A Guide to Protein Isolation

Clive Dennison

Kluwer Academic Publishers

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by

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Contents

Chapter 4

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With hindsight I can see that the scientific imperative of objectivity - of removing the man from the experiments - when it becomes a habit of life, may tend to remove the humanity from the man. I apologise to those near and dear to me who have suffered as a consequence.

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Preface

It is a truism of science that the more fundamental the subject, the more universally applicable it is. Nevertheless, it is important to strike a level of "fundamentalness" appropriate to the task in hand. For example, an in-depth study of the mechanics of motor cars would tell one nothing about the dynamics of traffic. Traffic exists on a different "level" - it is dependent upon the existence of motor vehicles but the physics and mathematics of traffic can be adequately addressed by considering motor vehicles as mobile "blobs", with no consideration of how they become mobile. To start a discourse on traffic with a consideration of the mechanics of motor vehicles would thus be inappropropriate.

In writing this volume, I have wrestled with the question of the appropriate level at which to address the physics underlying many of the techniques used in protein isolation. I have tried to strike a level as would be used by a mechanic (with perhaps a slight leaning towards an engineer) - i.e. a practical level, offering appropriate insight but with minimal mathematics. Some people involved in biochemical research have a minimal grounding in chemistry and physics and so I have tried to keep it as simple as possible.

Besides trying to find the right level, I have tried to show that the physical principles which can be employed in protein isolation are, in fact, ubiquitously applicable principles with which students may be well familiar, though perhaps in different contexts. These "ubiquitously applicable principles" - once identified as such - turn out to be old and familiar friends, with whom one can have a great deal of fun when applied to the challenges of protein isolation.

In an uncertain world one never knows what the future will bring - who knows whether the economy, the state of world politics, or the weather, will be better or worse this time next year than it is now? - but one of the enduring attractions of science is that, because of the labours of scientists thoughout the world, it is almost certain that, "this time next year we'll have greater understanding and insight". This book is offered in the spirit of sharing some of the insights that I have gained in my career in Biochemistry. In some instances, I might have got hold of the wrong end of the stick. Where this is the case, I would welcome comment so that we might all learn - as we always do - from the errors.

Clive Dennison

Chapter 1

An overview of protein isolation

Isolating a protein may be compared to playing a game of golf. In golf, the player is faced with a series of problems, each unique and yet similar to problems previously encountered. In facing each problem the player must analyse the situation and decide, from experience, which club is likely to give the best result in the given circumstances. Similarly, in attempting to isolate proteins, researchers face a series of similar-yet-uniqueproblems. To solve these they must dip into their bags and select an appropriate technique. The purpose of this book is thus to fill the beginnerís "golf bag" with techniques relevant to protein isolation, hopefully to improve their game.

Developing a protein isolation is also somewhat like finding a route up a mountainside. Different routes have to be explored and base-camps established at each stage. Occasionally it will be necessary to return to the base of the mountain for further supplies, and haul these up to the established camps, before the next stage can be attacked. A successful climb is always rewarding and if an efficient route is established, it may become a pass, opening the way to further discoveries.

1.1 Why do it?

This book is about the methods that biochemists use to isolate proteins, and so it may be asked, "why isolate proteins?" Looked at in one way, living organisms may be regarded as machines with features in common with the entities that we commonly think of as "machines". A typical machine is made of a number of parts which interact, transduce energy, and bring about some desired effect. Mechanical machines have moving parts, while electronic machines move electrons. "Engines" convert energy to mechanical motion. Internal combustion engines, for example, convert chemical energy to mechanical motion. Similarly, living organisms such as the human body are complex machines made up of many interacting systems. Proteins constitute the majority of the working parts of these systems and there are thus diverse reasons for isolating proteins, viz.;

- **To gain insight.** As with any mechanism, to study the way in which a living system works it is necessary to dismantle the machine and to isolate the component parts so that they may be studied, separately and in their interaction with other parts. The knowledge that is gained in this way may be put to practical use, for example, in the design of medicines, diagnostics, pesticides, or industrial processes.
- **For use in Medicine.** Many proteins may themselves be used as "medicines" to make up for losses or inadequate synthesis. Examples are hormones, such as insulin, which is used in the therapy of diabetes, and blood fractions, such as the so-called Factor VIII, which is used in the therapy of haemophilia. Other proteins may be used in medical diagnostics, an example being the enzymes glucose oxidase and peroxidase, which are used to measure glucose levels in biological fluids, such as blood and urine.
- **For use in Industry.** Many enzymes are used in industrial processes, especially where the materials being processed are of biological origin.

In every case a pure protein is desirable as impurities may either be misleading, dangerous or unproductive, respectively. Protein isolation is, therefore, a very common, almost central, procedure in biochemistry.

1.2 Properties of proteins that influence the methods used in their study

It must be appreciated that proteins have two properties which determine the overall approach to protein isolation and make this different from the approach used to isolate small natural molecules.

- Proteins are **labile.** As molecules go, proteins are relatively large and delicate and their shape is easily changed, a process called denaturation, which leads to loss of their biological activity. This means that only mild procedures can be used and techniques such as boiling and distillation, which are commonly used in organic chemistry, are thus *verboten.*
- Proteins are **similar** to one another. All proteins are composed of essentially the same amino acids and differ only in the proportions and sequence of their amino acids, and in the 3-D folding of the amino acid chains. Consequently processes with a high discriminating potential are needed to separate proteins.

The combined requirement for delicateness yet high discrimination means that, in a word, protein separation techniques have to be very **subtle.** Subtlety, in fact, is required of both techniques and of experimenters in biochemistry.

1.3 The conceptual basis of protein isolation

In a protein isolation one is endeavouring to purify a particular protein, from some biological (cellular) material, or from a bioproduct, since proteins are only synthesised by living systems. The objective is to separate the protein of interest from all non-protein material and all other proteins which occur in the same material. Removing the other proteins is the difficult part because, as noted above, all proteins are similar in their gross properties. In an ideal case, where one was able to remove the contaminating proteins, without any loss of the protein of interest, clearly the total amount of protein would decrease while the activity (which defines the particular protein of interest) would remain the same (Fig.1.).

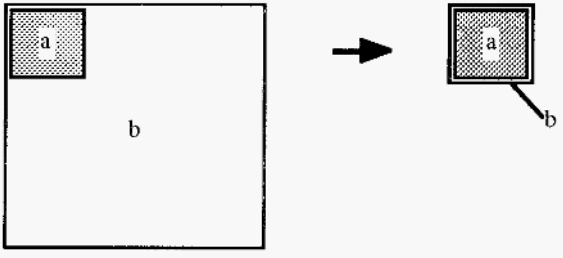


Figure 1. A schematic representation of a protein isolation.

Initially (Fig. 1A) there is a small amount of the desired protein "a" and a large amount of total protein "b". In the course of the isolation, ìbî is reduced and ultimately (Fig. 1B) only ìaî remains, at which point "a"="b". Ideally, the amount of "a" remains unchanged but, in practice, this is seldom achieved and less than 100% recovery of purified protein is usuallyobtained.

As a general principle, one should aim to achieve the isolation of a protein;-

- in as few steps as possible and,
- in as short a time as possible.

This minimises losses and the generation of isolation artefacts. Also, to further study the protein, the isolation will have to be done many times over and the effort put into devising a quick, simple, isolation procedure will be repaid many times over, in subsequent savings. The overall approach to the isolation of a protein is shown in Fig. 2.

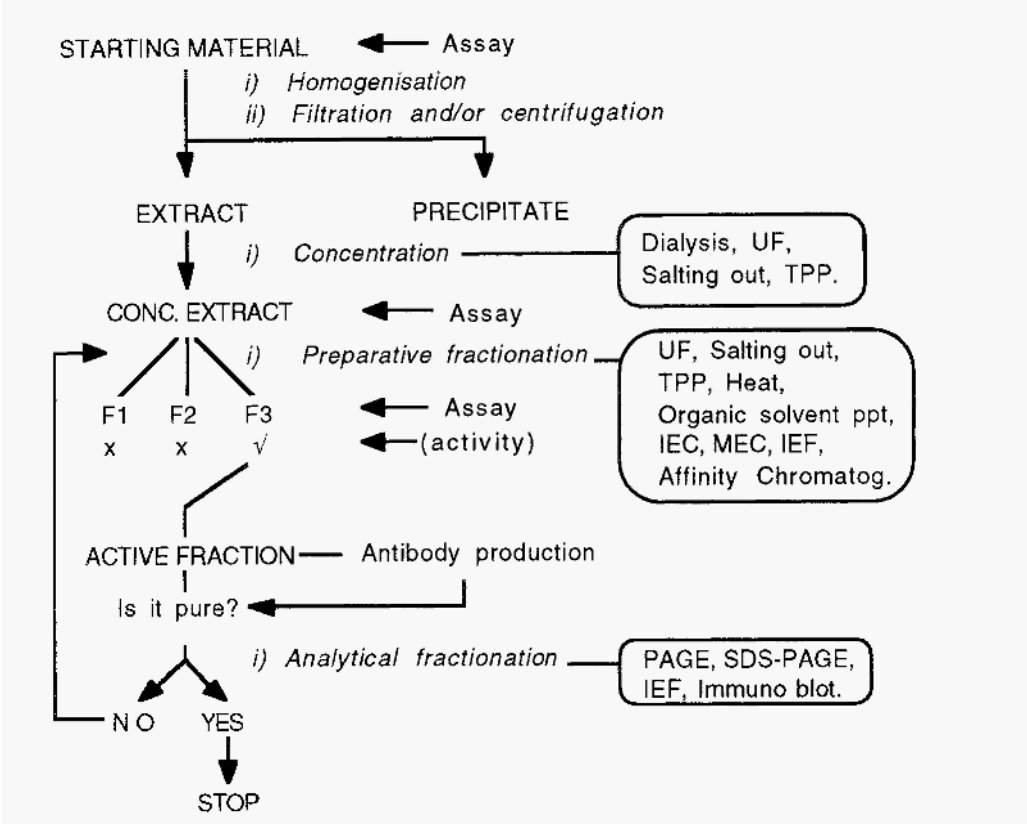


Figure 2. An overview of protein isolation.

1.3.1 Where to start?

To isolate a protein, one must start with some way of measuring the presence of the protein and of distinguishing it from all other proteins that might be present in the same material. This is achieved by a method which measures (assays) the unique activity of the protein. With such an assay, likely materials can be analysed in order to select one containing a large amount of the protein of interest, for use as the starting material.

Having selected a source material, it is necessary to extract the protein into a soluble form suitable for manipulation. This may be achieved by homogenising the material in a buffer of low osmotic strength (the low osmotic pressure helps to lyse cells and organelles), and clarifying the extract by filtration and/or centifugation steps.

The clarified extract is typically subjected to preparative fractionation, at this stage usually by salting out as this also usefully

serves to separate protein from non-protein material. It is necessary to assay the fractions obtained, in order to select the fraction(s) containing the protein of interest. The selected fraction(s) can then be subjected to further preparative fractionation, as required, until a pure fraction is obtained.

Experience has shown that there is an optimal sequence in which preparative methods may be applied. As a first approach it is best to apply salting out (or TPP) early in the procedure, followed by ion-exchange or affinity chromatography. Salting out can, with advantage, be followed by hydrophobic interaction chromatography, because hydrophobic interactions are favoured by high salt concentration, so desalting is obviated. The precipitate obtained from TPP, however, is low in salt and so can be applied directly to an ion-exchange system, without prior desalting. Generally, molecular exclusion chromatography should be reserved for late in the isolation when only a few components remain, since it is not a highly discriminating technique. Affinity chromatography often achieves the desirable aims of a rapid isolation using a minimum number of steps and so it should always be explored and preferentially used where possible.

1.3.2 When to stop?

How can one know when the fraction is pure, i.e. when to stop? To obtain this information it is necessary to analyse the isolated fraction using a number of analytical fractionation methods. If a number of such analytical methods reveal the apparent presence of only one protein, it may be inferred that the protein is pure, and that the isolation has been sucessfully completed. Note, however, that it is not possible to **prove** that the protein is pure; one can merely fail to demonstrate the presence of impurities. Future, improved, analytical methods may reveal impurities that are not detected using current technology.

If, on the other hand, any analytical fractionation method demonstrates the presence of more than one protein, it may be inferred that the preparation is not pure. In this case, the application of further preparative fractionation methods may be required before the protein is finally purified.

As illustrated in Fig. 1, the requirement is to remove as much contaminating protein as possible, while retaining as much as possible of the desired protein. Clearly then, to monitor the progress of an isolation, one needs two assays, one for the activity of the protein of interest (expressed in units of activity/ml) and another for the protein content (expressed as mg/ml). The activity per unit of protein

(units/mg) gives a measure of the so-called *specific activity.* In the course of a successful protein isolation, the specific activity should increase with each step, reaching a maximum value when the protein is pure. It is also desirable that a maximum *yield* of the protein is obtained. The protein of interest is defined by its activity and so information concerning the yield may also be obtained from activity assays.

1.4 The purification table

The results of activity and protein assays, from a protein purification, are typically summarized in a so called purification table, of which Table 1 is an example.

Table 1. A typical enzyme purification table

Step	Vol (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mp)	Purification (fold)	Yield (%)
Homogenate	900	43600	48000	1.1	(1)	(100)
pH 4.2 sínatant	650	4760	28000	5.9	5	58
$(NH_4)_2SO_4$ppt	140	1008	18667	18.5	17	39
S-Sepharose	57	7.1	7410	1044	949	15
SephadexG-75	35	2.45	3266	1333	1211	7

From an isolation of cathepsin L by R. N. Pike.

The figures in Table 1 are arrived at as follows:-

Volume (ml) this refers to the measured total solution volume at the particular stage in the isolation.

- *Total protein* (mg) - the primary measurement is of protein concentration, i.e. mg ml-1, which is obtained using a protein assay. Multiplying the protein concentration by the total volume gives the total protein (i.e. mg/ml x ml = mg).
- *Total activity* (units) the activity, in units ml-1, is obtained from an activity assay. Multiplying the activity by the total volume gives the total activity (i.e. units/ml x ml = units).
- *Specific–activity* (units/mg) - the specific activity is obtained by dividing the total activity by the total protein. Alternatively, the activity (units/ml) can be divided by the protein concentration (mg/ml), in which case the ìmlîs cancel out, leaving units/mg.
- *Purification* (fold) ìFoldî refers to the number of multiples of a starting value. In this case it refers to the increase in the specific activity, i.e. the purification is obtained by dividing the specific activity at any stage by the specific activity of the original

homogenate. The purification "per step" can also be obtained by dividing the specific activity after that step by the specific activity of the material before that step.

- *Yield* (%) - the yield is based on the recovery of the activity after each step. The activity of the original homogenate is arbitrarily set at 100%. The yield (%) is calculated from the total activity (units) at each step divided by the total activity (units) in the homogenate, multiplied by 100. The yield can also be calculated on a "per step" basis by dividing the total activity after that step by the total activity before that step and multiplying by 100.

  The efficiency of a step - is calculated as:-

$$\text{Purification (for that step)} \times \frac{\%\,\text{yield (for that step)}}{100}$$

1.5 Chapter 1 study questions

1. Why is protein isolation a common procedure in Biochemistry?
2. What distinguishes a protein isolation from the isolation of a small organic molecule?
3. What would one use as the starting material for the isolation of a particular protein?
4. In an ideal protein isolation, what is the yield of the desired protein?
5. Is such a yield ever achieved in practice?
6. If not, what yield should be aimed for?
7. Define the "specific activity" of a protein.
8 How does one know when to stop a protein isolation?

Chapter 2

Assay, extraction and subcellular fractionation

2.1 Buffers

Proteins have a pH dependent charge and many of the properties of proteins change with pH. Consequently, in working with proteins, it is important to control the pH. This is achieved by the use of buffers, and so at the outset it is important to have some insight into buffers, to know which buffer to use for any particular purpose, and how to make up the buffer.

Buffers are solutions of weak acids or bases and their salt(s), which resist changes in pH. Weak acids and bases are distinguished from strong acids and bases by their incomplete dissociation. In the case of a weak acid the dissociation is:-

$$HA \rightleftharpoons H^+ + A^-$$

and the dissociation constant is:-

$$Ka = \frac{[H^+][A^-]}{[HA]}$$

Now, $pKa = -\log Ka$

Thus, $$pKa = -\log \frac{[H^+][A^-]}{[HA]}$$

$$= -\log [H^+] - \log \frac{[A^-]}{[HA]}$$

$$= \text{pH} - \log\frac{[A^-]}{[HA]}$$

Hence, $$\text{pH} = \text{pKa} + \log\frac{[\text{salt}]}{[\text{acid}]} \qquad 2.1$$

For a weak base (e.g. Tris) the dissociation is:-

$$HB^+ \rightleftharpoons H^+ + B$$

Using similar arguments to those above, it can be shown that in this case,

$$\text{pH} = \text{pKa} + \log\frac{[\text{base}]}{[\text{salt}]} \qquad 2.2$$

Equations 2.1 and 2.2 are forms of the Henderson-Hasselbalch equation, which can be written in a general form as:-

$$\text{pH} = \text{pKa} + \log\frac{[\text{basic species}]}{[\text{acidic species}]} \qquad 2.3$$

From which it can be seen that, when [basic species] = [acidic species], then,

$$\text{pH} = \text{pKa}.$$

A simple monoprotic weak acid, such as acetic acid, yields a titration curve such as that shown schematically in Fig. 3. It will be noticed that when pH = pKa, the solution resists changes in pH, i.e. it functions best as a buffer in the range pH = pKa ± 0.5.

