies of the interactions. In this system, which uses microcomputer-assisted data acquisition in recycling partition QBAC, a slurry (approx 6 ml) of heparin-Sepharose affinity matrix (less than 1 mg) is placed in the recycling apparatus, which comprises a 10-ml Amicon ultrafiltration cell (devoid of membrane) positioned over a submersible magnetic stirrer in a water bath thermostatically maintained at the required temperature



X	Matrix-linked ligand (heparin)
A	Acceptor or partitioning solute (antithrombin III)
S	Ligand, in mobile phase (heparin)
$\bar{C}_X$	Effective concentration of matrix sites (matrix-bound ligand)
C_A	Total concentration of A (A plus AX and AS)
$\bar{C}_A$	Total (constituent) concentration of partitioning solute in mobile phase
C_A	Concentration of free A
$\bar{C}_S$	Total (constituent) concentration of S (S plus AS)
C_S	Concentration of free S
k_{AX}	Association constant for A binding to X (binding constant in terms of C_A)
$\bar{k}_{AX}$	Apparent (constitutive) association constant for A binding to X (binding constant in terms of $\bar{C}_A$)
k_{AS}	Association constant for S binding to A; binding constant in terms of C_S
V_A^*	Volume of mobile phase accessible to A
$(V_A^*)_o$	V_A^* prior to addition of an aliquot of partitioning solute and/or ligand.

Fig. 14.8. Summary of parameters used in the study of the interaction between heparin and antithrombin III by recycling partition quantitative bioaffinity chromatography (QBAC). Reproduced with permission from P. J. Hogg et al., Anal. Biochem., 192 (1991) 303-311.

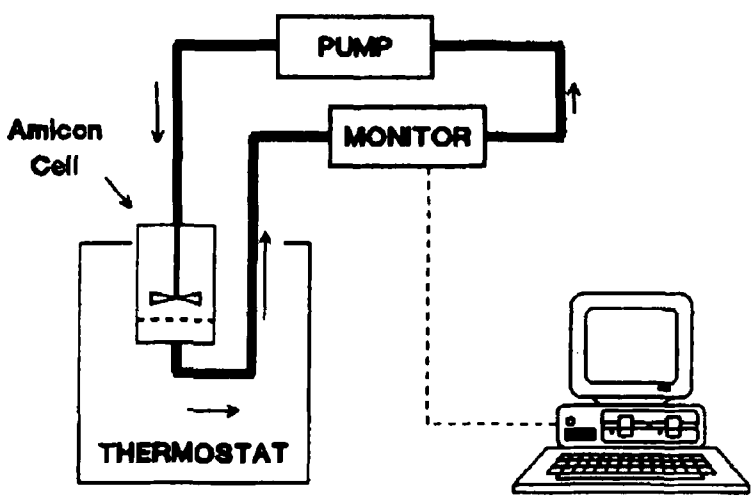


Fig. 14.9. Schematic representation of the recycling partition system used in studies of the interaction between heparin and antithrombin III by QBAC. Reproduced with permission from P. J. Hogg et al., Anal. Biochem., 192 (1991) 303-311.

(15, 25 or 35°C). An accurate value for the volume (V_T) is determined by weighing the slurry in the previously tared cell. Effluent from below the porous membrane support is returned to the recycling apparatus at a flow-rate of 1.4 ml/min *via* a peristaltic pump and the flow cell of a Pharmacia UV-M monitor operating at 280 nm and a full-scale deflection of 0.02, 0.05 or 0.10 absorbance unit. The monitor response is transferred at 1-s intervals to an IBM personal computer by means of a software package designed initially for the evaluation of progress curves in enzyme kinetic studies. Addition of an on-line data acquisition system to monitor the concentration of partitioning solute in the liquid phase as a function of time has permitted the adoption of an empirical approach to the determination of the liquid-phase concentration of acceptor in the system at partition equilibrium, a development which significantly decreases the time required to obtain a complete binding curve by QBAC.

The protocol for the characterization of antithrombin III - heparin interaction describes a subdivision into a series of sequential steps: (1) evaluation of the volume accessible to partitioning solute; (2) quantitation of the solute-biospecific matrix interaction; and (3) competition by high-affinity heparin.

Results of a recycling partition equilibrium experiment on the interaction between heparin and antithrombin III at 25°C are summarized in lines 1-8 of Table 14.2, about which the following points may be noted: (1) the first column lists the volume of liquid phase, V_A^*, which gradually increases due to the addition of aliquots of antithrombin. (2) The successive additions of antithrombin to the slurry of heparin-Sepharose give rise to a systematic increase in the total concentration of solute, $\overline{C}_A$ (column 2), calculated as the amount of added antithrombin divided by V_A^*. (3) The corresponding equilibrium concentrations of antithrombin in the recycling liquid phase, C_A, are given in column 4. (4) Since antithrombin is univalent in its interaction with heparin, the association equilibrium constant for the solute-matrix interaction (k_{AX}) and the effective total concentration of matrix sites ($\overline{C}_X$) are determined by Scatchard analysis. (5) In experiments of competition by heparin C_A varied between 3.70 and 3.68 and 0.5 and 3 μM; the corresponding range for C_S was 3.5 - 8.6 μM: these results are summarized

in the final four lines of Table 14.2. An operational (or constitutive) equilibrium constant, $\bar{k}_{AX}$, is calculated for the antithrombin-matrix interaction by means of the expression

$$\bar{k}_{AX} = (\bar{\bar{C}}_A - \bar{C}_A)/ [\, \bar{C}_A\{[(V_A^*)_0/V_A^*](\bar{C}_X)_0-(\bar{\bar{C}}_A-\bar{C}_A)\} \,] \qquad (14.20)$$

in which the concentration of partitioning solute in the liquid phase ($\bar{C}_A$) now includes the concentrations of free A and AS, the solute-ligand complex. The final term, $\{[(V_A^*)_0/V_A^*](\bar{\bar{C}}_X)_0-(\bar{\bar{C}}_A - \bar{C}_A)\}$, describes the concentration of free matrix sites. Values of k_{AX} so calculated comprise the entries in the final column of Table 14.2.

Table 14.2.

Results of a recycling partition equilibrium experiment for characterization of the binding of heparin to antithrombin III by QBAC[a]

V_A^* (ml)	$\bar{\bar{C}}_A$ (μM)[b]	$\bar{C}_S$ (μM)[b]	$\bar{C}_A$ (μM)[c]	$10\text{-}5\,\bar{k}_{AX}$ (M^{-1})
5.96	–	–	–	–
		Solute addition		
5.98	0.937	–	0.094	–
5.99	1.404	–	0.177	–
6.00	1.869	–	0.349	–
6.01	2.332	–	0.599	–
6.02	2.794	–	0.908	–
6.03	3.254	–	1.423	–
6.04	3.713	–	1.881	–
		Ligand addition		
6.06	3.700	3.465	2.937	1.855
6.07	3.694	5.189	3.225	0.944
6.08	3.688	6.908	3.357	0.540
6.09	3.682	8.621	3.439	0.370

[a] Experiment conducted at 25°C by the addition of aliquots of antithrombin III (0.28 mM) or high-affinity heparin (1.05 mM) to a slurry of heparin-Sepharose in 0.05 M Hepes-0.125 M NaCl, pH 7.4.

[b] Based on V_A^* and the amount of antithrombin or heparin added.

[c] Calculated from the absorbance of the liquid phase at partition equilibrium.

Association constants of 8.0 ± 2.2 x 10^7, 3.4 ±0.3 x 10^7, and 1.0 ± 0.2 x $10^7 M^{-1}$ were determined by Hogg et al. (1991) for the interactions of high-affinity heparin with antithrombin III at three temperatures: 15, 25, and 35°C. The standard enthalpy change of -4.2 ± 0.6 kcal/mol that is calculated from these data is in good agreement with a reported value obtained from fluorescence quenching measurements.

In as much as the magnitude of the binding constant for the interaction of heparin with antithrombin III had already been determined from fluorescence measurements, the most important factor to emerge from this investigation is the demonstration that the recycling partition adaptation of QBAC provides a simple means of characterizing solute-ligand interactions in instances where both reactants are macromolecular. Moreover, it does not require a specific signal, such as fluorescence, in order to evaluate the binding.

The use of a data acquisition system to identify the equilibrium distribution of solute from the time course of the change in absorbance after each addition of solute or ligand in the recycling partition adaptation of QBAC shortens the time required for acquiring the data to determine $\bar{\bar{C}}_x$, k_{AX} and k_{AS}. More importantly, this technological development paves the way for complete automation of the technique. In that regard there is not only scope for automating the delineation of liquid-phase concentrations of solute, but also scope for using the computer facility to determine the various interaction parameters from the collected partition equilibrium data. In addition to this technological development, the present investigation has also given rise to a significant advance in quantitative affinity chromatography theory by providing expressions that describe the partition of solute in terms of the total concentration of competing ligand. This is an important breakthrough for studies of high-affinity interactions, because it obviates the need to ascertain the magnitude of the free ligand concentration, the parameter in terms of which earlier quantitative relationships were expressed (Hogg and Winzor, 1984). A combination of quantitative affinity chromatography theory and partition equilibrium studies thus provides a means of assigning equilibrium constants not only to the adsorption phenomenon (k_{AX}) but also to the competing interactions (k_{AS}, k_{SX}) that may occur (Harris and Winzor, 1989). The stage thus seems to be set for the

characterization of many tight-binding interactions, in the blood-clotting cascade and elsewhere, that have hitherto been unquantifiable because of essentially stoichiometric complex formation at the reactant concentrations required for studies of ligand binding by classical methods. QBAC has clearly provided an inroad into the problem of characterizing the interplay of biphasic equilibria that effect the control of physiological, hormonal and immunological responses involving surface receptors (Winzor, 1992).

14.5 KINETIC ASPECTS OF MEMBRANE-BASED IMMUNOAFFINITY CHROMATOGRAPHY

In order to overcome mass transfer resistances in bioaffinity purifications, flat-sheet and hollow-fiber membranes have been used as an alternative to diffusion-controlled particles (Nachman et al.,1992). Conceptually, membrane-based purification systems have several advantages over conventional particle-based methods. The high porosity and minimal mass transfer resistance in membranes allow a high volumetric throughput, resulting in extremely short process times. The membrane structure is such that the adsorbent molecule is situated on the inner surfaces of membrane pores, in effect along the flow path of the soluble target protein, thereby minimizing the diffusion and accessibility problems associated with gel beads used in packed column operations. The end result is the maximum utilization of the immobilized adsorbent molecule. The high mass transfer capabilities of the membrane facilitate maximum utilization of the rapid adsorption kinetics of bioaffinity chromatography.

The kinetic aspects of membrane-based immunoaffinity chromatography (MIC) have been described by Nachman (1992). With a view to the efficient large-scale purification of recombinant proteins, factors influencing antigen-antibody adsorption kinetics were studied in a model hollow-fiber membrane-based immunosorbent. The antigen was recombinant human interferon-α2a (rIFN-α2a). The immunosorbent consisted of a 0.4 ml hydrazide-derivatized hollow-fiber membrane (produced by Sepracor, Marlborough, MA, USA) to which 7.02 mg of monoclonal antibody to rIFN-α2a was immobilized in an oriented fashion, *via* the sugar moieties in the F_c region of IgG molecule. The coupling reaction scheme is shown in Fig. 14.10.

The two potential rate-limiting factors in bioaffinity chromatography are mass transfer and adsorption kinetics. Both must be maximized for the system to be efficient. As immunoaffinity chromatography involves high affinity antigen-antibody (Ag . Ab) interactions, it is mass transfer which first becomes limiting in diffusion-controlled particle-based systems. Consequently, the fast Ag-Ab adsorption kinetics are often not utilized to the maximum. The diffusion time, t_D, must be smaller than the residence time, t_R, of the antigen in the support matrix. As t_D is directly related to the diffusional distance, L, $t_D = L^2/D$ the extremely short diffusional distance ($< 1\mu$m) in membranes results in negligible diffusional limitations. This enables the study of the kinetic limitations in MIC. In fact, non-diffusion-controlled solution kinetics are approached in membrane-based systems, the difference being that the antibodies are on a membrane surface.

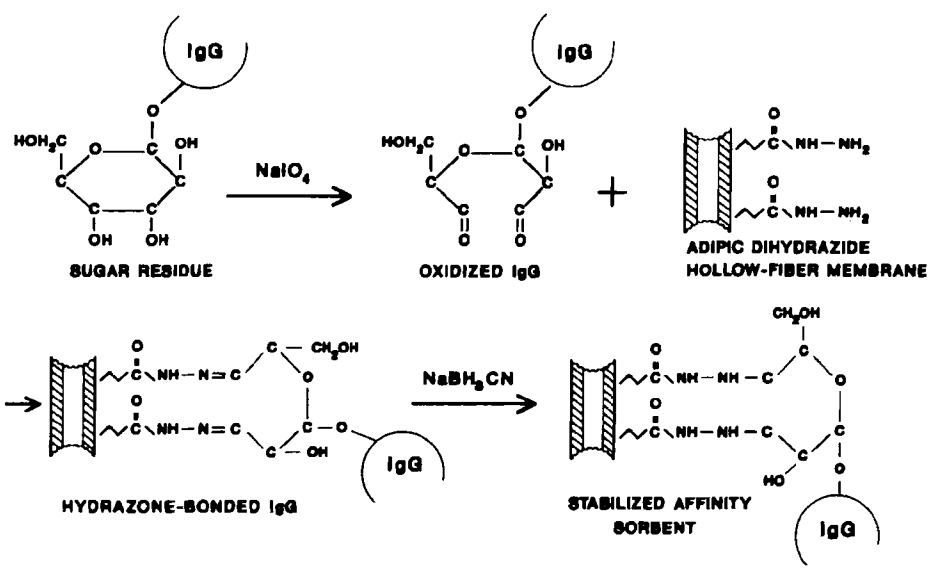


Fig. 14.10. Reaction scheme showing oriented coupling of IgG to hydrazide membrane. Reproduced with permission from M. Nachman, J.Chromatogr., 597 (1992) 167-172.

Assuming that membrane systems are analogous to free solution, antigen-antibody adsorption kinetics in MIC can be predicted by the rate expression

$$\frac{d[Ab.Ag]}{dt} = k_1[Ab][Ag] - k_{-1}[Ab.Ag] \tag{14.21}$$

Since k_{-1} is very small compared to k_1, the primary factors that determine the extent of antigen capture in an immunosorbent are [Ab], [Ag] and k_1.

Antigen-antibody adsorption kinetics in an immobilized system can also be described in terms of the effective dissociation constant, K_{de}, which is defined as

$$K_{de} = \frac{(q_m - q^*)\, C^*}{q^*} \tag{14.22}$$

where q_m is the immunosorbent's static binding capacity, q^* is the concentration of the immunocomplex formed ([Ab.Ag] in Eqn. 14.21) in equilibrium with C^*, which is the [Ag] remaining in solution. The concentration of functionally effective, unoccupied antibody binding sites is represented by q_m-q^* or [Ab] in Eqn.14.21.

In theory, for efficient antigen capture, [Ag] should be much greater than K_{de}. The capture efficiency would be expected to decrease when [Ag] < K_{de}. A typical K_{de} for antigen-antibody interaction in an agarose-based immunosorbent is *ca.* 1.5×10^{-8}M.

This model immunoaffinity system has been used to show that antigen-antibody adsorption kinetics, rather than mass transfer, is the initial rate-limiting factor in MIC. In the absence of diffusional limitations, immunoadsorption is at the limits of the antigen-antibody kinetics. Hence the high-affinity interactions which characterize immunoaffinity chromatography can be fully utilized in membranes. Antigen capture remains effective even at extremely short fluid residence times. Futher, binding is not kinetically limited at feed-stream antigen concentrations less than K_{de} and far below those usually associated with crude recombinant proteins. The membrane's mass transfer advantages are therefore effectively utilized.

Just the same as in Fig.4.18, the near-theoretical binding efficiency was achieved by Nachman (1992), only at low concentrations of immobilized antibody. The late antigen breakthrough point in membranes demonstrates that antigen-antibody adsorption kinetics are not mass transfer limited in the membrane system. However, the fact that antigen binding becomes less effective with increased antibody densities, together with the S-shaped character of the breakthrough curve (assuming uniform fluid flow), together point to the role of steric hindrance in reducing the absolute amount and overall rate of antigen capture. Steric hindrance, which can be expected in any immobilized system, is discussed in detail in Section 4.4.

REFERENCES

Anderson, D.J. and Walters, R.R., J. Chromatogr., 376 (1986) 69-85.

Andrews, P., Kitchen, B.J. and Winzor, D.J., Biochem. J., 135 (1973) 897-900.

Brodelius, P. and Mosbach, K., FEBS Lett., 35 (1973) 223-226.