CH_3COOH is the acidic species in this buffer and CH_3COO^- is the basic species. It may be observed that a solution of acetic acid itself (CH_3COOH) *will have* a pH less than the pKa of acetic acid. Conversely, a solution containing only sodium acetate *will have* a pH greater than the pKa of acetic acid. It is important to understand this point in order to appreciate how to make an acetate buffer using the approach described in Section 2.1.1.

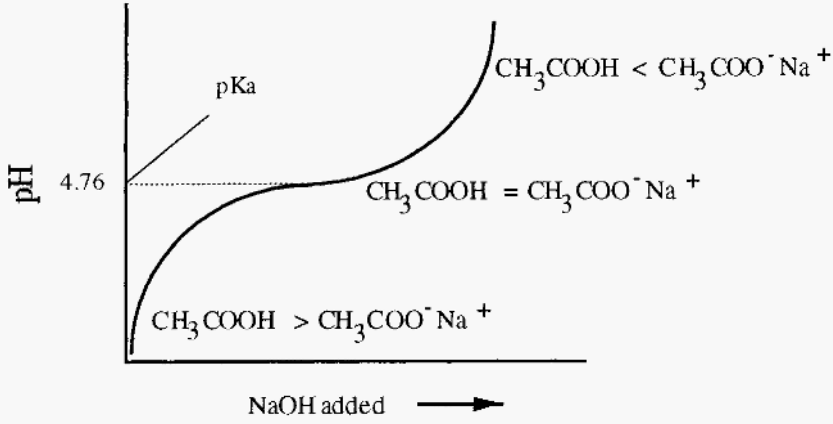


Figure 3. Schematic titration curve of a monoprotic acid, such as acetic acid.

A tri-protic acid, such as phosphoric acid will yield a titration curve having three inflexion points (Fig. 4), corresponding to the three pKa values of phosphoric acid.

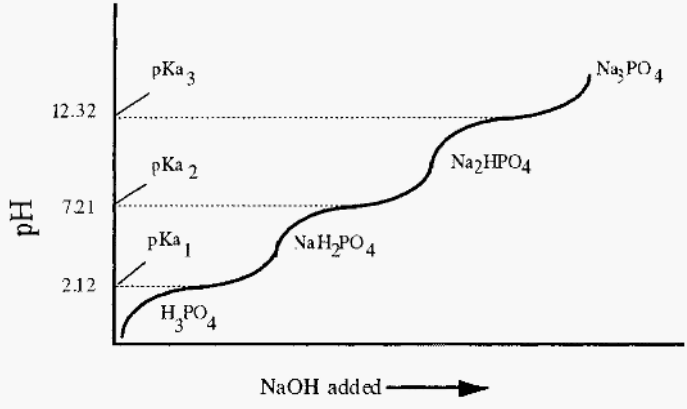


Figure 4. Schematic titration curve of phosporic acid.

For most biochemical purposes, pKa_2 is of greatest interest, since it is closest to the pH of the extracellular fluid of animals.

Note that:-

At pKa_2, $[NaH_2PO_4] = [Na_2HPO_4]$.

At $pH < pKa_2$, $[NaH_2PO_4] > [Na_2HPO_4]$.

At $pH > pKa_2$, $[Na_2HPO_4] > [NaH_2PO_4]$.

Put another way, a solution NaH_2PO_4 *will have a* pH less than pKa_2 and a solution of Na_2HPO_4 *will have* a pH greater than pKa_2. It is important to understand this point in order to appreciate how to make a phosphate buffer using the approach described below.

2.1.1 Making a buffer

A simple approach to the making of a buffer is described below. The advantage of this approach is that only *one* solution needs be made up. Several books suggest that buffers should be made up by adding "x" ml of a 1 M solution of "A" to "y" ml of a 1 M solution of "B". The problem with this approach is that it involves extra work (making up two solutions when one will do), waste (the unused volumes of "A" and "B" are discarded) and is usually inaccurate (the presence of extra salts and preservatives, for example, can change the pH due to common ion effects).

A simpler method follows the following stepsí:-

- *Choose the buffer.* A buffer works best at its pK, so the first step is to choose a buffer with a pKa as close as possible to the desired pH.
- *Identify the buffering species.* As described in Section 2.1, a buffer consists of two components: a weak acid and its salt or a weak base and its salt. The second step is thus to identify the species which will constitute the buffer. For example, in the case of an acetate buffer, the buffering species are CH_3COOH and CH_3COONa. In a phosphate buffer at pKa_2, the buffer species are NaH_2PO_4 and Na_2HPO_4.
- *Identify whether the buffer is made from an acid or a base.* The two buffer examples shown in Section 2.1 are made from acids, acetic acid or phosphoric acid. In the case of phosphate buffer at pKa_2, the acid is NaH_2PO_4. An example of a buffer made from a base is Tris/Tris-HCl, which buffers best at pH 8.1, the pKa of Tris.
- *Choose the species that gives no by-products when titrated.* Almost all buffers can be made up by weighing out one component, dissolving in a volume just short of the final volume, titrating to the right pH, and making up to volume. It is **not necessary** to make up separate solutions of the two buffer constituents - the required salt can be generated *in situ* by titrating the acid with an appropriate base - or *vice versa* in the case of a buffer made from a base. [Remember: Titrate an acid "up" (i.e. with a strong base) and titrate a base "down" (i.e. with a strong acid)].

Remember, acid + base = salt + water

and, a buffer = (acid + its salt) or (base + its salt).

The term "its salt" is important.
For example, if we wanted to make an acetate buffer, it is easy to identify that this buffer is made from acetic acid and its salt, say, sodium acetate. But,

Q: Could the required mixture of CH_3COOH and CH_3COONa be made by titrating a solution of CH_3COONa to the correct pH with HCl?

A: No! Because the reaction in this case is:-

$$CH_3COONa + HCl \rightarrow CH_3COOH + NaCl$$

and the resultant solution contains NaCl, which is an unwanted by-product and which is not a salt of acetic acid (i.e. it is not "its salt").
On the other hand,

Q: Could the required mixture be made by titrating a solution of CH_3COOH with NaOH?

A: Yes! The reaction in this case is:-

$$CH_3COOH + NaOH \rightarrow CH_3COONa + H_2O$$

which yields only the salt of acetic acid and water, i.e. there are no by-products.
Similarly, in the case of a phosphate buffer, if one chooses Na2HPO4, the pH of a solution of this salt *will be* higher than pKa, (see Fig. 4) and this will require titration with an acid. If one chooses HCl, the reaction will be:-

$$Na_2HPO_4 + HCl \rightarrow NaH_2PO_4 + NaCl$$

which yields NaCl as an unwanted by-product. (And if one chooses NaH_2PO_4, this will change the phosphate molarity.) However, if one starts with NaH_2PO_4, the pH of a solution of this salt *will be* lower than pKa, and this will require titration with a base. If one chooses NaOH, the reaction will be:-

$$NaH_2PO_4 + NaOH \rightarrow Na_2HPO_4 + H_2O$$

which yields only the desired salt (Na_2HPO_4) and water.
For a Tris buffer, one should start with the free base and titrate this with HCl to yield the salt of Tris, Tris-HCl.

- *Calculate the mass required to give the required molarity.* Having settled on the single buffer component to be weighed out, calculate the mass required to give the required molarity, when finally made up to

volume. For example, the molarity of a phosphate buffer is determined by the molarity of the phosphate moiety, $HPO_4^{=}$, which does not change when NaH_2PO_4 is titrated to Na_2HPO_4. If a litre of a 0.1 M buffer is required, then 0.1 moles of NaH_2PO_4 can be weighed out.

- *Add all other components, titrate and make up to volume.* Buffers often contain ingredients other than the two buffering species. For ion-exchange elution the buffer might contain extra NaCl, and buffers often contain preservatives such as NaN_3 or chelating agents such as EDTA. Except for NaN_3, these should all be added before the titration. All constituents should be dissolved in the same solution to just less than the final volume, i.e. a volume must be left for the titration but the final dilution after titration should be as small as possible. (The Henderson-Hasselbalch equation predicts that the pH of a buffer should not change with dilution, but this is only true over a small range, due to non-ideal behaviour of ions in solution.) Finally the solution is titrated to the desired pH and made up to volume. NaN_3 should be added after titration as it liberates the toxic gas, HN_3, when exposed to acid. Manganese salts should also be added after adjustment of the pH as these may form irreversibly insoluble salts at pH extremes.

2.1.2 Buffers of constant ionic strength

Besides pH, which influences the sign and magnitude of the charge on a protein, proteins are also influenced by the specific ions present in solution and by the solution ionic strength. In a buffer, the pH and the ionic strength are related. The Henderson-Hasselbalch equation, for a buffer made from an acid, is:-

$$pH = pKa + \log\frac{[salt]}{[acid]}.$$

The ionic strength of the buffer is a function of the [salt]. Therefore, in this case as the pH rises, the buffer ionic strength also rises. Ionic strength is also a function of the molarity of the buffer. One can picture the relationship between the three variables, molarity (M), pH and ionic strength (I) as a lever, for which any one of the three could be fixed as a fulcrum and the relative movements of the other two observed (Fig. 5).

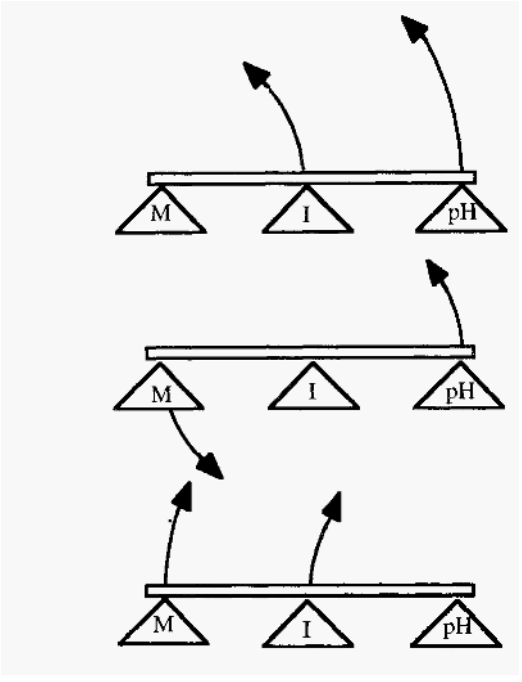


Figure 5. The relationship between molarity, pH and ionic strength for a buffer made from a weak acid.

For a buffer made from a weak base, the relevant form of the Henderson-Hasselbalch equation is:-

$$pH = pKa + \log\frac{[base]}{[salt]}$$

In this case, therefore, the ionic strength increases as the pH decreases and the relationship between "M" (molarity), "I" (ionic strength) and pH can be visualised by reversing the positions of M and I in Fig. 5.

The lever model shown in Fig. 5 must not be taken to imply a linear relationship between the variables. In fact, ionic strength changes sigmoidally with pH as shown in Fig. 6. The "rate" of change, i.e. d(ionic strength)/d(pH), is greatest at the pKa, pKa_2 in this case. The pKa itself also changes slightly with ionic strength[2,3]. The data in Fig. 6 were calculated according to Ellis and Morrison[2].

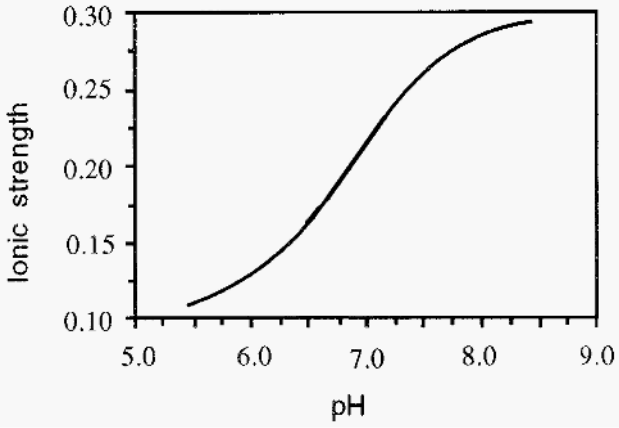


Figure 6. The relationship between ionic strength and pH for a 0.1 M phosphate buffer.

The relationship between pH, M and I is important when establishing the pH optimum of an enzyme. This is commonly done by using a range of buffers of constant M and varying pH. However, if the enzyme in question is affected by ionic strength (which is often the case) it is better to keep I constant and to vary M with pH (For an example, see ref. 4). The preparation of buffers of constant ionic strength is discussed by Ellis and Morrison[2]. An in depth discussion of buffers is provided by Perrin and Dempsey[5].

2.2 Assays for activity

Most proteins have some form of unique functional activity, which defines the specific protein and may be used to elaborate an assay for its detection and quantitation. A philosophical point to note is that it is necessary to conceive of an activity and to devise an appropriate assay, before the protein can be isolated. Ideally, the assay should be:-

- *specific,* to define the protein of interest and distinguish it from all others,
- *quantitative,* so that the success of the purification can be monitored, and,
- *economical* in terms of time and material.

The extent to which the assay meets these requirements has a major bearing on the difficulty, or otherwise, likely to be experienced in the subsequent protein isolation.

Assays for enzymes are usually specific although, for example, "proteolytic activity" may not be specific enough to be very useful by

itself. On the other hand, an activity like "toxicity" may not be specific and may not be due to a single component. Since a large proportion of proteins isolated are enzymes, enzyme assays will be used to illustrate some of the conceptual dimensions of assays. It must be appreciated, however, that many proteins are not enzymes and different assay methods will be required for these.

2.2.1 Enzyme assays

Enzymes are biological catalysts which speed up the rate of specific reactions. The activity of an enzyme is therefore defined, and measured, by the extent to which it speeds up a reaction.

2.2.1.1 The progress curve

The primary measurement in an enzyme assay is a progress curve, in which the amount of reaction that has taken place is plotted against time. The amount of reaction is defined as the amount of product formed or as the amount of substrate consumed. A typical progress curve, for an enzyme that is stable under the reaction conditions, is shown in Fig. 7. The velocity of the reaction is given by the slope of the progress curve. Initially, the relationship between the amount of reaction and time is linear and the slope of this linear portion gives the initial velocity (V_o). Eventually, the relationship becomes curvilinear and the reaction velocity (slope of the line) decreases, eventually reaching zero when the net reaction stops. At this point, forward and reverse reactions are in equilibrium.

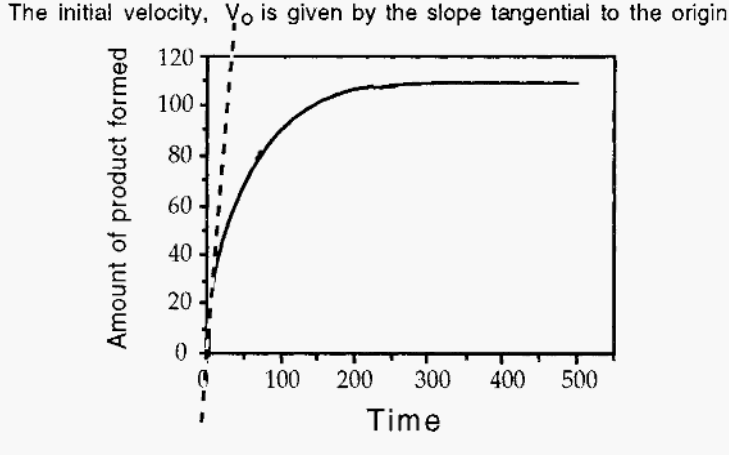


Figure 7. A progress curve for an enzyme-catalysed reaction.

The progress of an enzyme reaction may be visualised by considering the flow of water between two tanks, one initially empty and the other fairly full, with a pipe equipped with a tap connecting the two tanks at the bottom (Fig. 8).

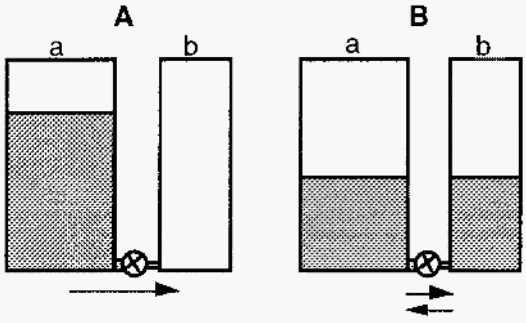


Figure 8. The water tank analogy of an enzyme-catalysed reaction.

In this analogy the volume of water in a tank is analogous to the concentration of reactant or product and the height (potential energy) is analogous to its chemical potential. Initially (A), there is a large amount of reactant (a) but no product (b). The reaction will therefore flow to the right until equilibrium is reached. The enzyme is equivalent to the tap in this model.