Brodelius, P. and Mosbach, K., Biochem. Soc. Trans., 2 (1974) 1308-1310.

Chaiken, I.M., J. Chromatogr., 376 (1986) 11-32.

Dunn, B.M. and Chaiken, I.M., Proc. Nat. Acad. Sci. U.S., 71 (1974) 2382-2385.

Dunn, B.M. and Chaiken, I.M., Biochemistry, 14 (1975) 2343-2349.

Harris, S.J. and Winzor, D.J., Biochim. Biophys. Acta, 999 (1989) 95-99.

Hogg, P.J., Jackson, C.M. and Winzor, D.J., Anal. Biochem., 192 (1991) 303-311.

Hogg, P.J., Reilly, P.E.B. and Winzor, D.J., Biochemistry, 26 (1987) 1867-1873.

Hogg, P.J. and Winzor, D.J., Arch. Biochem. Biophys., 234 (1984) 55-60.

Hogg, P.J. and Winzor, D.J., Biochim. Biophys. Acta, 843 (1985) 159-163.

Kasai, K. and Ishii, S., J. Biochem. Tokyo, 77 (1975) 261-264.

Kasai, K.I., Oda, Y., Nishikata, M. and Ishii, S.I., J. Chromatogr., 376 (1986) 33-47.

Kuter, M.R., Masters, C.J. and Winzor, D.J., Arch. Biochem. Biophys., 225 (1983) 384-389.

Kyprianou, P. and Yon, R.J., Biochem. J., 207 (1982) 549-556.

Lee, W.C., Tsai, G.J. and Tsao, G.T., J. Chromatogr., 504 (1990) 55-67.

Moore, R.M. and Walters, R.R., J. Chromatogr., 384 (1987) 91-103.

Nachman, M., J. Chromatogr., 597 (1992) 167-172.

Nachman, M., Azad, A.R.M. and Bailon, P., J. Chromatogr., 597 (1992) 155-166.

Nichol, L.W., Ogston, A.G., Winzor, D.J. and Sawyer, W.H., Biochem. J., 143 (1974) 435-443.

Winzor, D.J., in P.D.G. Dean, W.S. Johnson and F.A. Middle (Editors), Affinity Chromatography : A Practical Approach, IRL Press, Oxford, 1985, pp. 149-168.

Winzor, D.J., J. Chromatogr., 597 (1992) 67-82.

Chapter 15

Theory of bioaffinity chromatography

In contrast to the considerable number of papers in which the most varied factors affecting the results of bioaffinity chromatography are treated empirically, theoretical guidelines based on physicochemical properties and relationships have been presented rather rarely. The first papers dealing with this theory were published by Kasche (1970), Reiner and Walsch (1971), Lowe et al. (1974), Nishikawa et al. (1974) and Porath (1974). Of these, the elaboration by Graves and Wu (1974) of simple kinetic and equilibrium models of affinity sorption and desorption are most instructive. These authors analyzed the adsorption and desorption phases of bioaffinity chromatography separately, and in each treatment they first considered only the limitations based on equilibrium relationships and took no account of rate processes (diffusion and reaction). Since it is much easier to analyze the results of adsorption or desorption in a batchwise arrangement, Graves and Wu deduced the main relationships in a simple model consisting of batch adsorption, washing and elution, under the assumption that equilibrium is attained in each step. They considered an enzyme as the substance being isolated, but the same relationships are, of course, also valid for any other substance.

Cooperative bonding, considered in terms of the plate theory, was described by Okada et al. (1975), who accomplished a mathematical deduction and a simulation of elution profiles on the basis of a cooperative elution of oligoadenylic acid on agarose with attached polyuridylic acid. An application of the statistical theory of chromatography elucidated by Giddings and Eyring (1955, plate theory) to bioaffinity chromatography was elaborated by Denizot and De Laage (1975). They use a convenient expression of moments to establish the convergence towards a Laplace-Gauss distribution. The Gaussian character is not preserved if other causes of dispersion are taken into account, but expressions of moments can be obtained in a generalized form. The same authors also deduced a simple procedure for expressing the fundamental constants of the model in terms of purely experimental quantities. The papers mentioned above are discussed in more detail in the first edition of this book (Turková, 1978).

This chapter only deals with several examples of papers on the theory of bioaffinity chromatography which have been published recently.

15.1 THEORY OF MEMBRANE-BASED RECEPTOR BIOAFFINITY CHROMATOGRAPHY

A simple theoretical analysis of the rate of receptor-ligand binding in membrane - based receptor affinity chromatography (RAC) has been described by Nachman et al. (1992) based on the assumption that the interaction which takes place in bioaffinity chromatography is analogous to the molecular recognition reaction in free solution.

In order to achieve maximum efficiency in RAC, the rate at which the soluble protein ligand binds to the immobilized receptor must be maximized. The binding affinity between the ligand, L, and the receptor, R, and the rate of diffusion of the soluble ligand into the receptor binding site determine the rate of ligand binding to the receptor affinity sorbent. As the receptor-ligand binding affinity is sufficiently high, this is rarely a problem in RAC. Thus, binding rates are generally determined by diffusional limitations of the affinity support. One can only take full advantage of the fast receptor-ligand adsorption kinetics when the characteristic diffusion time, t_D (the time needed for the soluble ligand to reach the immobilized receptor), is much shorter than the ligand's residence time, t_R, in the affinity purification device, i. e., $t_D << t_R$.

A smaller diffusional distance should yield a shorter diffusion time. In membranes, the bulk of mass transfer occurs *via* convection through the pores rather than by diffusion. Because of their short diffusional distance ($< 1\,\mu m$), membranes afford a short diffusion time and, for all practical purposes, mass transfer resistances in membranes are negligible. These minimal mass transfer restrictions enable one to study the kinetic limitations of the bioaffinity membrane. The kinetic advantages of using membranes are discussed in detail in Section 14.5 and have been described by Klein (1991) in the first book on affinity membranes.

The theoretical treatise of Nishikawa et al. (1976) describing the equilibrium binding of an adsorbate to an affinity ligand, with Chase' s (1984) modification for antigen-anti-

body binding, is also applicable to receptor-ligand binding. The binding kinetics are described by the following rate expressions:

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R.L \tag{15.1}$$

$$\frac{d[R.L]}{d_t} = k_1[R][L] - k_{-1}[R.L] \tag{15.2}$$

$$K_{de} = \frac{k_{-1}}{k_1} = \frac{[R][L]}{[R.L]} = \frac{(q_m - q^*)C^*}{q^*} \tag{15.3}$$

where k_1 is the binding rate constant, k_{-1} is the rate constant for the desorption of ligand from the complex, [R] is the concentration of unoccupied receptor binding sites, [L] is the concentration of soluble ligand, [R.L] is the concentration of receptor-ligand complex, K_{de} is the effective dissociation constant for the binding reaction in the affinity system, q_m is the maximum experimentally determined binding capacity per unit volume of the affinity sorbent, and q^* is the binding capacity per unit volume of the sorbent in equilibrium with concentration C^* of the adsorbate in solution. The effective dissociation constant, K_{de}, takes into account only the receptor which remains functionally effective after immobilization, and this may differ from the normal K_d in solution.

In affinity membranes, t_D is sufficiently small compared with t_R, and since receptor-ligand interactions involve a low dissociation constant, k_{-1} is also very small. Therefore, k_1, [R] and [L] are the primary factors which affect the adsorption rate in membrane-based RAC. Under such conditions the adsorption kinetics approach homogeneous, non-diffusion-controlled reaction kinetics in solution, the difference being that the receptor is on a membrane surface. The concentration [R] is determined by the immobilization density, which depends on the membrane surface area and the efficiency of immobilization. Concentration [L] is the feed stream ligand concentration and k_1 is determined by the characteristics of the receptor-ligand interaction. With the aim of

efficiently using the multipurpose interleukin receptor (IL-2R) affinity membrane in the purification of interleukin, Nachman et al. (1992) studied the effects of [R], [L] and k_1 on the capture efficiency of a hollow-fiber membrane-based IL-2R affinity system.