2.2.1.2 The enzyme dilution curve

The initial velocity is proportional to the enzyme concentration, a relationship expressed in an enzyme dilution curve (Fig. 9). The linear enzyme dilution curve forms the basis of enzyme assays, in which the concentration of an enzyme is estimated from a measurement of its activity (i.e. from the initial velocity of the enzyme catalysed reaction), in the presence of an excess of substrate (to ensure that a substrate limitation does not restrict the initial velocity).

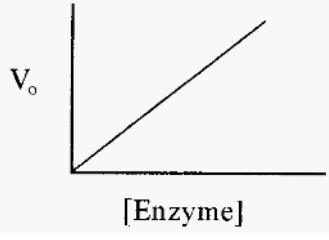


Figure 9. An enzyme dilution curve.

2.2.1.3 The substrate dilution curve

The concentration of substrate also affects the initial velocity, V_o, of an enzyme-catalysed reaction; in the simplest case, in a manner expressed by the so-called Michaelis-Menten equation:

$$V_o = \frac{V_{max}[S]}{[S] + K_m} \qquad 2.4$$

A plot of V_o versus [S] yields a so-called substrate dilution curve, such as shown in Fig. 10, which was calculated from the Michaelis-Menten equation, using values of V_{max} = 1000 and K_m = 90.

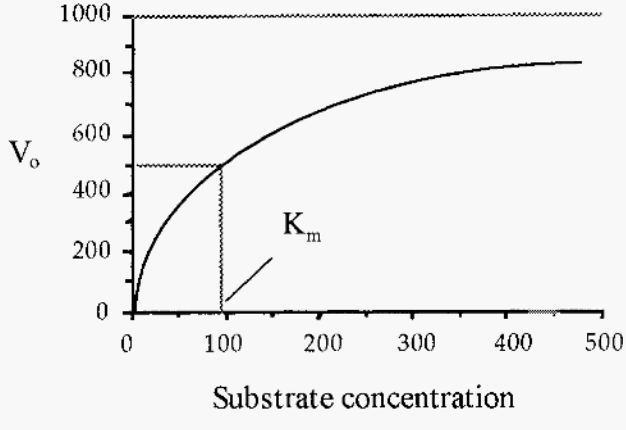


Figure 10. A substrate dilution curve.

Note: The substrate dilution curve must **not** be confused with the similarly-shaped progress curve.

The K_m, i.e. that substrate concentration which gives one half of the maximal velocity possible (at that enzyme concentration) is a constant. characteristic for a particular enzyme acting on a particular substrate. Knowledge of the K_m is useful when devising an enzyme assay as it enables one to use a substrate concentration where V_o will not be too sensitive to small changes in [S] due to experimental error. A good rule-of-thumb is that [S] should be as high as possible, preferably at a level where the substrate dilution curve is asymptotic to V_{max}. Often, however, [S] is constrained by cost or experimental practicability, and values of less than K_m may have to be used. For example the proteinase cathepsin B is routinely assayed at [S] = $\frac{1}{40}$ K_m, using a fluorogenic substrate.

2.2.1.4 The effect of pH on enzyme activity

Another factor which influences V_o is pH, which can exert its effect in different ways; on the ionisation of groups in the enzyme's active site, on the ionisation of groups in the substrate, or by affecting the conformation of the either the enzyme or the substrate, These effects are manifest in changes in the kinetic constants, K_m and k_{cat}.

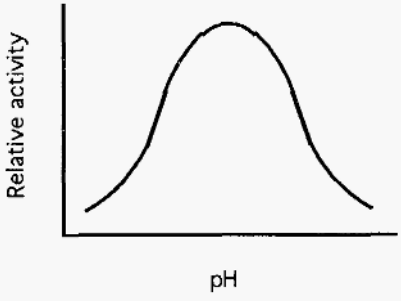


Figure 11. A typical pH-activity curve.

The net result is usually a bell-shaped pH-activity profile (Fig. 11). V_o reaches its maximum at the optimum pH, which is the pH that should be used when assaying the enzyme. (See the discussion of pH vs ionic strength in Section 2.1.2.)

In expressing pH-activity profiles, many authors plot k_{cat}/K_m against pH. Why, and what does this mean?.

For a reaction of the form:-

$$E + S = ES \rightarrow ES' \rightarrow E + P$$

the initial velocity, expressed as a function of the concentrations of **free** enzyme [E] and substrate, is described by the equation:-

$$V_o = \frac{k_{cat}}{K_m} .[E][S] \qquad 2.5$$

in which k_{cat}/K_m is readily recognised as a second order rate constant. k_{cat}/K_m is also known as the specificity constant as it is maximal with an optimum substrate.

Changes in pH will affect V_o, linearly, through effects on either (or both of) the enzyme's affinity for the substrate (K_m) or its turnover

number (k_{cat}), but will not affect [E] or [S]. The influence of pH is, therefore, essentially on k_{cat}/K_m and k_{cat}/K_m is maximal at the pH optimum.

The practical problem is that [E], the concentration of *free* enzyme, is not known. In the measurements involved in establishing a pH-activity profile, the total enzyme concentration, $[E]_o$, and the initial substrate concentration, $[S]_o$, are constant (and known), and in the measurement of V_o it can be assumed that [S] $[S]_o$. The concentration of free enzyme. [E], is not known but is a function of [S] and K_m as described by equation 2.6:-

$$[E] = \frac{[E]_o}{\frac{[S]}{K_m} + 1} \qquad 2.6$$

[E] is thus markedly influenced by the magnitude of [S] relative to K_m. The variation of [E] with Km is least when [S] is small relative to K_m (Modelling of equation 2.6 reveals that [S] must be $\leq K_m/40$). When this is true (and *only* when this is true):-

$[E] \approx [E]_o$,

- the shape of the pH-activity profile is linearly affected by changes in k_{cat} and/or K_m, brought about by the changes in pH, and
- "relative activity" is proportional to k_{cat}/K_m.

When these conditions apply:-

$$V_o = \frac{k_{cat}}{K_m} .[E]_o.[S] \qquad 2.7$$

which means that, if it is possible to use a substrate concentration I $K_m/40$, a pH-activity profile of k_{cat}/K_m versus pH can be constructed from measurements of V_o at different pH values and the known values of $[E]_o$ and [S].

However, it is not always practicable to use a substrate concentration of I $K_m/40$ and when [S] is *not* small relative to Km, $[E] \neq [E]_o$ and the more familiar Michaelis-Menten equation applies, i.e.

$$V_o = \frac{k_{cat}}{K_m + [S]} .[E]_o.[S].$$

In this case separate measures of k_{cat} and K_m have to be obtained in the classical way by measuring V_o at a number of levels of [S], at each pH.

The paired data can be used to obtain estimates of k_{cat} and K_m at each pH, preferably by the method of Eisenthal and Cornish-Bowden[6]. From these, k_{cat}/K_m values can be obtained and the pH-activity profile plotted.

2.2.1.5 The effect of temperature on enzyme activity

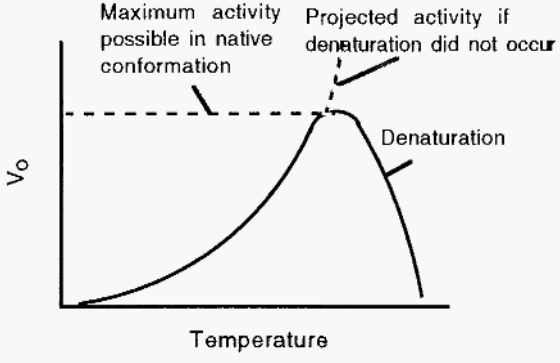


Figure 12. A typical temperature profile for an enzyme-catalysed reaction.

Finally, temperature also influences V_o. Two effects interact to give a resultant curve. On the one hand, like all chemical reactions, the velocity of enzyme-catalysed reactions increases with an increase in temperature, typically doubling for every 10 C rise in temperature. In the case of an enzyme-catalysed reaction, however, eventually a temperature is reached where the enzyme becomes unstable and begins to denature, at which point the reaction rate again declines. The resultant is usually an asymmetrical peak, which rises relatively slowly with an increase in temperature, and then drops rather suddenly (Fig. 12).

It must be realised that denaturation is itself a reaction, with a temperature-dependent rate constant. Denaturation is generally a first-order reaction, since each protein molecule simply unfolds, independently of interaction with any other protein molecules. A useful way of expressing the temperature stability of an enzyme is therefore to measure the half-life ($t_{1/2}$) of its activity as a function of temperature. The ìhalf-lifeî is the time taken for the enzyme activity to decrease from any value to half of that value. The half-life will be “infinite” until the temperature is reached at which the enzyme begins to denature. Thereafter, the half-life will decrease with an increase in temperature. The half-life of first-order reactions is discussed by Segel[7].

2.3 Assay for protein content

A number of methods are available for measuring protein concentration, each being based on a specific property of proteins, and each having certain advantages and disadvantages. Consequently, the different methods are more or less suitable for different applications and it is useful to have insight into these methods so that one can decide which one to use for a given application.

2.3.1 Absorption of ultraviolet light

UV-absorption is perhaps the most simple method for measuring the concentration of proteins in solution. A typical protein absorption spectrum has an absorption peak at 280 nm, due to the aromatic amino acids, such as tryptophan and tyrosine. Below 220 nm the absorption also increases strongly, due to peptide bonds, which absorb maximally at 185 nm. The extinction coefficients of different proteins tend to be different at 280 nm, due to their different aromatic amino acid contents, while below 220 nm the extinction coefficients are more similar. It is difficult to measure absorption accurately in this part of the spectrum, however, partly because oxygen forms begins to absorb in this region.

Because the extinction coefficients of proteins differ, UV-absorption is useful as a qualitative measure, for detecting the presence of protein, but is less useful for accurate quantitative measurements, except for pure proteins of known extinction coefficient. Because of its simplicity, UV-absorption is the method favoured for continuous (semi-quantitative) monitoring of the protein concentration in the eluate from chromatography columns.

One of the limitations of UV-absorbance, as a method for measuring protein, is that UV-absorbing, non-protein, compounds may interfere with the measurement. Nucleic acids, which are ubiquitously present in biological material, absorb UV radiation strongly, with a profile overlapping that of protein, but with a maximum at 260 nm. An elegant method for eliminating the absorption due to nucleic acids, thus allowing a measurement of protein in the presence of nucleic acid, has been proposed by Groves *et al.*[8].

In measuring the concentration of proteins by their UV-absorbance, remember that the extinction coefficient (or absorption coefficient) is given by the equation:-

$$A = a_m c l$$

where, A = absorbance
am = molar extinction coefficient
c = molar concentration of protein in solution
l = length of the light path through the solution (usually 1 cm).

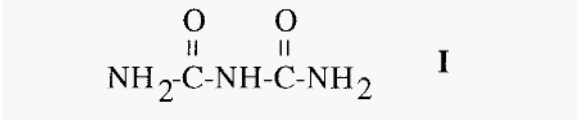
If the concentration is given in g/litre, then the equation becomes:-

$$A = a_s c l$$

where a_s = specific extinction coefficient.
Note that a_m = a_s x MW.

2.3.2 The biuret assay

In alkaline solution, proteins reduce cupric (Cu^{2+}) ions to cuprous (Cu^{1+}) ions which react with peptide bonds to give a blue coloured complex. This reaction is called the biuret reaction and is named after the compound biuret (I), which is the simplest compound that yields the characteristic colour.



I

Because the reaction is with peptide bonds, there is little variation in the colour intensity given by different proteins. The biuret method can be used for the measurement of protein concentration in the presence of polyethylene glycol, a common protein precipitant.

A disadvantage of the biuret method is that it is relatively insensitive, so that large amounts of protein are required for the assay. A more sensitive variant of the method, the micro-biuret assay, has been devisedí, which overcomes this limitation to some extent. Another limitation is that amino buffers, such as Tris, which are commonly used in the pH range *ca.* 8-10, can interfere with the reaction.

2.3.3 The Lowry assay

The Lowry assay[10] may be considered as an extension of the biuret assay. Initially, a copper-protein complex is formed, as in the biuret assay. The cuprous ions then reduce the so-called Folin-Ciocalteu

reagent[11], a phosphomolybdic-phosphotungstate complex, to yield an intense blue colour. An advantage of the Lowry over the biuret assay is that it is much more sensitive, and thus consumes much less of the protein sample. A disadvantage of the Lowry assay is that it is more sensitive to interference, a consequence of the more complicated chemistry involved. The Lowry assay has been reviewed by Peterson[12].

2.3.4 The bicinchoninic acid assay

Another development of the biuret reaction is the bicinchoninic acid (BCA) assay. Bicinchoninic acid forms a 2:1 complex with cuprous ions formed in the biuret reaction, resulting in a stable, highly coloured chromophore with an absorbance maximum at 562 nm[13,14]. The BCA assay is more sensitive than the Lowry method and is also less subject to interference by a number of commonly encountered substances. As the reaction is dependent, in the first instance, on the reduction of cupric ions to cuprous ions by the protein, it is sensitive to interference by strong reducing agents, e.g. ascorbic acid. This limitation also applies to the biuret and Lowry assays.

2.3.5 The Bradford assay

A protein assay which is rapidly becoming the most commonly used method, due to its simplicity, sensitivity and resistance to interference, is the dye-binding method described by Bradford[15]. Coomassie blue G-250, dissolved in acid solution, below pH 1, is a red-brown colour but regains its characteristic blue colour when it becomes bound to a protein. The concentration of protein can therefore be measured by the extent to which the blue colour, measured at 595 nm, is restored. Coomassieblue G-250 binds largely to basic and aromatic amino acids. Different proteins will differ in their content of these amino acids and so, ideally, a standard curve should be elaborated for each specific protein. A modification has been introduced by Read and Northcote[16] to overcome this problem to some extent. A disadvantage of the Bradford assay is that the reagent tends to stick to glass and plasticware. For this reason, the use of disposable cuvettes is recommended although, if necessary, the dye can be removed from surfaces by using SDS.

2.4 Methods for extraction of proteins

Once a promising source material has been identified using the activity assay described in Section 2.2, the next step is to extract the protein

from this source. The objective in extracting proteins is to get them from the site where they occur in the tissue, into solution where they can be more easily manipulated and separated out. Most tissue proteins occur within cells, and possibly within organelles in the cells, and in these cases it is necessary to break open the cells and organelles, to release their protein contents. The methods chosen to disrupt the cells and organelles should be such that the proteins themselves are minimally damaged.

If the desired protein occurs within an organelle, then a useful purification of the protein may be achieved by a sub-cellular fractionation, whereby the different organelles are separated, before the protein is extracted from the organelle. Sub-cellular fractionation may be effected by differential centrifugation as described in Section 2.6.

2.4.1 Osmotic shock

A useful technique, which may be used in conjunction with mechanical means of disrupting cells, is the use of a buffer with a low osmotic pressure. In such a buffer water will tend to flow into the cells and organelles by osmosis, promoting their lysis and release of their proteins. To further promote the disruption of cell membranes, a low concentration of organic solvent, e.g. 2% n-butanol, is often added to the extraction buffer.

Laminar flow. A number of the homogenisers described below are dependent on the principle of the laminar flow of fluids for their operation. Laminar flow may be illustrated by taking a sheaf of paper sheets and throwing them onto a stationary surface. It will be observed that the bottom-most sheet of paper travels the smallest distance and the top-most sheet travels the greatest distance, due to the friction between the layers.

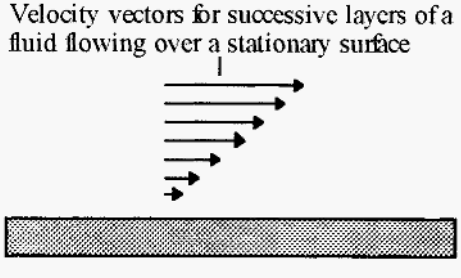


Figure 13. Laminar flow of a fluid.

Fluids, which may be liquids or gases, flow over stationary surfaces in a similar way; the layer of fluid against the surface (the so-called boundary layer) is virtually stationary relative to the surface and successive layers travel at increasingly greater speeds.

An everyday example of the effects of laminar flow is the well-known phenomenon that oneís voice can be heard to a greater distance downwind, than upwind. The speed of sound is about 1000 kph, which is high relative to common wind speeds, so the phenomenon is not due to the wind speed itself. Rather, the laminar flow of the wind distorts the sound waves, causing them to bend upwards into wind, and downwards downwind (Fig. 14), so that the sound will be heard at greater distances, downwind.

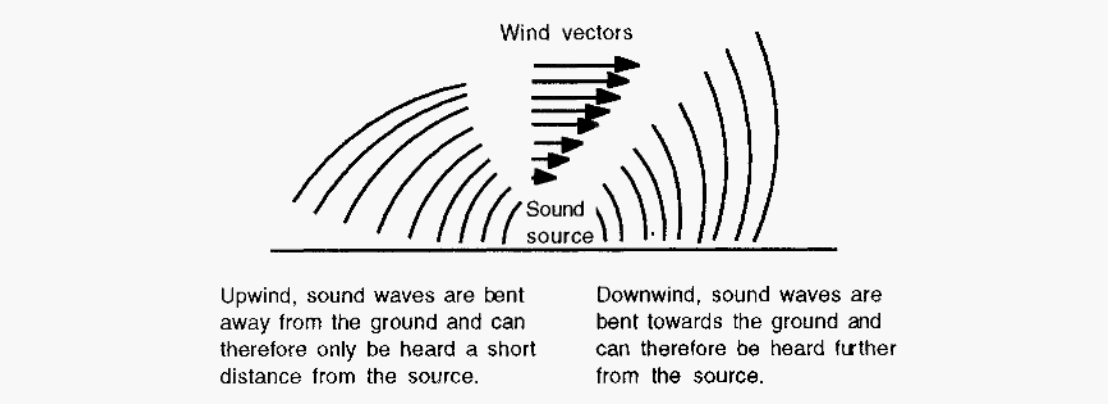


Figure 14. The effect of the laminar flow of the wind upon sound waves.

Pilots of light aircraft with slow flying speeds, have to be especially conscious of the effects of laminar flow when landing. Landing is always done into wind to reduce the speed relative to the ground but, as the aircraft descends its airspeed will decrease and it may be necessary to compensate for this by applying power or by approaching with extra speed. Pilots get information about the wind from the windsock, which indicates the wind direction and strength.

2.4.2 Pestle homogenisers

An effective and gentle method of disrupting animal cells is by the use of a pestle homogeniser, of which there are two main types, Dounce and Potter-Elvehjemem homogenisers. Pestle homogenisers generally disrupt whole cells but not organelles.

The Dounce homogeniser consists of a cylindrical glass tube, closed at one end, and two pestles (pistons) which fit into the cylinder with different clearances. Tissue is cut into small cubes, placed in the homogeniser with buffer and the "L" (loose) pestle is used first, to break the tissue into a fluid mixture. The "T" (tight) pestle is then used to disrupt the cells, releasing their contents. Typically, homogenisation is effected by a defined number of "passes" of the pestle, up and down the cylinder. Care should always be taken to support the end of the homogeniser against the bench, when it is being used, so that the end is not broken off by the hydraulic pressure within the cylinder.

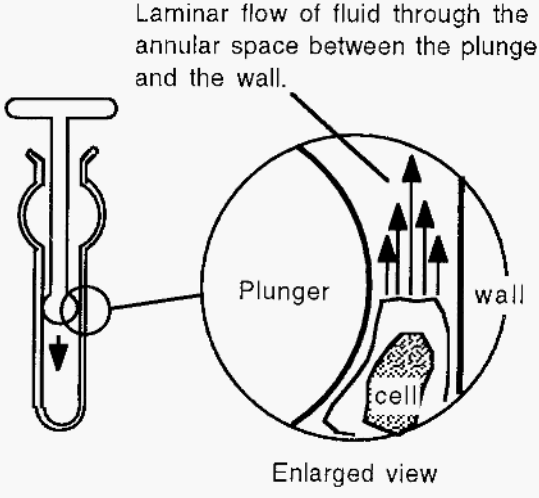


Figure 15. A Dounce homogeniser.

In a Dounce homogeniser, laminar flow of the fluid through the annular space between the pestle and the homogeniser wall results in different fluid speeds existing over the diameter of the cell, and the resulting shear forces disrupt the cell (Fig. 15).

A Potter-Elvehjem homogeniser works in a similar way, except that the pestle has a more cylindrical shape, which induces shear forces over a greater area. Potter-Elvehjem homogenisers are available in automated, motorised versions.

2.4.3 The Waring blendor and Virtis homogeniser

These devices consist of a high speed stirrer with cutting blades, mounted in a glass vessel, the walls of which are indented from top to bottom, forming a clover-leaf cross section. The speed of the blades'

motion generates strong shear forces, due to laminar flow, while the irregular outline of the vessel gives good overall mixing of the solution. The degree of disruption depends upon the speed of rotation of the blades. At high speeds, a blendor will disrupt mitochondria and nuclei and may even denature proteins. it is mostly used with plant and animal tissues but is less effective with micro-organisms.

Note that although it is a ìblenderî, the trade name is ìWaring blendorî.

2.4.4 The Polytron/UItra-Turrax-type homogeniser

ìPolytronî and ìUltra-Turraxî are trade names for a type of homogeniser which consists of a stationary vertical tube, equipped with serrated teeth and radially distributed holes at its lower edge. Fitting closely into the stationary tube is a motor-driven tube, also with radially distributed holes corresponding to those on the stationary tube.

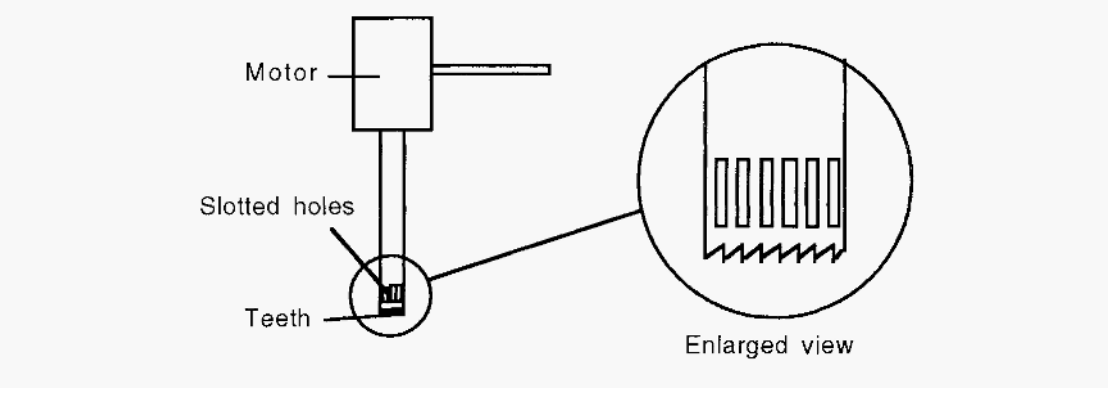


Figure 16. The Polytron/Ultra-Turrax type homogeniser.

Rotation of the inner tube causes the sample to be flung outwards, through the holes in the tubes. Because the two sets of holes continuously and rapidly come into and out of register, the sample gets chopped into small pieces and simultaneously homogenised by shear between the rotating and stationary tubes. Such homogenisers are very effective and only a short period of homogenisation is required, the sample being cooled in an ice bath during this period.

2.4.5 Grinding

Several types of apparatus are available for grinding. in the Edmund-Buhler disintegrator, bacterial cells are vibrated with glass beads in a

jacketed container. Cells are broken by impact, tearing and maceration between the hard surfaces. To avoid heating, cooling liquid is circulated through the jacket.

2.4.6 The Parr bomb

In the Parr bomb, the sample is subjected to nitrogen gas under very highpressure. Under these conditions, the nitrogen dissolves in the cell fluids. When the pressure is released, the explosive generation of nitrogen bubbles causes disruption of the cell, and less frequently of organelles.

2.4.7 Extrusion under high pressure

In an apparatus such as the French pressure cell (Fig. 17), cells are broken by extrusion through a narrow orifice at pressures of up to 8,000 p.s.i. Laminar flow causes intense shearing forces which disrupt the cells as they pass through the narrow orifice of the needle-valve.

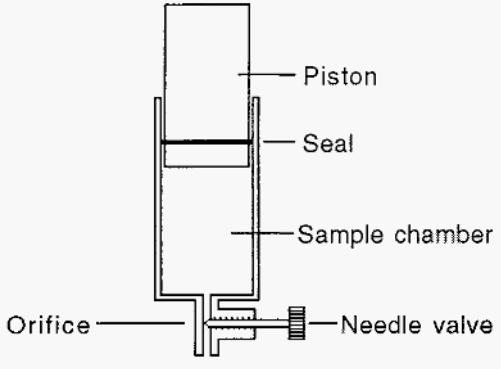


Figure 17. A French pressure cell.

Needle valves. Needle-valves arc devices used to adjust or regulate the flow of fluids. They consist of a tapering "needle", with a round cross-section, which fits into a corresponding round hole, called a "jet". When the needle is retracted slightly from the jet, an annular gap is formed, between the needle and the jet, and fluid can flow through this gap. The cross-sectional area of the annular gap can be altered by adjusting the degree to which the needle is retracted from the jet.

An every-day application of needle-valves is in carburettors, where they are used to control the flow of petrol and air, to ensure a correct mixture of the two. Adjusting the needle-valves is one of the steps involved in "tuning" a carburettor.

2.4.8 Sonication

Application of high frequency sound waves is an effective method of cell breakage which can be applied to micro-organisms. The mechanism is thought to involve "micro-cavitation", i.e. the production of very local transient pressure differences, which break cell walls. The efficiency of cell breakage is influenced by the power output of the instrument, the duration of exposure and the volume of material processed. In general, the volume which can be treated in a given time is not great - not as great, for example as that using high pressure extrusion. Cooling is necessary to prevent the build-up of heat.

Micro-cavitation. The formation of a bubble of vapour in a liquid, due to a local reduction in pressure to below the vapour pressure of the liquid at that temperature, is known as "cavitation". For example, in the case of a boat propeller, there is a pressure differential on either side of the blades - low pressure in front and high pressure at the back. If too much power is applied. the pressure in front becomes too low and the water vaporises. The propeller then spins in the vapour bubble formed, without generating thrust. "Micro-cavitation," is caused by the formation and collapse of very small bubbles of vapour in the liquid, due to the passage of sound (pressure) waves. Micro-cavitation can be very corrosive and is a major cause of the erosion of ships' propellers, for example.
(See p47, "How hard can one "suck" on water")

2.4.9 Enzymic digestion

Enzymes provide a very gentle and specific means of disrupting cells to release their contents. For example, the cell walls of bacteria may be digested with the enzyme, lysozyme. Similarly plant cell walls may be digested with cellulases and fungal cell walls with chitinases.

2.5 Clarification of the extract

The cellular extract prepared by one of the methods described above may be clarified, by filtration through a nylon mesh or cheese-cloth, to remove the larger tissue debris, and centrifuged at relatively low speed to remove insoluble cell components.

2.6 Centrifugal sub-cellular fractionation

Centrifugal = ìfleeing the centreî

The Svedberg Equation. As an introduction to the topic of centrifugation and to gain insight into the forces acting upon a molecule undergoing centrifugation, it is useful to consider the derivation of the so-called Svedberg equation.

Newton's law of motion states that any body in motion at a constant speed in a straight line will continue in that motion unless acted upon by a force. This force will cause the body to accelerate in the direction of the force, according to the equation:

Force = Mass x acceleration

e.g. the passengers in a cornering motor car.

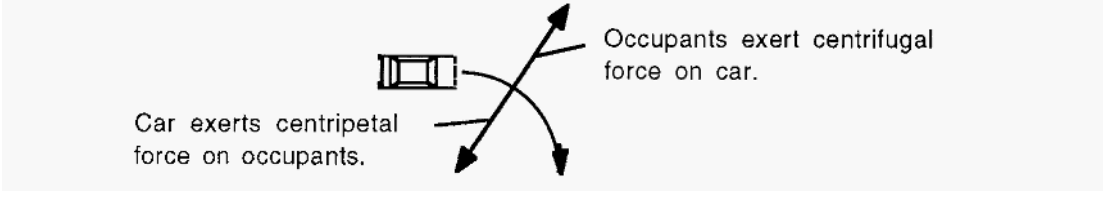


Figure 18. The equal and opposite forces acting on a cornering car.

The occupants of the car experience a gravitational field, due to their centripetal acceleration "a", described by the equation:-

$$a = \varpi^2 r$$

where, ϖ = the angular velocity in radians sec-1 (remember one revolution = 2π radians)

r = radial distance from axis of rotation.

The centripetal force (F) which the car exerts on the occupants and the equal-and-opposite centrifugal (inertial) force which the occupants exert on the car is a function of their mass.

i.e. $F = M\varpi^2 r$ 2.9

From equation 2.9 we can see why a car will skid if it is turned too rapidly;

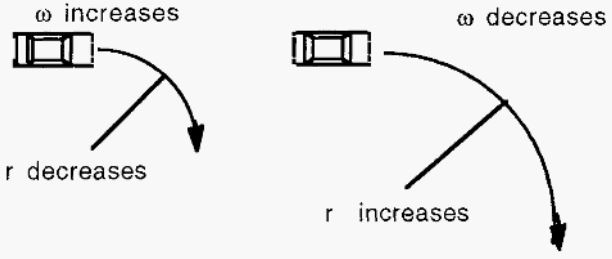


Figure 19. The effect of increasing the sharpness of a turn.

Increasing the sharpness of a turn increases ϖ, but decreases r. In equation 2.9, however, a is squared, while r is linear, so the increase in ϖ has the greater effect. The centrifugal force is thus increased in a sharp turn and the car is more likely to skid, which it will do when the centrifugal force becomes greater than the frictional grip of the tyres.

If we now consider molecules of molecular weight M, undergoing centrifugation, the centrifugal force acting upon them is given by;-

Centrifugal force $= M\varpi^2 r$ 2.10

This Centrifugal force causes the molecules to sediment down the centrifuge tube. As they start to move, however, they encounter a frictional resistance to their movement, given by:-

Frictional force $= f\left(\frac{dr}{dt}\right)$ 2.11

Where f = frictional coefficient

$\frac{dr}{dt}$ = the rate of sedimentation expressed as the change in radius with time (t).

The sedimenting molecule must also displace the solvent into which it sediments and this gives rise to a buoyant force:-

$$\text{Buoyant force} = M\omega^2 r\nu\rho \qquad 2.12$$

Where ν = partial specific volume of the molecules (cm^3 volume increase caused by 1g of solute),

ρ = density of the solution.

Note that the buoyancy force increases with the radius in the same way as the gravitational force does. To grasp what this means, conduct the following thought experiment:- Imagine a little ship floating on the surface of the water in a centrifuge tube, undergoing centrifugation, with its plimsoll line on the water line. If "r" was increased, by removing some of the water, the gravitational force $(M\omega^2 r)$ would increase but the ship would not float lower in the water because the buoyancy force $(M\omega^2 r\nu\rho)$ would increase by the same amount (Fig. 20).

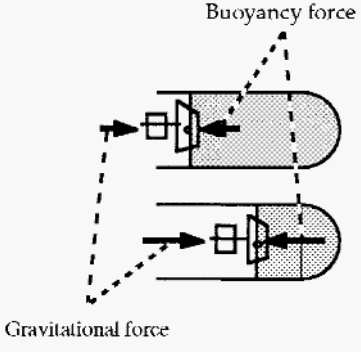


Figure 20. The effect of radius of rotation on gravitational and buoyancy forces.

Equations 2.6 to 2.8 can be combined in the expression:-

Centrifugal force = Buoyant force + Frictional force

i.e. $$M\omega^2 r = M\omega^2 r\nu\rho + f\left(\frac{dr}{dt}\right)$$

Hence, $$M = \frac{f}{(1-\upsilon\rho)} \cdot \frac{\left(\frac{dr}{dt}\right)}{\omega^2 r} \qquad 2.13$$

i.e. if all other factors are kept constant, the rate of sedimentation depends upon the molecular weight.

A new term, "s", the sedimentation coefficient, can be defined:-

$$S = \frac{\left(\frac{dr}{dt}\right)}{\omega^2 r}$$

(i.e. it is the rate of sedimentation per unit of gravitational field)

Hence, $$M = \frac{f S}{(1-\upsilon\rho)} \qquad 2.14$$

f can be determined from the diffusion coefficient (D), measured by molecular exclusion chromatography, immunodiffusion or ultra-centrifugation:

$$D = \frac{RT}{f} \text{ and, therefore } f = \frac{RT}{D}.$$

Substituting into equation 2.10, gives the so-called *Svedberg equation* named in honour of Professor The Svedberg, of Sweden:-

$$M = \frac{RTs}{D(1-\upsilon\rho)} \qquad 2.15$$

Note: "s" has the range 1 x 10^{-13} to 500 10^{-13}. A new unit, "the Svedberg", abbreviated "S", can be defined to obviate the (x 10^{-13}),

i.e. $S = s \times 10^{13}$

Fractionation of sub-cellular organelles is usually effected by centrifugation. Assuming one is dealing with rigid, spherical particles, the time required to sediment a specific particle, subjected to centrifugation, is given by the equation[17]; -

$$t = \frac{9}{2}\frac{\eta}{\omega^2 r_p^{\,2}(\rho - \rho_0)}\ln\frac{R_m}{R_b} \qquad 2.16$$

where,

t = time required for particle to sediment from meniscus to bottom of the tube,
η = viscosity of the medium,
ω = angular velocity,
r_p = radius of sedimenting particle,
ρ = density of sedimenting particle,
ρ_o = density of medium,
R_m = radius from centre of rotation to solution meniscus,
R_b = radius from centre of rotation to the bottom of the tube.

If the experimental set-up is established and the angular velocity is kept constant, Eqn 2.16 can be simplified to:-

$$t = K\frac{1}{r_p^{\,2}(\rho - \rho_o)} \qquad 2.17$$

Note that if the angular velocity is not constant, equation 2.17 must be modified to:-

$$t = K''\frac{1}{\omega^2 r_p^{\,2}} \qquad 2.18$$

So, for a given particle in a given system:-

$$t\omega^2 = \frac{K''}{r_p^{\,2}} \qquad 2.19$$

Hence, if we wish to change the time of sedimentation, the term $(t\omega^2)$ must be kept constant,

i.e. specified time x (specified rpm)^2 = new time x (new rpm)^2.