The other aspect of bioaffinity purification which must be considered is the desorption phase. Elution of the adsorbed ligand from the receptor sorbent requires complete dissociation of ligand from the receptor-ligand complex. Commonly used methods of elution involve nonspecific eluents which alter the three-dimensional structure of either the ligand, the receptor or both, thereby reducing or completely eliminating the binding avidity. Applying the expression of Lowe and Dean (1974) for the dissociation of an antigen-antibody complex to receptor-ligand dissociation, the elution kinetics can be described by Eqn. 15.4

$$\frac{d[R \,.\, L]}{d_t} = k_{-2}[R \,.\, L] \tag{15.4}$$

where k_{-2} is the rate constant for dissociation. The fast and complete elution by non-specific eluents, such as low pH buffers, chaotropes and denaturants, provides evidence that these eluents actually promote breakdown of the complex rather than prevent rebinding of the dissociated ligand. Overall, the elution conditions are chosen on the basis of protein stability, receptor-ligand binding strength and the types of forces involved in the receptor-ligand binding. Membrane bioaffinity chromatography cartridges are produced e.g. by Millipore (Bedford, Ma 01730, USA) or Amicon Corporation (Lexington, MASS.02173, USA).

15.2 INTERACTIONS IN COMPLEMENTARY BINDING SITES IN BIOAFFINITY CHROMATOGRAPHY

It is necessary to insert here a note on the terminology concerning biospecific intermolecular interactions. Different authors use a different nomenclature. Nachman et al. (1992) described types of forces in the receptor-ligand binding as electrostatic and hydrophobic interactions, van der Waals and London dispersion forces and hydrogen

bonding. Katchalski-Katzir (1983) stated that the interactions prevailing in biopolymer systems are: Coulomb forces, van der Waals forces, hydrogen bridges and hydrophobic binding. However, Hobza and Zahradník (1988) have used the term van der Waals interactions for all noncovalent bonds. The role of van der Waals systems in physical chemistry and in the biodisciplines has been described in their monograph "Intermolecular Complexes". Their classification of van der Waals systems (also called vdW molecules, complexes, associates) is that vdW complexes are systems in which parts (subsystems) are held together by forces other than chemical bonds. In contrast to the covalent interactions the noncovalent interactions occur between quite distant subsystems, where the overlap of the electron clouds is apparently negligible. Because of the negligible overlap between the subsystems, the molecular orbitals of individual subsystems are practically unaffected.

Four main types of noncovalent interactions based on quantum chemical perturbation theory are described by Havlas et al. (in press).

Coulombic interaction between two molecules is caused by the interactions of partial charges formed in the molecules due to the noncontinuous distribution of electrons in the molecules. Regions with opposite charge attract each other and regions with the same charge repeal each other. The strength of the Coulombic interaction depends on the distance between regions bearing positive and negative charge and therefore also on the orientation of the two molecules. The Coulombic energy (E^C) can be either attractive or repulsive.

A molecule with a noncontinuous distribution of electrons forms an electric field which can redistribute electrons in another molecule if it is within a short distance of the other one. This effect induces a dipole moment which is oriented in such a way that the interaction is always attractive. Such a contribution to the total interaction energy is called the induction energy E^I.

Another attractive interaction is called the dispersion interaction (or London interaction). The dispersion interaction is difficult to interpret in terms of classical physics. It is connected with fluctuations of electrons which temporarily form local dipole moments which interact in both molecules. The dispersion energy (E^D) forms a very

important contribution to the total interaction energy between large biological molecules. The interactions between the electrons of all bonds cooperate, resulting in a force which operates over very large distances (even up to 100 Å).

The last contribution to the total interaction energy is called the exchange-repulsion energy (E^{REP}). This reflects the fact that electron clouds cannot penetrate each other. This interaction is repulsive and is effective only at very small distances, where electron clouds overlap.

One specific type of attractive force is very common in biological interactions: the hydrogen bond. In the language of perturbation theory the hydrogen bond is caused mainly by Coulombic interaction, exchange-repulsion interaction and dispersion interaction (Umeyama and Morokuma, 1977; Jeziorski and van Hemert, 1976). The coulombic nature of the interaction is clear. Hydrogen bonds occur between a proton donor AH, where A is an electronegative atom (O, N, S, Cl, Br, I), and an acceptor group B, which is a lone electron pair or the π-electron orbital of an unsaturated bond. The importance of the dispersion interaction can be seen from a numerical analysis of different hydrogen bonds.

Another type of attractive forces is the so-called hydrophobic effect (or hydrophobic interaction). This takes place especially in solution and the role of the solvent is essential. The dissolution of hydrocarbons in water is an entropically unfavourable process. Thus, if two new hydrocarbon molecules associate with each other, part of the "frozen water" surrounding the hydrocarbons is liberated and the ensuing increase in entropy is the driving force behind the process of hydrophobic binding. The interaction energy between two hydrophobic molecules (e.g. hydrocarbons) is rather small, but when immersed in aqueous media they break down the structure of water and are forced to associate. As already mentioned above, the change in the water structure is reflected by a positive entropy change. This is in contrast to, e.g., the hydrogen bond, where the interaction energy appears in the enthalpy term. The biospecific adsorbents suitable for the determination of equilibrium constants by quantitative bioaffinity chromatography, described in Chapter 14, can therefore be a good model for the study of biospecific complex formation in living cells. Another way to study this problem can be the use of

the enzyme thermistor reported by Danielsson et al. (1981) as a specific detector for chromatographic procedures.

The effects of the microenvironment on the specific interaction between α-chymotrypsin and soybean trypsin inhibitor coupled to Sepharose 4B have already been studied by Kasche (1973). The aim of this study was to obtain data on the relative importance of the perturbation of reversible processes due to different microenvironmental effects. The heterogeneity of the microscopic association constants was found to increase with the pH used in the coupling procedure. The microenvironmental effect due to the nature of the matrix is discussed in more detail in Section 4.2.

Complementarity, specificity and dynamics in protein recognition have been described by Katchalski-Katzir (1983). Complementarity is revealed by: (a) the observation that the interface between an enzyme and its substrate, between an antibody and its corresponding antigen, or between two proteins bound together, is stabilized by the same types of interactions that stabilize globular protein molecules; i.e., there is close packing of atoms, a maximum of hydrogen bonds, complementary pairing of charges and buried hydrophobic groups;

(b) the shapes of enzyme and substrate, or antigen and antibody, which give a close-packed interface without internal holes; and

(c) pairing of oppositely charged groups, this is important for reasons of specificity.

Specificity is achieved through favourable interactions between a ligand and the specific high molecular weight compound to which it binds. The nature of interatomic forces places certain constraints on specificity. Charged substrates can be recognized with high specificity, since pairing between the wrong charges is very unfavourable for interaction. Because the force between charges decreases slowly with separation, unfavourable interactions cannot be avoided as small atomic shifts occur. Large substrates will not fit into pockets designed for small substrates, giving good discrimination. The reverse, however, is not true, since small substrates can fit into a large pocket.

All atoms move as an inescapable consequence of their thermal energy at room temperature. Precise recognition occurs in spite of atomic motion, not because of it. It should be noted, however, that charge interactions which are not sensitive to the precise

atomic positions are only slightly affected by thermal vibrations. In certain cases dynamics can play a constructive role by allowing access of substrates to an active site that is blocked in the static structure. Under appropriate conditions, marked conformational alterations can occur in proteins and probably also in other biopolymers; these might play an important biological role in some cases.

According Katchalski-Katzir (1983) evidence for mobility within proteins comes from a variety of physical methods: single crystal X-ray or neutron diffraction, electron microscopy, and spectroscopic techniques such as NMR, fluorescence depolarization, Mössbauer spectroscopy and hydrogen exchange studies. Theoretical approaches, such as potential-energy minimization and molecular-dynamics calculations, may also be used to study flexibility.

Even when attention is restricted to examples of protein flexibility characterized by the motion of large parts of the molecule, the functional roles of flexibility are quite diverse, as suggested by Huber and Bennett (1983).

(a) Flexibility might serve a regulatory function by controlling biospecific complex formation. Interaction with a rigid, stereochemically complementary surface would be stronger than binding to a flexible segment that must be stabilized before it can provide optimal noncovalent interactions. Biospecific binding could in some cases be regulated by an allosteric linkage of the transition between flexible and rigid states to other environmental factors. (b) Some proteins are composed of domains that are structurally separated but covalently linked by segments that allow them to move and to function independently. (c) For example, in some enzymes domains may move in relation to one another during the catalytic cycle. These enzymes occur both in an open form which presumably allows substrate binding and product release, and in a closed form in which the substrate is shielded from solution and is properly aligned in relation to catalytic groups.

The observation that kinases generally have a bilobal structure roughly similar to that found in hexokinase has led to the suggestion that analogous domain motions are a common feature of this class of enzymes.

Conformational changes of the last type have been beautifully demonstrated by Remington, Wiegand and Huber (1982) in the case of citrate synthase, a condensing enzyme that catalyzes the reaction between acetyl coenzyme A and oxaloacetate to form citrate. The molecule is a dimer of two identical subunits each containing 437 amino acid residues. It is a large globular molecule formed almost entirely of α-helices. The two subunits pack tightly *via* eight helices in an antiparallel arrangement. Each subunit consists of two well-characterized domains: a large one mediating the dimer aggregation, and a small one of about 110 residues comprising the rest of the molecule. The small domain has a much less rigid structure than the large one, and appears to respond to the domain arrangement and to functional states of the enzyme by changes in tertiary structure. Citrate is bound in a cleft between the large and small domains, and upon domain closure is completely enveloped in a highly polar pocket. CoA-SH is bound to the small domain, and the cysteamine part comes very close to the bound citrate. Only in the closed conformational form of the enzyme is the CoA binding site completely formed. Domain closure thus provides a better binding site for the cofactor. The importance of electrostatic forces in the binding of citrate and oxaloacetate by citrate synthase is revealed by the fact that both of these negatively charged substrate molecules are surrounded by four histidine residues and three arginine residues. Kinetic studies show that citrate synthase has an ordered mechanism in which oxaloacetate is bound first, whereupon a conformational change occurs, leading to very strong binding of acetyl CoA. Catalytic action, condensation of oxaloacetate and acetyl CoA to form citryl CoA, and hydrolysis of citryl CoA to citrate and CoA all proceed in the closed form.

An understanding of the atomic and molecular forces prevailing in the conformational changes illustrated above might pave the way for prediction of the conditions favouring tight binding between a biologically active compound and its corresponding ligand on the one hand, and conditions under which the ligand is weakly bound and the biospecific adsorbed compound can be readily released. Macromolecular conformational fluctuations as well as reversible conformational changes seem to play an important role in many of the biological reactions in which recognition by and of biopolymers occurs. The detailed understanding of such forces is of the greatest interest,

since it promises to lead to a better understanding of many life processes and provide sound guidelines for further development in the art of bioaffinity chromatography. Stabilizations of biologically active compounds by suitable immobilization or modification are in detail discussed in Sections 4.3.2, 4.3.3 and 4.3.4.

15.3 ENERGY OF BIOSPECIFIC COMPLEX FORMATION

Total interaction energy between two molecules depends on the distance between them, as shown in Fig. 15.1. The minimum on the curve represents a region of equilibrium distance with maximum bonding of components.

The interacting molecules tend to release energy by shortening the distance between them. In the region of small distances the force is repulsive, repelling the molecules. The optimum distance is in the region where repulsive and attractive forces are in equilibrium.

It is valuable to analyze the nature of the forces acting on the interacting molecules. In this respect the interaction energy is partitioned into several contributions. Note that, from the physical point of view, only total interaction energy is correctly defined (it is observable) and any partitioning is arbitrary. There are two requirements for a partitioning. First, the sum of all contributions ought to describe, at least approximately, the total interaction energy; and second, each contribution should be physically well understandable.

One very popular partitioning is based on quantum chemical perturbation theory. The total interaction energy is divided into four main contributions called Coulombic (or electrostatic) interaction energy, E^C, induction energy, E^I, dispersion energy, E^D, and exchange-repulsion energy, E^{REP}. All these types of energy have already been characterized in Section 15.2.

The binding of a substance to be isolated to a biospecific sorbent in the bioaffinity chromatography process can be described, from the thermodynamic point of view, by the equilibrium constant K.

$$A + B \rightleftharpoons A \ldots B, \qquad K = \frac{[A...B]}{[A][B]} \tag{15.5}$$

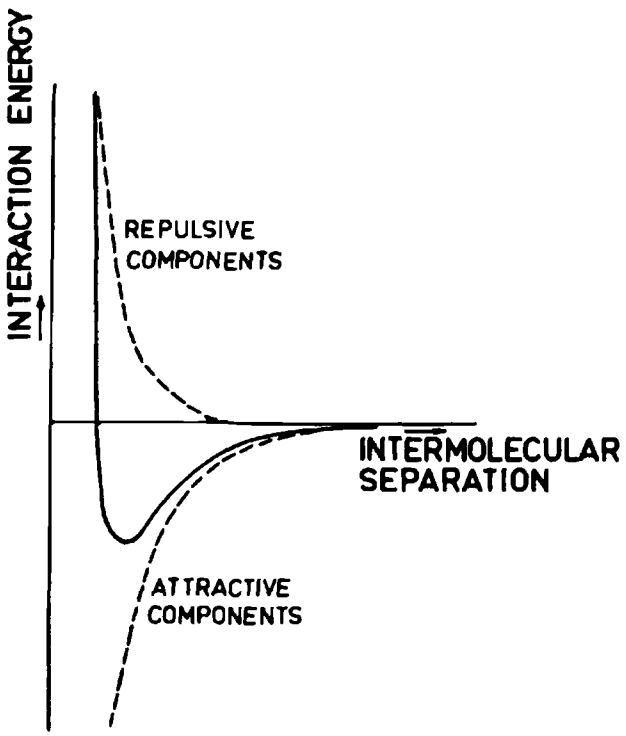


Fig. 15.1. A typical dependence of total interaction energy on intermolecular distance (full curve). The dashed lines represent the sum of repulsive and attractive components of the total energy.

where A and B represent for the substance and the biospecific sorbent, respectively, and A...B is the complex formed. The equilibrium constant has to be high enough to indicate sufficiently strong binding of the substance molecules to the biospecific sorbent, but not too high, because it would then be hardly possible to elute the substance.

The equilibrium constant bears a simple relationship with the standard change of free (Gibbs) energy (Havlas et al., in press),

$$\Delta G^{o} = - RT\ln K \qquad (15.6)$$

The term "standard change" refers to the change of Gibbs energy in reaction (15.5), when reactants and products are in their standard states; R is the gas constant and T is

absolute temperature. The change of Gibbs energy can be expressed in terms of two components, enthalpy and entropy changes,

$$\Delta G^{o} = \Delta H^{o} - T\Delta S^{o} \qquad (15.7)$$

ΔH^{o} and ΔS^{o} stand for standard changes of enthalpy and entropy. At low temperature $T\,\Delta S^{o}$ is often small and so $\Delta G^{o} \sim \Delta H^{o}$. This approximation, which is quite good for low temperatures and large ΔH^{o}, gave rise to the erroneous view that equilibria occur because the reactions tended to occur in the direction that lowered the energy (the enthalpy) of the mixture. At higher temperatures, or when ΔH^{o} is small, the $T\,\Delta S^{o}$ term may dominate, and $\Delta G^{o} \sim -T\,\Delta S^{o}$. This is negative if ΔS^{o} is positive, and so the reaction then tends to occur in the direction that maximizes the entropy of the reaction mixture. Sometimes ΔH^{o} and $T\,\Delta S^{o}$ are of similar importance in determining the sign of ΔG^{o}, sometimes they are in opposition, and sometimes they reinforce each other. The situation may be illustrated by the study of Barlow and Burtson (1979) on adsorption of enantiomeric pairs of esters of phenylcyclohexylglycollic acid onto muscarimic receptors in the guinea pig ileum. The enthalpy and the entropy changes vary from negative to positive values, always giving a negative change of Gibbs energy.

The enthalpy change (ΔH^{o}) is experimentally accessible either directly, by calorimetry measurements, or from the temperature dependence of the equilibrium constant *via* the van' t Hoff equation

$$\frac{d\,(\ln K)}{d\,(1/T)} = -\Delta H^{o} / R \qquad (15.8)$$

The entropy term ($T\,\Delta S^{o}$) is usually estimated from the value of the equilibrium constant and the enthalpy change using Eqns 15.6 and 15.7.

In order to understand Eqn. 15.7 better, we have to interpret it at the molecular level. The enthalpy term, ΔH^{o}, is closely related to forces between all the components forming the complex on the biospecific sorbent (including solvent molecules). A

knowledge of the interaction energy between all pairs of molecules included in the adsorption is sufficient for an estimation of the enthalpy change (Hobza and Zahradník, 1980). Because all the interactions in bioaffinity chromatography are of a noncovalent nature, the theory of weak intermolecular interactions can help our understanding of the process. The experimentalist can then deliberately change the conditions of the experiment with the aim of increasing the efficiency of the chromatographic separation.

In contrast to the enthalpy term, which can be divided into a set of individual interactions between pairs of molecules involved in the process, entropy measures the dispersal of energy of the whole system and is a purely statistical quantity. No simple partitioning into physically well-defined contributions exists. Entropy is a very complex and complicated function of molecular constants.