In the case of a sub-cellular fractionation, most particles are of about the same density and so equation 2.17 may be reduced to:-

$$t = K' \frac{1}{r_p^2} \qquad 2.20$$

i.e. the time taken to sediment a particle is inversely related to the square of its radius. This fact underlies the technique of fractionation by differential centrifugation, such as the example shown in Fig. 21. In a differential centrifugation fractionation, the "pellet" is unlikely to be pure because, initially, all particles of the homogenate are distributed evenly throughout the centrifuge tube. Upon sedimentation, the heaviest particles sediment first but other less dense material is dragged along and that originally near the bottom of the tube is co-precipitated. Repeated "washings" can improve the purity.

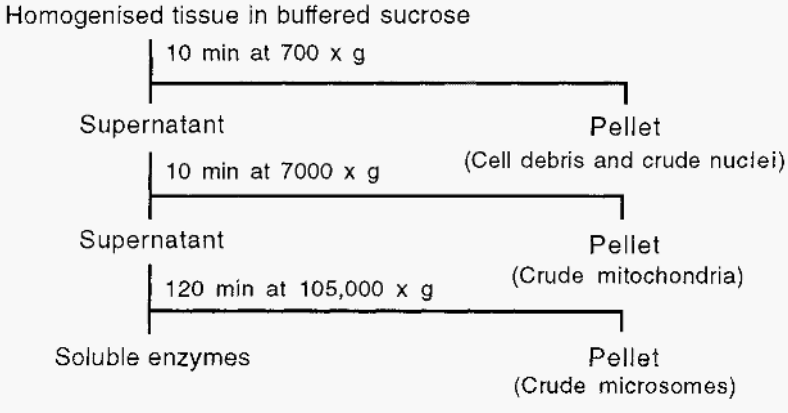


Figure 21. An example of sub-cellular fractionation by differential centrifugation.

2.6.1 Density gradient centrifugation

Re-consider equation 2.17, i.e.:-

$$t = K \frac{1}{r_p^2 (\rho - \rho_o)} \qquad 2.17$$

If, $(\rho - \rho_o) = 0$, $t = \infty$ and particle will not sediment,
if $(\rho - \rho_o) < 0$, $t < 0$ and particle will have negative sedimentation in real time, i.e. it will rise to the top,
and if $(\rho - \rho_o) > 0$, $t > 0$ and particle will sediment.

Using this information, one of two strategies for isopycnic (equal density) centrifugation can be adopted to separate particles by virtue of their differences in density:-

The sample can be suspended in a solution having a density equal to that of the sample protein of interest. Proteins that are more dense or less dense will sediment or float to the top of the solution, respectively, leaving the protein of interest in solution. A difficulty with this approach, however, is that often the density of the protein of interest is not known beforehand.

The sample can be layered on top of a continuous density gradient. If the gradient extends to densities exceeding the density of the sample proteins, these will sediment through the gradient until they reach points where their density is equal to that in the gradient, at which point sedimentation ceases, i.e. the proteins become focused at their isopycnic points.

Density gradients may be generated using sucrose dissolved in buffer. Disadvantages of sucrose are:-

It interferes with the Lowry and Bradford protein assays, though less with the latter.

It can penetrate cells. This, of course, is a problem which only applies to the fractionation of cells.

Advantages of sucrose are:-

It is biologically inert.

- It is inexpensive.
- It is dialysable and is thus easy to separate from the sample proteins.

"Ficoll", a Pharmacia product, consists of sucrose crosslinked with epichlorhydrin, and has a molecular weight of about 400,000. Like sucrose it is biologically inert, but in some respects it is opposite to sucrose, e.g. it cannot penetrate cells and so is suitable for fractionation of cells, but it is not dialysable and so is difficult to separate from proteins.

With sucrose or Ficoll, a density gradient can be generated using a two chamber gradient generator, with an insert in the low density chamber to compensate for the lower density. To generate a linear gradient, the solutions in the two chambers must have the same volume and they must be in hydrostatic equilibrium, i.e. when there is no flow out of the apparatus, there must be no tendency for fluid to flow from one compartment to the other.

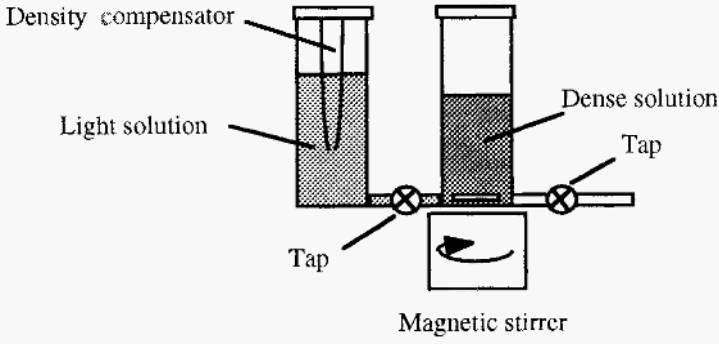


Figure 22. A gradient generator for use with sucrose or Ficoll.

An elegant new approach to gradient making, the tilted tube method, was introduced by Coombs and Watts[16]. In this method, the light solution is layered on top of the heavy solution directly in the centrifuge tube. The tube is then tilted and rotated, resulting in the rapid formation of a smooth gradient (Fig. 23).

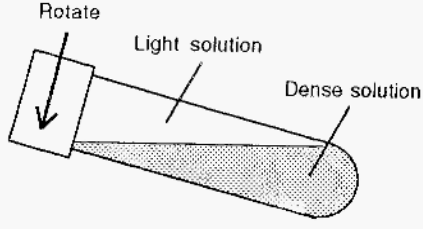


Figure 23. Rapid gradient generation by rotating a tilted tube.

"Nycodenz", a product of Nyegaard Diagnostica, of Norway, consists of a substituted tri-iodobenzene ring linked to a number of hydrophilic groups. It has a MW of 821, is non-ionic and has a density of 2,1 g/ml. With Nycodenz, or caesium chloride, a technique known as equilibrium isodensity centrifugation (isopycnic focusing) can be used. In this technique, the sample is mixed homogeneously with a concentrated solution of Nycodenz (or caesium chloride) and centrifuged. The Nycodenz (or caesium chloride) tends to sediment out slightly, generating a density gradient. The sample molecules distribute themselves in the gradient, the density field driving them to a region where the solution density is equal to their own buoyant density. This is comparable to isoelectric focusing (IEF), and the Nycodenz plays a role analogous to that of ampholytes in IEF.

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2.7 Chapter 2 study questions

1. What is the primary measurement in the assay of an enzyme?
2. How can V_o be determined from this primary measurement?
3. Left to itself, when will an enzyme catalysed reaction stop?
4. If [enzyme] is doubled, what happens to V_o?
5. Is V, affected by the amount of substrate present?
6. What [S] is best in an enzyme assay?
7. Describe the effect of temperature and pH on V_o.
8. What is the object of extracting a protein? (Note ìex tractionî ≠ iisolationî)
9. How may cells be lysed by osmosis?
10. Why is a Dounce homogeniser provided with two plungers?
11. Describe the physical principle common to the operation of both pestle and blender type homogenisers.
12. Why is the turbine of a water-pump windmill is usually located on top of a tower?
13. What limits the height of the tower used?
14. A car travelling at speed over a gravel road gets dusty. Why doesnít the wind, resulting from the carís forward motion, blow the dust away?
15. What does the word "acceleration" mean?
16. What is the difference between a gravitational field and a gravitational force?
17. Give an equation which relates the particle radius with the time taken to sediment a rigid, spherical particle.
18. Two identical particles are in a solution undergoing centrifugation, one near the top of the solution and one halfway down. Which one of these will reach the bottom first? Will they both be completely sedimented in the time described in the equation given in question 17?
19. You are following a centrifugation method, which prescribes centrifugation for 1 h at a given rpm. You wish to leave the lab in 30 min time for an important date. What can you do about this?
20. Why is it usually necessary to ìwashî the pellet obtained in a differentialcentrifugation? How might this "washing" be effected?
21. In real time, dairy cream has a negative sedimentation in a gravitational field. Explain.
22. Describe two methods of forming a density gradient, one being by the use of a gradient generator.

Chapter 3

Concentration of the extract

Proteins are most efficiently extracted into dilute solution, whereas subsequent handling is more convenient if the protein is present in a relatively small volume. The first step, following the extraction of the protein into solution is, therefore, usually to concentrate it into a smaller volume. The concentration method may be non-specific, in which case only the water is removed and all non-volatile molecules are concentrated. Alternatively, it may be non-specific with respect to large molecules, i.e. the water and all small molecules are removed and all large molecules, including all the proteins, are concentrated. Finally, the concentration may be more-or-less specific, i.e. a particular protein may be concentrated in relation to the water and other molecules, including some protein molecules.

3.1 Freeze drying

Freeze drying is a method for the removal of water from a sample kept at low temperature, the water being removed directly from ice into the vapour phase by sublimation. It is a non-specific method as all of the non-volatile solutes are concentrated.

A major use of freeze-drying is for long term storage (preservation) of proteins or other biological samples. By reducing the water content to a very low level, microbial growth is inhibited and spoilage of the stored material is prevented. Aqueous-phase chemical reactions are also inhibited and this helps to preserve the sample. It may be noted that if the water is not removed, a temperature of *ca.* -70 C is required in order to stop aqueous-phase reactions, i.e. a deep-freeze at -20 C is not cold enough.

Freeze drying may destroy the activity of some enzymes and, if it is important to retain the activity, this should always be checked before freeze-drying is used to preserve a particular protein.

3.1.1 Theoretical and practical considerations in freeze-drying

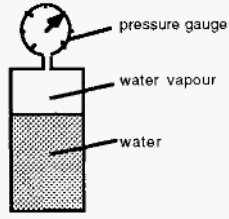


Figure 24. Measurement of the vapour pressure of water.

In order to understand how a freeze-dryer works, it is important to understand the concept of vapour pressure. What is "vapour pressure"? Consider the set-up shown in Fig. 24; a sealed container containing only a liquid (water) and its vapour, i.e. with no other gases present, and with a pressure gauge to monitor the (vapour) pressure. In this way, the vapour pressure can be measured as the temperature of the set-up is changed. If this is done, it will be noticed that the measured pressure (the vapour pressure) changes with the temperature as shown in Fig. 25. If there are other gases present then the vapour will contribute a part of the total pressure, i.e. the total pressure will be the vapour pressure at that temperature plus the (partial) pressures of the other gases.

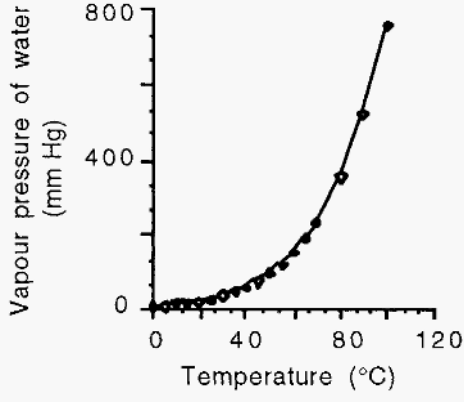


Figure 25. The vapour pressure of water as a function of temperature.

Notice that at 100 C, the standard boiling point of water, the vapour pressure of water is 760 mm Hg, which is the standard atmospheric pressure. This illustrates the important principle that a liquid will boil

when its vapour pressure becomes equal to the environmental atmospheric pressure.

At *ca.* 0 C (at pressures of ambient or below), water undergoes a phase change from a liquid to a solid, ice. The vapour pressure of ice is relatively low (compared to that of water) and is asymptotic to zero as shown in Fig. 26.

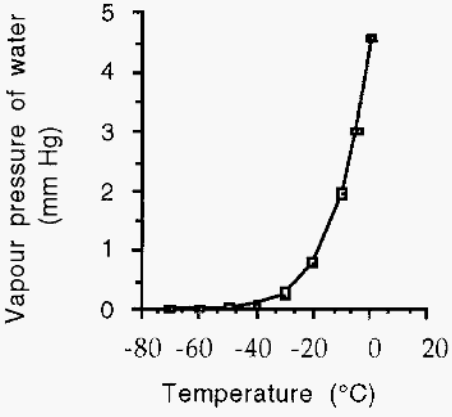


Figure 26. The vapour pressure of ice as a function of temperature.

Just as a liquid will boil when its vapour pressure becomes equal to the environmental pressure, so a solid will **sublime** from the solid state to the vapour state when its vapour pressure becomes equal to the environmental atmospheric pressure. The salt, *sal ammoniac,* for example, has a vapour pressure higher than 760 mm Hg and thus sublimes from the solid to the vapour at ambient atmospheric pressures. In order for ice to sublime, however, it is necessary to reduce the pressure to which it is exposed to values less than or equal to its vapour pressure at its particular temperature, e.g. the pressure must be reduced to $\leq$ 1.95 mm Hg, if the ice is at -10 C (Fig. 26).

Therefore, consider the situation illustrated in Fig. 27, where flask A is at a temperature T_1 and condenser B is at a temperature T_2, both T_1 and T_2 being below the freezing point of ice but $T_1 > T_2$. It follows then that if P, the overall pressure within the system, is less than V_pT_1 (the vapour pressure of ice at temperature T_1) and more than V_pT_2 (the vapour pressure of ice at temperature T_2), ice will sublime in flask A and condense in B.

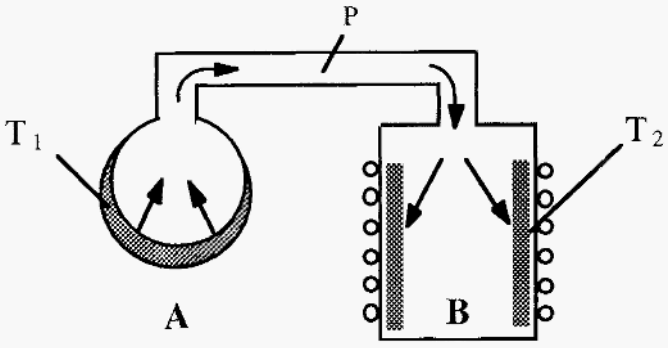


Figure 27. A simple freeze-dryer.

In a practical freeze-dryer, T1 will be about -10 C and T_2 will be about -50 C. V_pT_1 will thus be about 1.95 mm Hg and V_pT_2 will be about 0.029 mm Hg (Fig. 26). "P", the pressure measured on the vacuum gauge, must therefore be between 1.95 mm Hg (1,950 microns) and 0.029 mm Hg (29 microns): in practice it is usually between 500 and 100 microns, when the freeze dryer is operating properly.

With regard to the net transport of water vapour from A to B an analogy can be drawn with electricity, i.e. where in electricity,

$$\text{Current} = \frac{\text{Voltage}}{\text{Resistance}},$$

In freeze-drying,

$$\frac{\text{Mass of water vapour transported}}{\text{unit time}} \quad \frac{\text{Vapour pressure difference}}{\text{Constant x Pressure of permanent gases}} \qquad 3.1$$

The constant in the "resistance" term in equation 3.1 describes in part the geometry of the piping system connecting flask A and condenser B and is minimal when this is short and wide. To achieve a maximal rate of freeze–drying,therefore, it is necessary to establish:-

- A maximal vapour pressure difference between the sample and the condenser,
- a minimum pressure of permanent gases, and,
- a minimum value of the ìpiping geometry constantî.

Most of these factors are fixed by the design of a particular machine but it is useful for the researcher to have an appreciation of their influence. For example, the first item, considered in conjunction with

Fig. 26, suggests that there is no advantage to be gained in using a condenser temperature much below -50 C, since below this temperature there is little change in the vapour pressure of ice (since it is asymptotic to zero). A temperature of -50 C can be achieved with single-stage refrigeration systems and there is thus little benefit in using expensive two-stage systems to reach a lower condenser temperature.

As ice sublimes from flask A it removes latent heat of sublimation, which keeps the remaining ice cold. The heat removed by sublimation is replaced by heat from the atmosphere, especially the latent heats of condensation and freezing of ice condensing on the outside of the flask. A thermal gradient is thus formed through the layers of ice and the wall of the freeze-drying flask (Fig. 28), and a dynamic equilibrium is established in which the rate of heat input to the system is balanced by the rate of heat loss. At equilibrium, the rate of heat input is the factor limiting the rate of freeze-drying. However, a limit to this rate of input is determined by the point at which the sample melts.

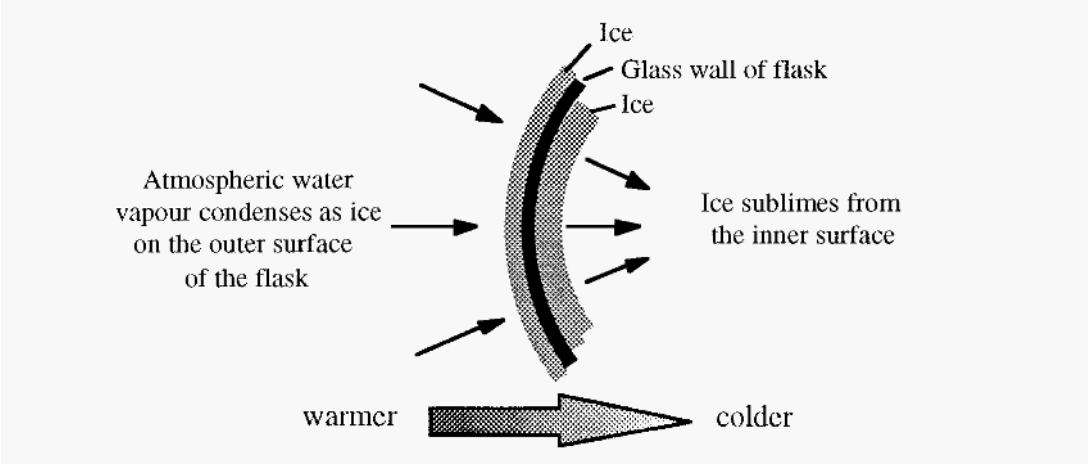


Figure 28. Thermal gradient across a freeze-drying flask and its associated ice layers.

The nature of the system has the following practical ramifications:-

- The greater the area over which the sample is spread, the greater will be the rate of heat input and the faster will be the rate of freeze–drying;
- The sample layer should be as thin as possible since if it is too thick there is a risk of the sample melting on its outermost surface, due to the thermal gradient. (Note: If this should happen, the sample should not be re-frozen as the melted sample, being trapped between the flask

wall and the frozen part of the sample, might crack the flask as it expands upon re-freezing.)

- The flask should be made of a material with a high thermal conductivity: thus glass is commonly used.

When the freeze-dryer is in operation, there is a flow of heat through the system, as illustrated in Fig. 29.

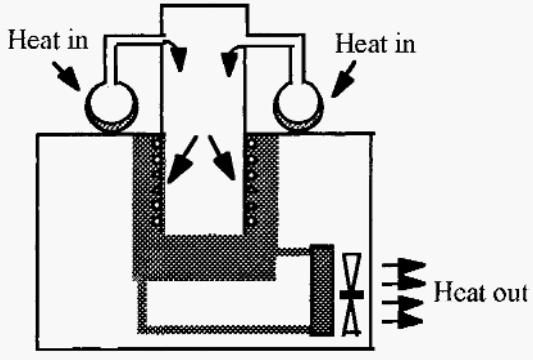


Figure 29. The heat flow through a freeze dryer in operation.

Atmospheric heat enters the freeze drying flask, via the ice condensing on the outside of the flask. The heat is transferred through the flask and is removed by the subliming water vapour. When this water vapour condenses, in the condenser, the heat is transferred to the refrigerant gas and is ultimately returned to the atmosphere via the radiator of the refrigeration unit.

3.1.2 Some tips on vacuum

Many people have confused thoughts about vacuums and so a few words on the subject may be useful. A vacuum may be defined as any pressure less than the prevailing ambient pressure. Since vacuums are defined in terms of a pressure differential, clear thinking is easier if one thinks only in terms of pressure, which has a range from zero to "infinity".

Ambient pressure is usually about one atmosphere, or 760 mm Hg, or about 15 p.s.i. ("p.s.i." or pounds per square inch is an old measure but one which is easy to visualise. 1 p.s.i. $\approx$ 6.89 kPa). An absolute vacuum, which is practically unattainable, corresponds to a pressure of zero. The theoretical maximum pressure differential across the walls of a vessel in the atmosphere but "containing" an absolute vacuum is therefore one atmosphere, i.e. 760 mm Hg or about 15 p.s.i. This is not a large

differential, as pressures go, and there is clearly no truth in the belief that, "if one sucks hard enough, almost any vessel can be made to collapse!"

How hard can one ìsuckî on water?

Everyone is familiar with the process of drinking water from a container by using a straw.

Q: Is there a limit to the length of the straw, i.e. to the height that the water can be sucked up?

A: Yes. In the case of water the limit is about 7 metres, which is the height to which the atmospheric pressure can lift a column of water. The equivalent height of a column of mercury is 760 mm (do this only as a "thought experiment", though, since mercury is very toxic!)

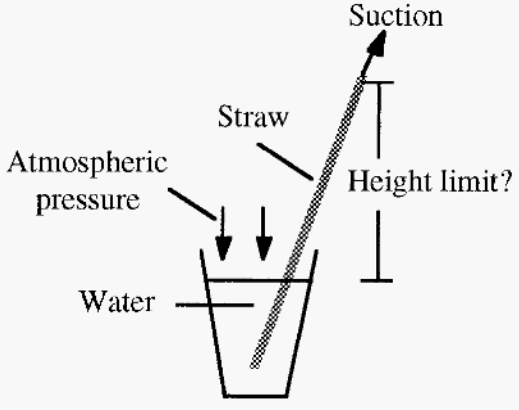


Figure 30. How far can water be sucked up?

This is why farmers have their water pumps near the bottom of the valley, near the river. The pump can only "suck" the water up a limited distance (about 7 metres vertical height) but it can ìpushî it up much further than this.

Q: What limits the water from being sucked up higher than 7 meters?

A: The water will boil (i.e. be converted to vapour) when the pressure applied to it becomes less than its vapour pressure at the prevailing temperature.

Refer back to the discussion on cavitation on p30.

In engineering terms the point to remember about pressure differentials is that the resultant *force* is a function of the *area* over

which the differential exists. Consider, for example, the force acting upon the perspex lid of a typical laboratory freeze dryer. If the lid is 8 inches in diameter (1 inch = 2.54 cm) it will have an area of *ca.* 50 square inches. At a pressure differential of 15 p.s.i., the force acting on the lid is equivalent to ca. 750 pounds weight (ca. 340 kg): no wonder it bowsinward. If one could get a good enough grip, one could lift the entire machine by its lid once the vacuum was established!

On the other hand, what is a high vacuum and how is it different to a "non -high vacuum"? An atmosphere corresponds to a pressure of 760 mm Hg. A pressure of 1 mm Hg is not really in the high vacuum range but theforce applied to the system would be $^{759}/_{760}$ of the ultimate force. High vacuum pumps "split" the last mm of Hg and 0.5 mm Hg (500 microns) requires a high-vac pump, but the forces on the system will increase by only 0.5/760 or 0.07%, a negligible amount!

A generalisation can therefore be made: that if a system is structurally strong enough to withstand a ìmoderateî vacuum of 1 mm Hg (1000 microns) it will probably withstand any possible high vacuum! (Remember that attaining high vacuums is like splitting hairs!). In practical terms this means that one should not be too nervous of flasks imploding under vacuum. A flask is more likely to break due to thermal stress or mechanical abuse (point impacts) than under vacuum loads perse. Nevertheless one should be aware that the likelihood of a flask failing, from whatever cause, increases with the size ofthe flask.

3.2 Dialysis

Dialysis is the term used to describe the *diffusion* of solutes through semi-permeable membranes when the membrane forms the boundary between solutions of different concentrations. The membrane acts as an inert sieve with a certain average pore size. The pores result from the random distribution ofthe fibres making up the dialysis membrane.

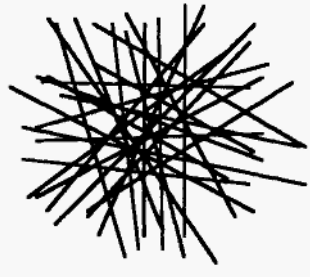


Figure 31. The random distribution of (cellulose) fibres in a dialysis membrane.

A "pore" corresponds to a space bounded by fibres. Clearly the pores are not all of the same size: there is a normal distribution of pore sizes. Molecules with a molecular radius larger than the largest pore size of the membrane will be completely retained while those with smaller radii will pass through more or less easily depending on their size. Fig. 31 shows a 2-dimensional representation but it must be realised that pores are 3-dimensional.

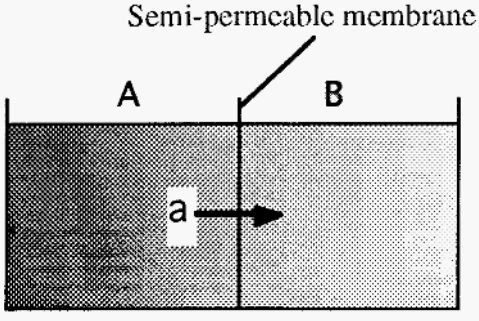


Figure 32. Dialysis across a semi-permeable membrane.

With reference to Fig. 32, consider a small solute "a", initially in compartment "A" which is separated from compartment "B" by a semi-permeablemembrane. As the initial concentration of "a" in A is greater than its concentration in B, ìaî will diffuse from A→B.

The rate of diffusion will be affected by the following factors:-

- *The concentration differential across the membrane.* Stirring of both solutions, if possible, and regular changing of solution B will ensure that [a]A >> [a]B and thus the rate of diffusion will be kept at a maximum.
- *Surface area.* The larger the surface area of the membrane, the faster the overall rate of diffusion. Therefore the membrane area should always be kept at a maximum.
- *Solution volume.* If the solute molecules have to diffuse a long distance before reaching the membrane, then the rate of dialysis will be relatively slow. Stirring can speed up the rate of transfer to the membrane, but the distance should also be kept to a minimum, i.e. the surface area:volume ratio should be large.

Dialysis is typically used to desalt protein solutions, or to effect a buffer exchange, i.e. to get the protein from one buffer solution into another (Note that "desalting" and "buffer exchange" are really the same process, in the former the second ìbufferî is simply distilled water).

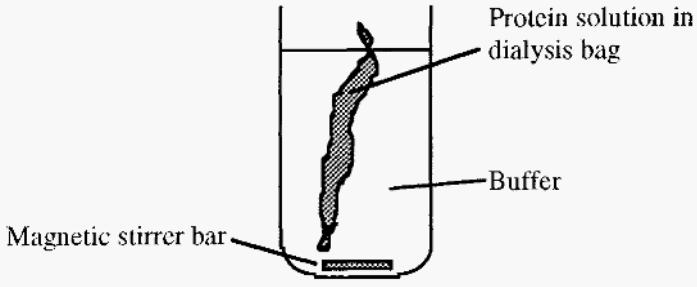


Figure 33. Dialysis using a visking dialysis bag.

Dialysis can be done in various ways, but in the laboratory it is most commonly done using "Visking" tubing. This is a cellulosic material reconstituted into tubular form, dried, and supplied in rolls. A length can be cut from the roll, hydrated by immersion in water for several minutes, and clamped or knotted at one end to form a sealed "dialysis bag". The protein is introduced into this bag and the open end is sealed by clamping or knotting. The dialysis bag is immersed in a large volume of distilled water or buffer for several hours at 4 C to effect exchange of the permeable ions and molecules (Fig. 33), the dialysis solution being changed at intervals (every few hours).

During dialysis, water enters the dialysis bag due to the osmotic pressure of the protein solution. For this reason a dialysis bag must not be filled, but a potential space must be left to accommodate the increasing volume of the protein solution (see Section 3.2.3). Note that if the dialysis bag is sealed with knots, the knot should be tightened by pulling only on the outside, not on the bag side of the knot, to avoid stretching the bag and thus distorting the pores.

3.2.1 The Donnan membrane effect

The Donnan membrane effect[1] describes the phenomenon whereby a charged macromolecule, constrained by a semi-permeable membrane, causes an asymmetrical distribution of permeable ions on either side of the membrane. The net effect is to cause an apparent movement of ions, having the same charge as the protein, away from the protein, i.e. if the protein is positively charged, there will be a lower concentration of small cations in the compartment containing the protein than in the compartment on the other side of the membrane, and *vice versa.* In buffers, the Donnan effect is not very significant, but when a protein is dialysed against distilled water the Donnan effect can cause significant pH

differences on either side of the membrane. This may or may not be significant, depending on the circumstances.

Similarly, ion-exchange resins repel ions of like charge and attract ions of opposite charge. In buffers of low ionic strength, this may cause the pH to be significantly different in the immediate vicinity of the resin substituent groups, compared to that in the bulk of the solution, i.e. cation exchangers, which are negative, will attract cations, including H+ ions, and this will cause a decrease in pH in the immediate vicinity of the resin substituents. With anion exchangers, which are positive, hydroxyl ions are attracted and the pH around the substituents is consequently higher than in the bulk solution.

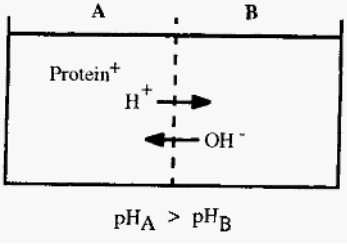


Figure 34. The Donnan membrane effect.

3.2.2 Counter-current dialysis

A very efficient form of dialysis, often used in automatic analysers, is counter-current dialysis (CCD). In this, a stream of the solution to be dialysed is arranged to flow through a thin, convoluted, channel on one side of a dialysis membrane. and the dialysing solution is arranged to flow in the opposite direction through a corresponding thin channel on the other side of the dialysis membrane. In CCD, a maximal concentration difference is thus maintained between the solution being dialysed and the dialysingsolution. Since thin channels are used, the diffusion distance is small and so there is little need to stir the solutions. A stirring effect can be induced, by flowing the solutions at a high speed so that laminar flow breaks down into turbulent flow, but the period of dialysis per pass is reduced and the benefits, if any, have to be assessed for each case by empirically establishing the optimal flow rate.

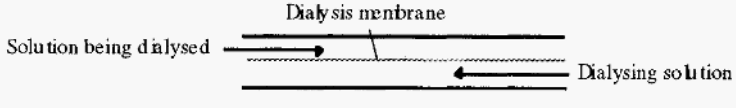


Figure 35. Counter current dialysis.

3.2.3 Concentration by dialysis (concentrative dialysis)

As mentioned above, a "complication" of dialysis is *osmosis,* which is the movement of water through a semi-permeable membrane from a solution of low osmotic pressure to a solution of high osmotic pressure. Normally the flow is into the protein solution, so that the protein solution becomes diluted during dialysis against distilled water or a buffer solution: for this reason a dialysis bag is never filled when a protein solution is dialysed. However, the flow can be reversed and the protein solution concentrated, by dialysing the protein against a solution with a higher osmotic pressure.

The dialysis bag may be simply surrounded by granular sucrose. The water flowing out of the bag will dissolve the sucrose, generating a concentrated sucrose solution with a high osmotic pressure, and this will cause further egress of water from the bag. Alternatively, the dialysis bag may be suspended in a solution of polyethylene glycol (PEG), a hydrophilic polymer.

It will be appreciated that, because water is flowing *out* of the dialysis bag in such a case, the bag can be filled completely with protein solution before concentrative dialysis. Concentrative dialysis is a specific method in the sense that only macromolecules are concentrated - all buffer salts etc. are not concentrated - but it is non-specific with respect to proteins.

An effect similar to that of concentrative dialysis can be achieved by adding a dry, reversibly hydratable gel (i.e. one that can be dried and reconstituted to have the same structure) such as Sephadex. The Sephadex xerogel will absorb water and, provided it is larger than the exclusion limit of the gel, the protein will be concentrated in the fluid between the swollen gel particles.

3.2.4 Perevaporation

A method of concentration using dialysis bags but which is not used much today, is perevaporation. In this method a dialysis bag containing the protein solution to be concentrated is suspended in a stream of air. Water evaporates from the outside of the bag, keeping the bag and its remaining contents cool. As the water evaporates, all of the non-volatile contents of the dialysis bag are concentrated.

An application of perevaporation which is frequently used today is in the drying of polyacrylamide gels after electrophoresis. Dried gels are mechanically strong and are more easily stored than hydrated gels. To dry the gel, a cellophane membrane is placed on either side of it and the

sandwich is suspended in a stream of warm, dry, air until it is completely dry.

3.3 Ultrafiltration

Ultrafiltration is a technique related to dialysis, and can also be used to desalt protein solutions, effect buffer exchange, or concentrate protein solutions. It is more expensive than dialysis, however, as special equipment and membranes are required.

In this technique, pressure is applied to the solution to cause a bulk flow of water and dissolved low molecular weight solutes, through the membrane, while high molecular weight solutes are retained.

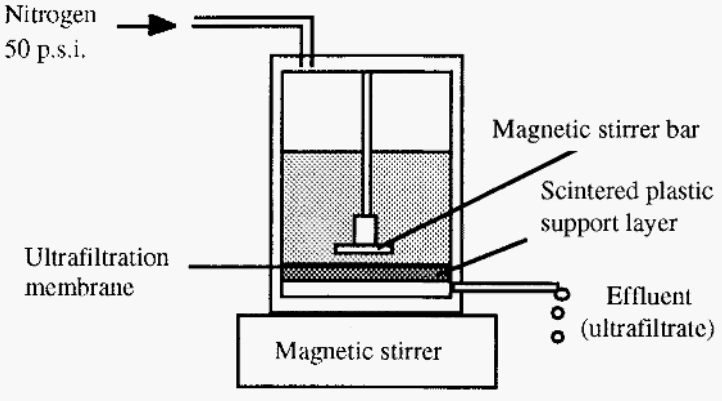


Figure 36. An ultrafiltration cell.