As mentioned above, in Section 15.2, interactions in chemistry are traditionally divided into two groups. The first group of interactions leads to the formation of chemical bonds, either covalent or ionic, and these are called strong interactions. Interaction energy falls to the range of 80 - 400 kJ/mol. The second group of interactions is termed weak and its interaction energy amounts to 3 - 60 kJ/mol. Although there are weak covalent bonds and strong noncovalent bonds, it is still reasonable to distinguish these two types of binding. As mentioned in Section 3.8 the binding of the water-soluble vitamin biotin to the egg-white protein avidin is paralleled by one of the greatest drops in free energy ever observed for noncovalent interactions. The ΔH-value for the binding of four biotin molecules was determined by Green (1966) as to be - 334 kJ/mol avidin. The change in entropy for this reaction was found to be zero. However, in the case of bioaffinity chromatography, as in all biochemical processes, weak intermolecular interactions play a dominant role.

15.4 MODELS SUITABLE FOR EFFICIENT LARGE-SCALE BIOAFFINITY CHROMATOGRAPHY

Appropriate theoretical models are needed in order to predict the dynamic behaviour, to design, to scale-up, to optimize, and to control bioaffinity chromatography systems. Suitable theoretical aspects of bioaffinity chromatography have been discussed

by Liapis (1989). He considered four stages in affinity separations: (a) the adsorption stage during which adsorbate is brought into contact with the adsorbent to allow the adsorption interactions to occur; (b) the wash stage in which loosely or non-specifically adsorbed components are removed; (c) the elution stage during which the adsorbate is released from the adsorbate-ligand complex; and (d) the re-equilibration or regeneration stage which prepares the adsorbent for reuse in another cycle of operation.

The above four stages of affinity chromatography may occur in a finite bath (batch) system, a fixed bed, a periodic countercurrent bed, a fluidized bed, or in a magnetically stabilized fluidized bed. The most commonly used mode of operation in bioaffinity chromatography separations is to pack the adsorbent particles into a fixed bed and carry out the various stages by pumping the relevant liquid streams through the bed with axial or radial flow. The fixed bed operation provides reasonable adsorption, wash, and elution performance, minimizes the physical handling of the adsorbent material, and can be easily automated. Batch adsorption systems may be appropriate where the fluid to be processed is of high viscosity or contains particulate material.

Currently there is significant industrial interest in the design, control, and optimization of large-scale bioaffinity chromatography systems. Liapis and coworkers have constructed and presented models which may be used to predict and study the dynamic behaviour of the adsorption, wash, and elution stages in batch systems, fixed beds, and periodic countercurrent beds. The mass transfer mechanisms and rate steps involved in the formation and dissociation of the adsorbate-ligand complex have been reviewed by Liapis (1989). Fig. 15.2 shows the information flow of data used in batch and column models to predict the dynamic behaviour of bioaffinity chromatography.

An examination of the literature strongly suggests that there is a need for researchers to consider and perform experimental and theoretical studies in order to develop expressions from which the free molecular diffusivities of biological macromolecules in different solution environments (single and multicomponent mixtures) and temperatures may be accurately estimated (the solution environment of the elution stage is most often significantly different from that of the adsorption stage).

Liapis et al. (1989) presented experimental equilibrium and dynamic column data on nonporous silica particles with immobilized anti-lysozyme monoclonal antibody. On the basis of experimental equilibrium data they estimated that the heat of adsorption of lysozyme onto immobilized monoclonal anti-lysozyme ligand was 12.22 kJ/mol lysozyme when the ligand density was low, and ΔH was equal to 10.38 kJ/mol lysozyme when the density of the anti-lysozyme ligand was high. It was also shown that the biospecific interaction rate constants between lysozyme and monoclonal anti-lysozyme ligand are many times larger than those of the non-specific interactions involving lysozyme molecules and a silica surface. The conclusion of this paper is that Langmuir's model was found to give a satisfactory representation of the equilibrium experimental data.

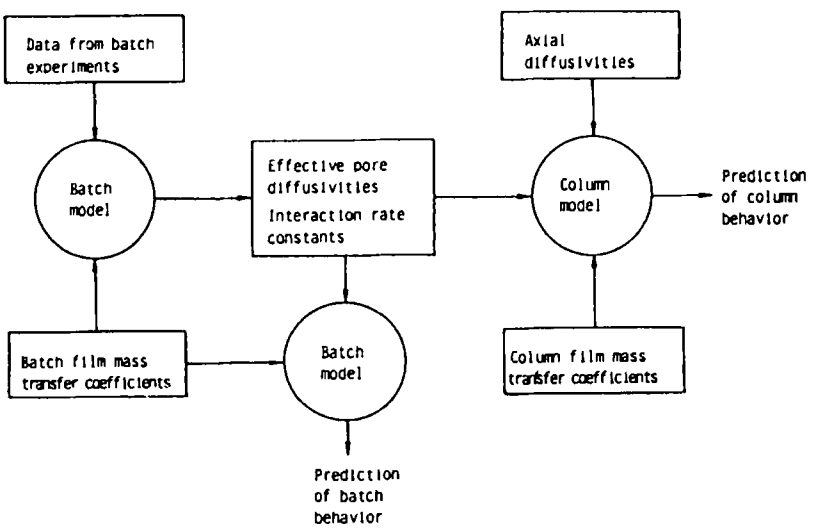


Fig. 15.2. Information flow of data used in batch and column models to predict the dynamic behaviour of bioaffinity chromatography. Reproduced with permission from A. I. Liapis, J. Biotechnol., 11 (1989) 143-160.

However, it is necessary to suggest that, in biospecific adsorption systems, the units of the experimental equilibrium adsorptivity should be in terms of the number of moles of adsorbate interacting per mole of ligand.

The influence of temperature on equilibrium adsorption is different for the different systems studied. Some investigators have reported a decrease of adsorption on raising the temperature (Liapis et al., 1989) and this implies that ΔH is negative (exothermic process). Some authors (Arnold and Blanch, 1986) have reported an increased adsorption on raising the temperature, and this implies an endothermic adsorption process where ΔH is positive; therefore, in such cases, an increase of entropy must be the driving force behind the adsorption process. The bioaffinity chromatography processes are reported to be isothermal since the heat of adsorption apparently does not change the temperature of the liquid stream, even in large-scale systems. This occurs because the total amount of adsorbed material is small and the heat capacity of the liquid stream is high.

A very important feature of macromolecular surfaces is the degree of irregularity of protein surfaces and its effect on macromolecular interactions (Liapis et al., 1989). Evidence has been presented that the fractal surface of lysozyme plays a role in the biological performance of this protein.

The employment of bioaffinity chromatography as an efficient and competitive separation process, when compared to other purification methods, requires the effective use of the ligands immobilized on the internal surface of porous adsorbent particles or on the external surface of nonporous adsorbents. The most efficient mode of column operation, with respect to ligand utilization, would theoretically be continuous countercurrent operation, where the adsorbent particles move in a direction opposite to the direction of motion of the flowing liquid stream (continuous countercurrent movement). However, this mode of operation would have practical problems because of the mechanical complexity of the equipment, gradual attrition of the solid adsorbent, and channeling (nonuniform flow) of either fluid or solid. Therefore, it may be easier to use a periodic countercurrent mode of operation since, if a column is divided into a infinite number of infinitesimally-sized beds operating in a periodic countercurrent

mode, this would give the same results as the continuous countercurrent mode of operation. In practice one has to deal with finite bed sizes, and therefore the original column of length L was divided into two columns, each of length L/2, which operated in a periodic countercurrent mode during the adsorption stage. Using this experiment, which is described in Fig. 15.3, Arve and Liapis (1988) have shown that the utilization of an adsorbent may be substantially increased if periodic countercurrent operation is employed in the adsorption process.

The column results indicate that when short beds are employed, then the choice of ligand, with respect to its rate of interaction with the adsorbate, may be of paramount importance. It was also shown that when a single column is divided into two beds operating in a periodic countercurrent mode the ligand utilization is higher than that obtained in a column of the same length operating in a fixed bed mode. This can be observed in Fig. 15.3 where, for beds of 0.5 m and longer, the utilization is very close to 100% when the columns are operated in a periodic countercurrent mode. It is also observed that for bed lengths shorter than 0.5 m there is a substantial difference in the utilization for the two modes of operation. In fact, for a bed length of 0.1 m the ligand utilization is almost four times greater than when the column is operated in a periodic countercurrent mode. This result should be of great importance in the evaluation of biospecific adsorption systems since the operation is often carried out in short beds. The ligand utilization in Fig. 15.3 is defined as the ratio of the amount of ligands that have formed adsorbate-ligand complexes at the end of the adsorption stage to the total amount of ligands available at the beginning of the adsorption stage. The percentage utilization is obtained by multiplying this ratio by one hundred. For the system shown in Fig. 15.3 the column switch occurred when the outlet concentration of the adsorbate had reached 1% of its inlet value. The advantage gained with a reversed flow increases as the bed length decreases.