If a conventional dialysis membrane were used for ultrafiltration, it would soon become blocked with proteins trapped within the membrane. To overcome this problem, special membranes are used. These have a unimolecular sieving layer (a layer one molecule thick), supported by a much thicker support layer having a larger pore size.

Such a membrane is called an anisotropic membrane, since it is not the same in all parts. The sieving side can be distinguished from the support side because the sieving side is shiny while the support side is dull.

Figure 37. An anisotropic ultrafiltration membrane.

Whether or not a particular molecule will pass through an ultrafiltration membrane is determined at the unimolecular sieving layer. Proteins which are unable to pass through are rejected on the surface where they can easily be removed and the filter is therefore resistant to blocking.

The pressure exerted on the solution causes a flow of solvent through the membrane but immediately flow commences, a phenomenon known as *concentration polarisation* occurs. This refers to the process whereby a secondary membrane layer of retained protein is formed.

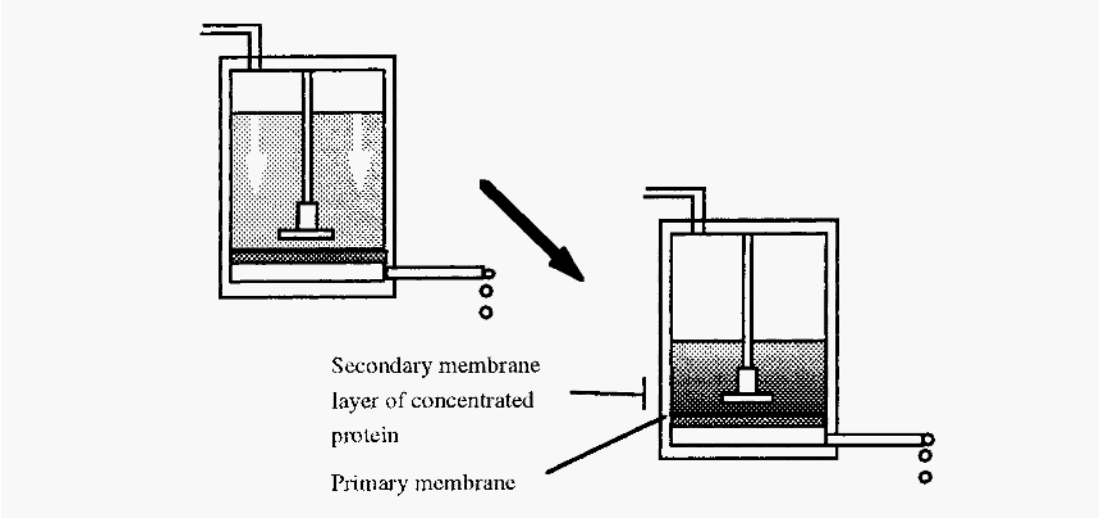


Figure 38. Concentration polarisation: the formation of a secondary membrane layer.

The secondary membrane layer (SML) constitutes the major resistance to flow and thus determines the flow rate. At any given pressure, an equilibrium is rapidly set up whereby the transport of macromolecules into the SML, by bulk flow of solvent, is counterbalanced by diffusion of macromolecules out of the SML. The SML thus attains a stable thickness and the flow rate remains constant.

If the applied pressure is increased, the flow rate initially increases, but this results in more macromolecules being transported into the SML. The thickness of the SML thus increases, its resistance to flow increases, and the flow rate drops, virtually to the original value. Thus the flow rate is essentially independent of the applied pressure.

Since the resistance is determined by the thickness of the SML, reducing this thickness will result in an increased flow rate. One way of decreasing the thickness of the SML is to stir the solution and thus

increase the effective rate of back diffusion. This is the purpose of the stirring bar illustrated in Fig. 36.

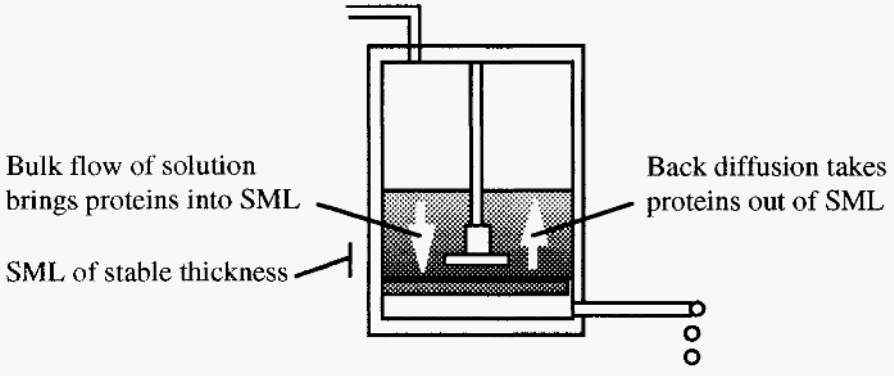


Figure 39. Dynamic nature of the secondary membrane layer (SML), at equilibrium.

An alternative way is by the use of a thin channel ultrafiltration module, in which the UF-membrane is sandwiched between two blocks of perspex◊, with corresponding thin channels milled into each (Fig. 40). By pumping the solution at a high flow rate, turbulent flow can be induced in the channel and this stirs up the SML. The pressure applied to the membrane can be adjusted by restricting the outlet pipe on the high-pressure side of the membrane.

Industrial scale UF is usually accomplished by such flow-through UF modules. The modules may be stacked, with the flow arranged in series or in parallel, and very high total membrane surface areas and overall flow rates can be obtained. An advantage for industrial scale applications is that such UF systems are one of the few protein fractionation methods that can run continuously, all other methods requiring batchwise operation.

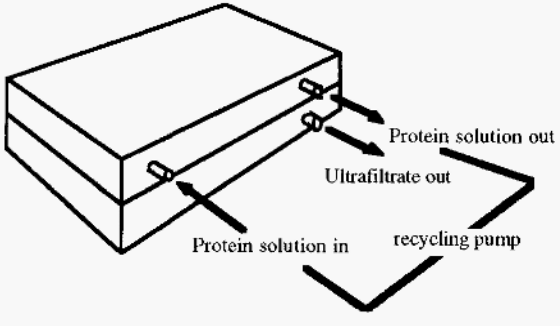


Figure 40. A thin-channel flow-though ultrafiltration module.

The secondary membrane determines the flow rate but the primary membrane (the anisotropic membrane) determines the *selectivity,* i.e. the size of molecules that will be retained. Primary membranes can be purchased with different "exclusion limits", ranging from $500 \rightarrow 100{,}000$ Daltons. Conceptually, the "exclusion limit" is the molecular weight of a globular protein which will just be retained by the membrane - which is the same as the molecular weight of a protein which will just pass through the membrane. In practice, however, there is not a clear-cut distinction between the size of molecule which will be retained and that which will pass through the membrane, since the pore sizes in any membrane have a normal (Gaussian) distribution.

3.3.1 Desalting or buffer exchange by ultrafiltration

Ultrafiltration may be used to change the buffer in which a protein is suspended, either for another buffer or for distilled water. In either case the approach used is the same. The solution is reduced to a small volume, rediluted in the new buffer (or distilled water), reduced to a small volume again etc., the process being repeated several times, until the protein is in the new buffer only. The process is analogous to the washing of the retentate on a paper filter and the same equation applies, i.e.:-

$$x_n = x_o\left[\frac{u}{u+v}\right]^n$$

Where
- n = number of "washings"
- x_o = conc. of original salts before desalting
- x_n = conc. of original salts after "n" washings
- u = volume to which sample is reduced
- v = volume of new solution added for each washing

Consequently, to achieve buffer exchange (or desalting) in the minimum time, the factors "u" and "v" should be kept to a minimum, since the time taken depends upon the total volume of washing solution used. (This is a useful equation to remember when rinsing oneís laundry).

3.3.2 Size fractionation by ultrafiltration

Proteins may be fractionated into size groups by ultrafiltration, by passing the solution, successively, through membranes of decreasing pore size. The largest proteins will be retained by the membrane with the largest pore size, etc.

3.4 Concentration/fractionation by salting out

Salting out using ammonium sulfate is one of the classical methods in protein biochemistry. Formerly it was widely used for the fractionation of proteins, but it is not a highly discriminating method and it is unusual to get a pure fraction, using this method. Today it is rather used as an inexpensive way of concentrating a protein extract, while leaving non-protein material in solution, and any purification with respect to protein is generally regarded as a bonus.

3.4.1 Why ammonium sulfate?

Polyvalent anions are more effective at salting out than univalent anions, while polyvalent cations tend to negate the effect of polyvalent anions. The best combination is therefore a polyvalent anion with a univalent cation. Anions can be arranged in a so-called "Hofmeister series", which describes their relative effectiveness in salting out at equivalent molar concentrations[2]. In decreasing order of effectiveness, the series is: citrate > sulfate > phosphate > chloride > nitrate > thiocyanate. This series also describes a decreasing tendency for the anions to stabilise protein structure. Citrate and sulfate are thus "kosmotropes", which tend to stabilise protein structure, while thiocyanate and nitrate are "chaotropes" which tend to destabilise protein structure. An ideal salt would, therefore, be citrate or sulfate combined with a univalent cation. Ammonium sulfate is most popular because it meets these criteria, is available in a pure form at low cost and is highly soluble, so that high solution concentrations can be attained.

The sulfate ion has been viewed in a number of ways, regarding how it salts out proteins, including, ionic strength effects, kosmotropy, exclusion-crowding, dehydration, and binding to cationic sites, especially when the protein has a net positive charge (denoted Z_H+)[3]. All of these may play a role, depending upon the salt concentration and the pH-dependent charge on the protein.

Ionic strength effects. It will be noticed that the Hofmeister series goes from multivalent to univalent ions. This largely reflects the fact that the Hofmeister series is based on molarity, while ionic strength is a factor in salting out. The valency of the ion has an effect on ionic strength as can be illustrated by comparing NaCl with $(NH_4)_2SO_4$.

Ionic strength is defined as:-

$$I = \frac{1}{2}\sum c_i(z_i)^2$$

Where, c_i = concentration of each type of ion (moles/litre)
z_i = charge of each type of ion.

Thus in the case of 1 M NaCl,

$$I = \frac{1}{2}[(1 \times 1^2) + (1 \times 1^2)]$$
$$= \frac{1}{2}(1 + 1)$$
$$= 1$$

and for 1 M $(NH_4)_2SO_4$,

$$I = \frac{1}{2}[(2 \times 1^2) + (1 \times 2^2)]$$
$$= \frac{1}{2}(2 + 4)$$
$$= 3$$

Ionic strength effects come into play at low salt concentrations (0→0.2 M) and, as the name implies, they are not specific to ammonium sulfate. At low ionic strength, protein solubility is at its minimum at the proteinís pI (Fig. 41). At this pH, intramolecular electrostatic forces between oppositely charged side chains are at a maximum, protein conformation is maximally tightened and protein hydration is least. On either side of the pI, titration of ionisable groups leads to a lessening of intramolecular ionic interactions. In consequence, protein structure becomes more relaxed and hydration and solubility are increased[3].

Addition of low concentrations of salt causes a similar weakening of intramolecular ionic bonds, with similar consequences of more relaxed protein structure and greater solubility. As shown in Fig. 41, addition of salt and altering of the pH, away from the pI, have similar, and additive, effects. The increase in solubility of protein upon addition of modest amounts of salt is known as “salting in”.

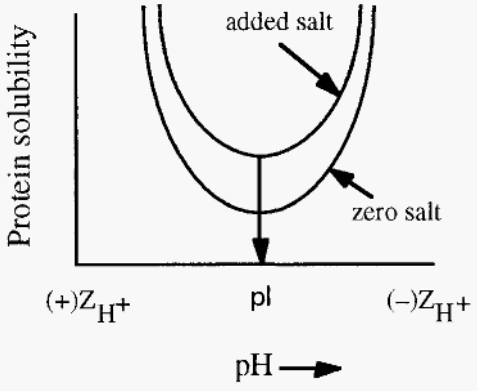


Figure 41. "Salting in" of proteins the interaction of pH and ionic strength (adapted from Dennison and Lovrien ref. 3).

Kosmotropy. At concentrations above 0.2 M the sulfate ion acts as a Hofmeister kosmotrope, i.e. it stabilises protein structure, and concomitantly reduces its solubility. The effect of a kosmotrope, in stabilising protein structure, can be described by the reaction:-

Relaxed, open protein structure (more soluble, less stable) → compact, tight structure (less soluble, more stable)

Kosmotropes may be described as "pushing" if they act on the left of this reaction and "pulling" if they act on the right, in either case driving the reaction to the right.

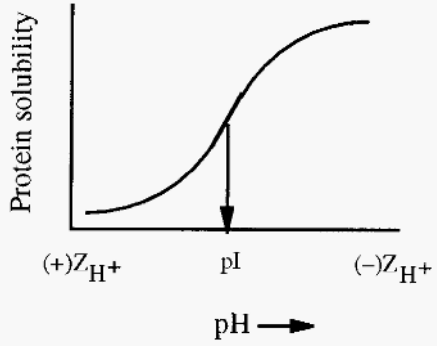


Figure 42. The effect of pH on the salting out of a protein by ammonium sulfate.

Sulfate can act as a pulling kosmotrope by virtue of its interaction with protein cationic sites. Consistent with this, the precipitation of proteins

is usually promoted at pH values below the pI (Fig. 42), where the protein has a maximal number of cationic sites[3]. Reinforcing its pulling effect is the fact that the sulfate ion is divalent, and so can bind to more than one cationic site at a time, and that it has a tetrahedral structure, with four oxygen atoms that can hydrogen bond to multiple sites on the protein.

Sulfate also acts as a pushing kosmotrope by virtue of its extraordinary hydration. By virtue of its hydration, the sulfate ion can act as a dehydrating agent and, in its hydrated form, as an exclusion-crowdingagent. The sulfate anion has 13 or 14 water molecules in its first hydration layer and possibly more in a second layer[4]. Consequently, in salting out at, say, 3 M ammonium sulfate, the sulfate anion will have accreted to itself 40 to 45 M out of the total of 55 M H_2O in neat water. In salting out, therefore, a large proportion of the water will be involved in hydrating the sulfate ions and increasing their effective radius. The large, hydrated, $[SO_4.(H_2O)n]^{2-}$ ions crowd and exclude the proteins, pushing them into tighter, more ordered (less soluble) conformations, with lower entropyí. The preferential accretion of the water molecules to the sulfate ions excludes the proteins from a proportion of the water (the proportion increasing with the salt concentration), ultimately bringing them to their solubility limit.

No other salt has the combination of properties which make ammonium sulfate so effective at salting out. Consequently, when the word ìsaltî is used in the context of salting out, it invariably means ammonium sulfate. Similarly, the term "ionic strength" is often used loosely, when what is really meant is ìthe concentration of ammonium sulfateî.

3.4.2 Empirical observations on protein salting out.

Starting from zero, increases in salt concentration initially increase the solubility ofthe proteins, due to salting in. With further increases in ammonium sulfate concentration, the protein solubility passes through a maximum and then decreases (Fig. 43).

The salting out relationship is described by an empirical equation[6];-

$$\text{Log } S = \beta - K_s I$$

where, S = protein solubility (g/l)
I = ionic strength
fl and K_S are constants.

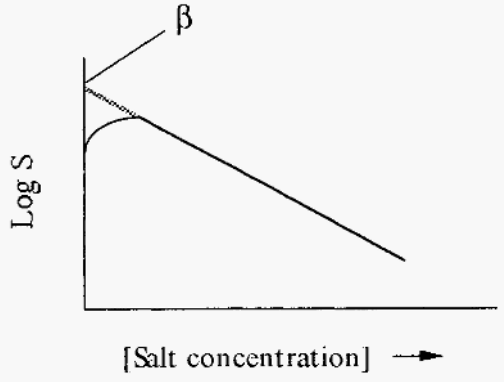


Figure 43. Solubility of a typical protein vs concentration of ammonium sulfate.

KS, the so-called "salting out constant" (the slope of the plot in Fig. 43), is essentially independent of temperature and pH but varies slightly with the nature of the protein. fl is markedly dependent upon the pH and temperature (Fig. 44) and also varies markedly with the nature of the protein.

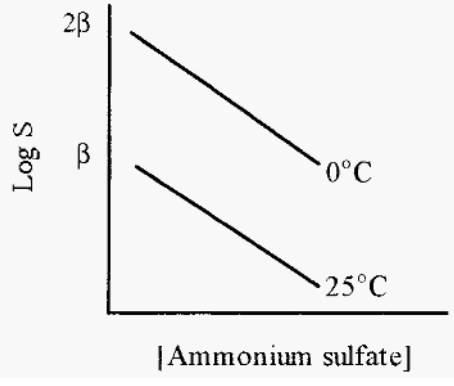


Figure 44. The effect of temperature on the salting out of carboxyhaemoglobin (adapted from ref. 7).