In the wash stage it was shown that a certain amount of the product may be removed from the adsorbent particles and enter the washing solution. The washing medium, time, and mode of operation should be selected so that the loss of product is small and within an acceptable level (Arve and Liapis, 1988). Recently McCoy and Liapis (1991) have

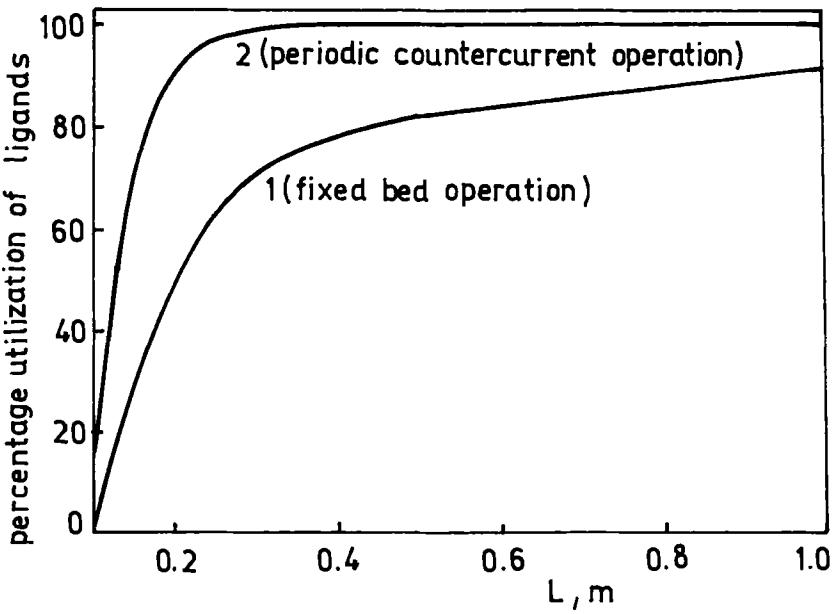


Fig. 15.3. Percentage of ligand utilization versus column length for a system involving a monovalent adsorbate. Curves: 1, fixed bed operation with a column of length L; 2, periodic countercurrent bed operation with two columns, each of length L/2, and total operating length L. Reproduced with permission from A. I. Liapis, J. Biotechnol., 11 (1989) 143-160.

reported the evaluation of kinetic models for biospecific adsorption and its implications for finite bath and column performance. Biospecific adsorption systems have been studied by Liapis and McCoy (1992) in the paper on theory of perfusion chromatography. Two different adsorption systems were examined: one system consider the adsorption of β-galactosidase on immobilized monoclonal anti-β−galactosidase, and the other system involves the adsorption of lysozyme on immobilized monoclonal anti-lysozyme.

Mattiason and Ramstorp (1984) developed a system for continuous separation where the adsorbent macromolecules are retained by ultrafiltration membranes and circulated within the lumens of ultrafiltration hollow fibers. The wash and elution steps take place within individual hollow-fiber modules in which the phase containing the ligand is circulated. The adsorption step is accomplished by either mixing the feed solution with the ligand and processing the mixture through the recirculating system, or by permea-

tion of the species through the membrane of a hollow-fiber module to the circulating ligand within the fibers. Heat-killed yeast cells were used as affinity sorption material for the isolation of concanavalin A from a crude extract of *Canavalia ensiformis*. The advantages of membrane-based receptor affinity chromatography shown by Nachman et al. (1992), or membrane-base immunoaffinity chromatography (Nachman, 1992), are discussed in Sections 15.1 and 14.5.

Research studies involving both experimental and theoretical investigations are very important for the development not only of bioaffinity chromatography and the oriented immobilization of biologically active compounds, but also for the better understanding of biospecific complex formation in nature. The theory of bioaffinity chromatography has recently been revisited by Winzor (1992). We believe that it is a great challenge and great joy to work in an area which is not only of considerable theoretical interest but also promises to achieve many important new practical applications.

REFERENCES

Arnold, F.H. and Blanch, H.W., J. Chromatogr., 355 (1986) 13-27.

Arve, B.H. and Liapis, A.I., Biotechnol. Bioeng., 32 (1988) 616-627.

Barlow, R.B. and Burston, K.N., Brit. J. Pharmac., 66 (1979) 581-585.

Chase, H.E., Chem. Eng. Sci., 39 (1984) 1099-1125.

Danielsson, B., Buelow, L., Lowe, C.R., Satoh, I. and Mosbach, K., Anal. Biochem., 117 (1981) 84-93.

Denizot, F.C. and De Laage, M.A., Proc. Nat. Acad. Sci. U.S., 72 (1975) 4840-4843.

Giddings, J.C. and Eyring, H., J. Phys. Chem., 59 (1955) 416-421.

Graves, D.J. and Wu, Y.T., Methods Enzymol., 34 (1974) 140-163.

Green, N.M., Biochem. J., 101 (1966) 774-779.

Havlas, Z., Hobza, P., Vondrášek, J., Turková, J. and Zahradník, R., Collect. Czech. Chem. Commun., in press.

Hobza, P. and Zahradník, R., Weak Intermolecular Interactions in Chemistry and Biology, Academia, Prague, 1980, pp. 1-307.

Hobza, P. and Zahradník, R., Intermolecular Complexes, Elsevier, Amsterdam, 1988, pp. 1-307.

Huber, R. and Bennett, W.S., Biopolymers, 22 (1983) 261-279.

Jeziorski, M. and van Hemert, M., Mol. Phys., 31 (1976) 713-719.

Kasche, V., Biochem. Biophys. Res. Commun., 38 (1970) 875-881.

Katchalski-Katzir, E., in I.M. Chaiken, M. Wilchek and I. Parikh (Editors), Affinity Chromatography and Biological Recognition, Academic Press, Inc., Orlando, 1983, pp. 7-26.

Klein, E., Affinity Membranes : Their Chemistry and Performance in Adsorptive Separation Processes, Wiley, New York, 1991, pp. 1-152.

Liapis, A.I., J. Biotechnol., 11 (1989) 143-160.

Liapis, A.I., Anspach, B., Findley, M.E., Davies, J., Hearn, M.T.W. and Unger, K.K., Biotechnol. Bioeng., 34 (1989) 467-477.

Liapis, A.I. and McCoy, M.A., J. Chromatogr., 599 (1992) 87-104

Lowe, C.R. and Dean, P.D.G., Affinity Chromatography, Wiley, New York, London, 1974, pp. 1-272.

Lowe, C.R., Harvey, M.J. and Dean, P.D.G., Eur. J. Biochem., 42 (1974) 1-6.

Mattiasson, B. and Ramstorp, M., J. Chromatogr., 283 (1984) 323-330.

McCoy, M.A. and Liapis, A.I., J.Chromatogr., 584(1991) 25-60.

Nachman, M., J. Chromatogr., 597 (1992) 167-172.

Nachman, M., Azad, A.R.M. and Bailon, P., J. Chromatogr., 597 (1992) 155-166.

Nishikawa, A.H., Bailon, P. and Ramel, A.H., Advan. Exp. Med. Biol., 42 (1974) 33-42.

Nishikawa, A.H., Bailon, P. and Ramel, A.H., J. Macromol. Sci. Chem., A10 (1976) 149-190.

Okada, S., Husimi, Y., Tanabe, S. and Wada, A., Biopolymers, 14 (1975) 33-49.

Porath, J., Methods Enzymol., 34 (1974) 13-30.

Reiner, R.H. and Walch, A., Chromatographia, 4 (1971) 578-587.

Remington, S., Wiegand, G. and Huber, R., J. Mol. Biol., 158 (1982) 111-152.

Turková, J., Affinity Chromatography, Elsevier, Amsterdam, 1978, pp. 13-34.

Umeyama, H. and Morokuma, K., J. Amer. Chem. Soc., 99 (1977) 1316-1332.

Winzor, D.J., J. Chromatogr., 597 (1992) 67-82.

SUBJECT INDEX *

* For some general terms which occur throughout the book, reference is given only to the page where the particular term is explained or to the chapter or paragraph dealing with the term in question.

JOURNAL OF CHROMATOGRAPHY LIBRARY

A Series of Books Devoted to Chromatographic and Electrophoretic Techniques and their Applications

Although complementary to the *Journal of Chromatography*, each volume in the Library Series is an important and independent contribution in the field of chromatography and electrophoresis. The Library contains no material reprinted from the journal itself.

Other volumes in this series

Volume 1	**Chromatography of Antibiotics** *(see also* Volume 26) by G.H. Wagman and M.J. Weinstein
Volume 2	**Extraction Chromatography** edited by T. Braun and G. Ghersini
Volume 3	**Liquid Column Chromatography.** A Survey of Modern Techniques and Applications edited by Z. Deyl, K. Macek and J. Janák
Volume 4	**Detectors in Gas Chromatography** by J. Ševčík
Volume 5	**Instrumental Liquid Chromatography.** A Practical Manual on High-Performance Liquid Chromatographic Methods *(see also* Volume 27) by N.A. Parris
Volume 6	**Isotachophoresis.** Theory, Instrumentation and Applications by F.M. Everaerts, J.L. Beckers and Th.P.E.M. Verheggen
Volume 7	**Chemical Derivatization in Liquid Chromatography** by J.F. Lawrence and R.W. Frei
Volume 8	**Chromatography of Steroids** by E. Heftmann
Volume 9	**HPTLC – High Performance Thin-Layer Chromatography** edited by A. Zlatkis and R.E. Kaiser
Volume 10	**Gas Chromatography of Polymers** by V.G. Berezkin, V.R. Alishoyev and I.B. Nemirovskaya
Volume 11	**Liquid Chromatography Detectors** *(see also* Volume 33) by R.P.W. Scott
Volume 12	**Affinity Chromatography** by J. Turková
Volume 13	**Instrumentation for High-Performance Liquid Chromatography** edited by J.F.K. Huber
Volume 14	**Radiochromatography.** The Chromatography and Electrophoresis of Radiolabelled Compounds by T.R. Roberts
Volume 15	**Antibiotics.** Isolation, Separation and Purification edited by M.J. Weinstein and G.H. Wagman

Volume 16	**Porous Silica.** Its Properties and Use as Support in Column Liquid Chromatography by K.K. Unger
Volume 17	**75 Years of Chromatography – A Historical Dialogue** edited by L.S. Ettre and A. Zlatkis
Volume 18A	**Electrophoresis.** A Survey of Techniques and Applications. **Part A: Techniques** edited by Z. Deyl
Volume 18B	**Electrophoresis.** A Survey of Techniques and Applications. **Part B: Applications** edited by Z. Deyl
Volume 19	**Chemical Derivatization in Gas Chromatography** by J. Drozd
Volume 20	**Electron Capture.** Theory and Practice in Chromatography edited by A. Zlatkis and C.F. Poole
Volume 21	**Environmental Problem Solving using Gas and Liquid Chromatography** by R.L. Grob and M.A. Kaiser
Volume 22A	**Chromatography.** Fundamentals and Applications of Chromatographic and Electrophoretic Methods. **Part A: Fundamentals and Techniques** edited by E. Heftmann
Volume 22B	**Chromatography.** Fundamentals and Applications of Chromatographic and Electrophoretic Methods. **Part B: Applications** edited by E. Heftmann
Volume 23A	**Chromatography of Alkaloids. Part A: Thin-Layer Chromatography** by A. Baerheim Svendsen and R. Verpoorte
Volume 23B	**Chromatography of Alkaloids. Part B: Gas–Liquid Chromatography and High-Performance Liquid Chromatography** by R. Verpoorte and A. Baerheim Svendsen
Volume 24	**Chemical Methods in Gas Chromatography** by V.G. Berezkin
Volume 25	**Modern Liquid Chromatography of Macromolecules** by B.G. Belenkii and L.Z. Vilenchik
Volume 26	**Chromatography of Antibiotics. Second, Completely Revised Edition** by G.H. Wagman and M.J. Weinstein
Volume 27	**Instrumental Liquid Chromatography.** A Practical Manual on High-Performance Liquid Chromatographic Methods. **Second, Completely Revised Edition** by N.A. Parris
Volume 28	**Microcolumn High-Performance Liquid Chromatography** by P. Kucera
Volume 29	**Quantitative Column Liquid Chromatography.** A Survey of Chemometric Methods by S.T. Balke

Volume 30	**Microcolumn Separations.** Columns, Instrumentation and Ancillary Techniques edited by M.V. Novotny and D. Ishii
Volume 31	**Gradient Elution in Column Liquid Chromatography.** Theory and Practice by P. Jandera and J. Churáček
Volume 32	**The Science of Chromatography.** Lectures Presented at the A.J.P. Martin Honorary Symposium, Urbino, May 27–31, 1985 edited by F. Bruner
Volume 33	**Liquid Chromatography Detectors. Second, Completely Revised Edition** by R.P.W. Scott
Volume 34	**Polymer Characterization by Liquid Chromatography** by G. Glöckner
Volume 35	**Optimization of Chromatographic Selectivity.** A Guide to Method Development by P. J. Schoenmakers
Volume 36	**Selective Gas Chromatographic Detectors** by M. Dressler
Volume 37	**Chromatography of Lipids in Biomedical Research and Clinical Diagnosis** edited by A. Kuksis
Volume 38	**Preparative Liquid Chromatography** edited by B.A. Bidlingmeyer
Volume 39A	**Selective Sample Handling and Detection in High-Performance Liquid Chromatography. Part A** edited by R.W. Frei and K. Zech
Volume 39B	**Selective Sample Handling and Detection in High-Performance Liquid Chromatography. Part B** edited by K. Zech and R.W. Frei
Volume 40	**Aqueous Size-Exclusion Chromatography** edited by P.L. Dubin
Volume 41A	**High-Performance Liquid Chromatography of Biopolymers and Bio-oligomers. Part A: Principles, Materials and Techniques** by O. Mikeš
Volume 41B	**High-Performance Liquid Chromatography of Biopolymers and Bio-oligomers. Part B: Separation of Individual Compound Classes** by O. Mikeš
Volume 42	**Quantitative Gas Chromatography for Laboratory Analyses and On-Line Process Control** by G. Guiochon and C.L. Guillemin
Volume 43	**Natural Products Isolation.** Separation Methods for Antimicrobials, Antivirals and Enzyme Inhibitors edited by G.H. Wagman and R. Cooper
Volume 44	**Analytical Artifacts.** GC, MS, HPLC, TLC and PC by B.S. Middleditch

Volume 45A	**Chromatography and Modification of Nucleosides. Part A: Analytical Methods for Major and Modified Nucleosides – HPLC, GC, MS, NMR, UV and FT-IR** edited by C.W. Gehrke and K.C.T. Kuo
Volume 45B	**Chromatography and Modification of Nucleosides. Part B: Biological Roles and Function of Modification** edited by C.W. Gehrke and K.C.T. Kuo
Volume 45C	**Chromatography and Modification of Nucleosides. Part C: Modified Nucleosides in Cancer and Normal Metabolism – Methods and Applications** edited by C.W. Gehrke and K.C.T. Kuo
Volume 46	**Ion Chromatography.** Principles and Applications by P.R. Haddad and P.E. Jackson
Volume 47	**Trace Metal Analysis and Speciation** edited by I.S. Krull
Volume 48	**Stationary Phases in Gas Chromatography** by H. Rotzsche
Volume 49	**Gas Chromatography in Air Pollution Analysis** by V.G. Berezkin and Yu. S. Drugov
Volume 50	**Liquid Chromatography in Biomedical Analysis** edited by T. Hanai
Volume 51A	**Chromatography, 5th edition.** Fundamentals and Applications of Chromatography and Related Differential Migration Methods. **Part A: Fundamentals and Techniques** edited by E. Heftmann
Volume 51B	**Chromatography, 5th edition.** Fundamentals and Applications of Chromatography and Related Differential Migration Methods. **Part B: Applications** edited by E. Heftmann
Volume 52	**Capillary Electrophoresis.** Principles, Practice and Applications by S.F.Y. Li
Volume 53	**Hyphenated Techniques in Supercritical Fluid Chromatography and Extraction** edited by K. Jinno
Volume 54	**Chromatography of Mycotoxins.** Techniques and Application edited by V. Betina
Volume 55	**Bioaffinity Chromatography** by J. Turková