Note that a rise in temperature causes a decrease in fl. Note also that, since fl is inunits of log S, a unit change in fl represents a ten-fold change in solubility. Therefore, a protein will be markedly less soluble at higher temperatures and in practice it is better to conduct salting out at, say, 25 C rather than at 4 C. The sulfate ion is kosmotropic, so proteins are stabilised by the presence of $(NH_4)_2SO_4$ and a high salt concentration also

inhibits microbial growth. For these reasons, also, it is less necessary than usual to work at a low temperature.

The initial concentration of a protein in solution has a major influence on the amount of $(NH_4)_2SO_4$ required to precipitate it. Proteins appear to fall into two categories, denoted type I and type II[2], depending upon how their concentration affects their salting out behaviour. For type I proteins, each protein has a characteristic precipitation curve (e.g. Fig. 45).

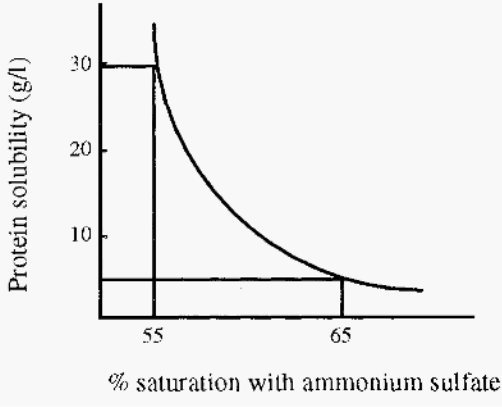


Figure 45. The salting out curve of carboxymyoglobin (adapted from ref. 7).

The lower the concentration of protein in solution, the more salt is required to precipitate it. In the example given in Fig. 45, if carboxymyoglobin is present at an initial concentration of 30 g/l, it will begin to precipitate at about 55% saturation with $(NH_4)_2SO_4$, whereas at an initial concentration of 4 g/l, 65% saturation is required to begin precipitation.

Not all proteins behave in this simple way. Type II proteins[2], such as BSA and α-chymotrypsin, precipitate to an extent dependent upon their initial concentration, i.e. such proteins manifest a family of precipitation curves, each curve arising from a particular initial protein concentration (Fig. 46).

Type I proteins have a single precipitation curve, regardless of the initial protein concentration. Type II proteins precipitate in a manner dependent upon their initial concentration.

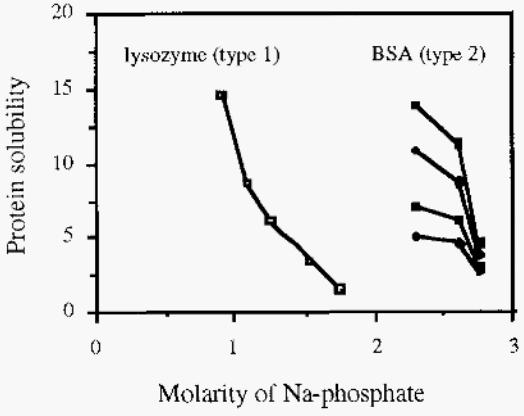


Figure 46. Differential salting out behaviour of type I and type II proteins. (Data reworked from Shih *et al.,* ref. 2).

Clearly, therefore, proteins do not precipitate between fixed and characteristic limits of ammonium sulfate concentration, as is implied in much of the older literature. Also, there is little point in repeating a precipitation, from the same volume and at the same ammonium sulfate saturation. Since the first precipitation will not have been quantitative, the concentration will be less if the protein is reconstituted in the same volume. To repeat the precipitation, the protein concentration should be readjusted to the same value as previously, which is not always practicable. In general, it is not worth repeating the precipitation as the cost, in terms of protein lost, is not justified by the increase in protein purity obtained.

Proteins may be purified from a mixture by differential precipitation at different saturations of ammonium sulfate (e.g. Fig. 47). The protein solubility curve (Fig. 47) has two steps, due to the precipitation of the serum albumin, followed by the carboxymyoglobin. Such perfect separation is rare, however, and it is more usual to obtain mixed fractions with, possibly, only slight enrichment of a desired protein. By altering the protein concentration it is sometimes possible to improve the separation, e.g. by diluting the solution, the points of precipitation (the peaks in the first derivative curve) will be moved to the right, to higher saturation levels. Due to differences in K_s, however, the peaks due to different proteins might move to different extents and the separation will thus be improved.

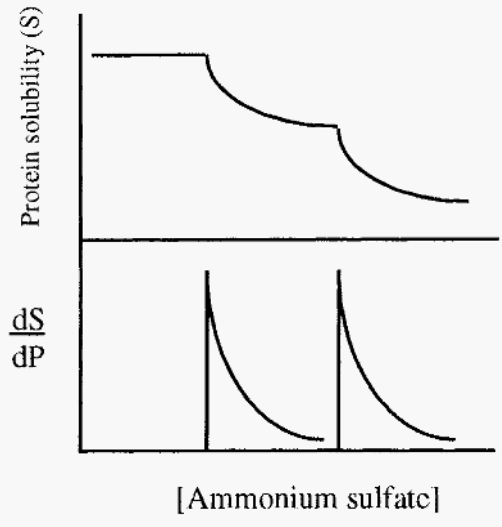


Figure 47. Separation of human serum albumin and carboxymyoglobin by salting out (from Dixon and Webb - ref. 7).

To summarise, in salting out the following can be manipulated;-

- *pH.* It is best to use a pH below the pI of the desired protein where precipitation is maximal.
- *Temperature.* Theoretically, the best temperature is the highest temperature at which the protein is stable. fl decreases as the temperature is increased and there is therefore greater precipitation at higher temperatures. For practical purposes, room temperature (25 C) is adequate.
- *Protein concentration.* The difficulty here is that the direction of any effects cannot be predicted in advance.

The resolving power of salting out is not high and so it is now commonly used mainly as a means of concentrating proteins from dilute extracts, while leaving non-protein molecules in solution. It is also generally used early in an isolation, immediately after preparation of the extract.

3.4.3 Three-phase partitioning (TPP)

Three-phase partitioning (TPP) is a method in which proteins arc salted out from a solution containing a mixture of water and t-butanol[3,8,9]. t-Butanol is infinitely miscible with water but upon addition

of sufficient ammonium sulfate the solution splits into two phases, an underlying aqueous phase and an overlying t-butanol phase. If protein was present in the initial solution, three phases would be formed, protein being precipitated in a third phase between the aqueous and t-butanol phases (Fig. 48). The amount and type of protein precipitated is dependent upon the ammonium sulfate concentration, as in conventional salting out. Unlike in conventional salting out, however, the protein precipitate is largely dehydrated and has a low salt content. Desalting before a subsequent ion-exchange step, which is a time-consuming necessity after conventional salting out, is therefore generally not necessary with TPP.

Conventional salting out is effected by adding $(NH_4)_2SO_4$ to a purely aqueous solution of protein. In this case the protein is initially hydrated and is thus soluble, and the addition of the salt, serves to dehydrate the protein and eventually brings it to its solubility limit.

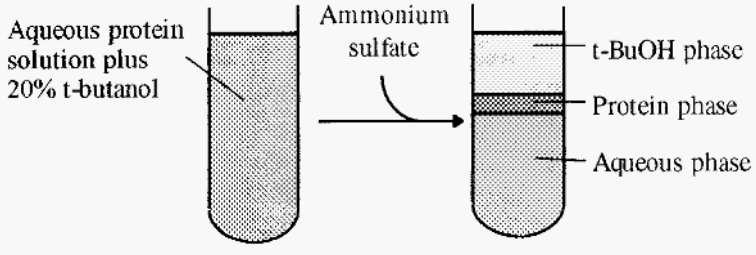


Figure 48. Three-phase partitioning.

In TPP, t-butanol may be first added to the aqueous solution of protein to about 20%. It is believed that this results in the protein equilibrating with the solvent (water) and the co-solvent (t-butanol). The protein thus becomes partially hydrated and partially t-butanolated, in proportion to the relative abundance of the solvents in the mixture.

Upon addition of $(NH_4)_2SO_4$, water is abstracted by the salt ions as these become hydrated. The salt apparently has a higher affinity for water than for t-butanol, and thus preferentially sequesters the water. In the absence of protein, this results in the solution dividing into two phases, as some of the water is made "unavailable" to the t-butanol. If protein is present, the protein equilibrates with the new proportions of solvent and co-solvent available to it. Upon addition of further $(NH_4)_2S0_4$, eventually the amount of water available to the protein becomes insufficient to keep the protein in solution, and it precipitates. At this point, however, the protein will be largely t-butanolated. This results in the protein having a reduced density and so, when it precipitates

with the situation in conventional salting out where the dehydrated protein normally sinks, indicating that it is *more* dense than the solution. In conventional salting out, as the concentration of $(NH_4)_2SO_4$ increases, the solution density increases and the difference in density between the solution and the precipitate decreases, until the point is reached where it is no longer possible to sediment the precipitate. In the case of TPP, however, the precipitate is *less* dense than the solution and so, with increasing $(NH_4)_2SO_4$ concentration, the precipitate floats more and more easily.

In non-aqueous environments, a-helices are favoured and so, during TPP, as the proportion of t-butanol increases, the protein conformation may become distorted as it acquires a greater proportion of a-helices. This distortion leads to the denaturation of many proteins, which may be adisadvantage. On the other hand, if the protein of interest is able to survive TPP, then it is likely that TPP will effect a purification by the denaturation of impurities, as well as by its fractionating ability. Selective denaturation of contaminating protein has been put to good effect in the isolation of cathepsin D[10] and a number of erythrocyte proteins[11], where the major problem was the presence of a large excess of haemoglobin.

To effect a fractionation of a protein mixture by TPP, about 20% t butanol can be added to the protein mixture in aqueous solution and increments of $(NH_4)_2SO_4$ are added, the interfacial precipitate being concentrated by centrifugation and removed for analysis after each increment of $(NH_4)_2SO_4$.

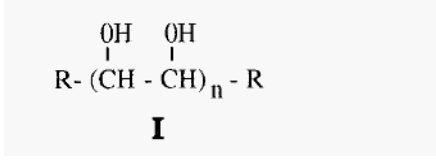
Empirically, it has been found that, in TPP;-

- Proteins precipitate in order of their molecular weight, i.e. larger proteins precipitate before smaller proteins, present at the same concentration.
- Proteins are most readily precipitated into the third phase when they have a positive charge,
- Proteins are most soluble, after TPP (i.e. denaturation is minimal), when TPP is done at the pI of the protein.
- The protein concentration has a marked influence: generally the greater the concentration, the more easily the protein will precipitate.
- Temperature has little effect, in the range 0 C to 25 C.

It should be noted that t-butanol is unusual in that it is an organic solvent which tends not to denature proteins. TPP can therefore be done at room temperature, which is fortunate because t-butanol solidifies at about 25 C, and is most conveniently used above this temperature.

3.5 Fractional precipitation with polyethylene glycol

Polyethylene glycol (PEG) (I) is a hydrophilic polymer. It is commercially available as preparations of different average molecular weights, the 6,000 and 20,000 materials being most often used for protein isolation.



I

It is thought that PEG precipitates proteins by virtue of excluding the proteins from a relatively large volume of water per PEG molecule. With an increase in PEG concentration the protein is thus eventually brought to its solubility limit. It has been empirically observed that, at equimolar concentrations, large proteins are precipitated at lower PEG concentrations than small proteins. For this reason PEG precipitation is especially suited to the isolation of large proteins and even particles, such as viruses. PEG-precipitation may thus be thought of as "molecular exclusion precipitation" [12].

The effect of protein concentration has not been explored in detail but it would appear from limited data that in PEG precipitation proteins behave much as type I proteins do in salting out, i.e. the higher the protein concentration, the less precipitant is required to initiate precipitation.

After precipitation, it is difficult to separate the PEG from the protein. PEG is acetone soluble, however, and acetone precipitation of the protein can be used to separate it from the PEG. It may not always be necessary to remove the PEG since many of the subsequent uses of the protein may be tolerant of the presence of PEG. It should be noted, though, that PEG can alter the behaviour of proteins during molecular exclusion chromatography [13].

A practical difficulty in the use of PEG is that it absorbs UV light at 280 nm, and also interferes with the Lowry protein assay. Protein can, however, be measured in the presence of PEG using the biuret assay.

3.6 Precipitation with organic solvents

Proteins are maintained in solution by the interaction of surface hydrophilic groups with the water solvent. Consequently, if the polarity of the solvent is reduced by the addition of an organic solvent less polar than water, the protein will tend to become less soluble. Concurrently,

the protein usually becomes less stable because a major contributor to protein stability is the distribution of hydrophilic and hydrophobic groups into the lowest energy conformation, based on the solvent being water. To minimise denaturation of the protein in the less polar solvent, it is usually necessary to conduct the fractionation at a low temperature.

Two solvents that have been commonly used in the fractional precipitation of proteins are ethanol and acetone. Ethanol is widely used in the low temperature fractionation of blood proteins[14], for example, while acetone was formerly commonly used to make "acetone powders", a means of preserving proteins.

To effect the precipitation of a protein with an organic solvent, the protein solution should be chilled close to 0 C and the solvent to at least -20 C. The solvent is slowly but smoothly added to the protein solution, with constant stirring to avoid the formation of high local concentrations of solvent. Occasionally, addition of the solvent leads to the formation of a milky colloidal suspension, rather than a precipitate. If this happens, addition of a drop of NaCl solution may be necessary to induce flocculation.

For the preparation of an "acetone powder", the protein precipitated with acetone is harvested by centrifugation and spread out to air dry. The latent heat of vaporisation of the acetone keeps the protein cool as it dries to a powder. Alternatively, the protein precipitate may be stored at low temperature and subsequently reconstituted by dissolving in chilled buffer solution. Some denaturation of the protein during organic solvent precipitation is unavoidable.

3.7 Dye precipitation

For the reaction:-

Protein in solution $\rightarrow$ Protein precipitate

most of the methods discussed above may be called "pushing" methods, in that the properties of **the solution** are changed, making these unsuitable for protein dissolution, i.e. the protein is ìpushedî out of solution. If the protein is present at a very low concentration, in a large volume of solution, "pushing" methods can be quite uneconomical as the amount of precipitant required is proportional to the total solution volume, and often inversely proportional to the protein concentration. The alternative is a "pulling" method, in which properties of **the protein** are changed so that it comes out of solution. The ìpush/pullî terminology is due to Dr Rex Lovrien, of the University of Minnesota.

An example of a pulling method is dye precipitation or, as it has been called, "matrix co-precipitation"[15]. Proteins are kept in solution by the disposition of charged, hydrophilic, groups on their surfaces. At low pH these are mainly positive and at high pH mainly negative. Dyes, on the other hand, are typically salts of strong acids or bases with attached aromatic groups having extended conjugation, which gives rise to their colour. If a dye having a negatively charged sulfonic acid group is added to a positively charged protein, ionic bonds will form between the dye and the protein. As a result, bulky, hydrophobic groups will become attached to the protein at its previously positive sites and the protein will be precipitated out of solution. An advantage of this method is that the amount of dye required is proportional to the moles of protein present, not to the volume of solution, so it is particularly suitable for harvesting proteins from dilute solutions.

After precipitation, it is necessary to separate the protein from the complexed dyestuff. This can be accomplished either by ion-exchange or by TPP. In TPP, the high salt concentration breaks the ionic bonds and the released dye is extracted into the t-butanol layer. Since dye precipitation is a ìpullingî method and TPP is a "pushing" method, the sequential application of these two could be described as a "pull-push" method.

An example of dye precipitation and a discussion of the mechanism is provided by Wu *et al.*[16]

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3.8 Chapter 3 study questions

1. What are the two major uses of freeze-drying?
2. What is meant by the "vapour pressure" of water?
3. Should a freeze drier have: a) short, wide-bore tubing, or, b) long, thin-bore tubing between the sample and the condenser? Explain.
4. A freeze-drier condenser usually operates at about -50 C. Is there any benefit in using a lower temperature? Explain.
5. What is a "vacuum"? and an "absolute vacuum"?
6. Imagine this situation. A freeze-drier is placed on top of a building 4 storeys high and switched on. A glass tube, connected to one of its ports reaches down into a beaker of water on the ground floor. Explain what you think would happen when the tap on that particular port is opened, so that the glass tube becomes evacuated.
7. Define "dialysis" and describe three factors that affect its rate.
8. Explain how dialysis can be used to concentrate a protein solution.
9. In what way does an ultrafiltration membrane differ from a dialysis membrane?
10. What is meant by "concentration polarisation"?
11. How is the flow rate of ultrafiltration affected by the applied pressure?
12. Why is ammonium sulfate a popular choice for salting out?
13. Describe the effects of, a) temperature, b) pH, and, c) protein concentration on the salting out of a protein:
14. Why do proteins float on the aqueous phase in TPP, yet sink in conventional salting out?
15. Describe one advantage of TPP over conventional salting out.

Chapter 4

Chromatography

Several of the methods discussed in the previous chapter - ultrafiltration, salting out and TPP - besides being concentrating methods, can also be used for preparative fractionation. Similarly, ion-exchange chromatography can be used to concentrate a dilute solution. The division of the chapters between concentrating and fractionating methods is therefore somewhat arbitrary, but is based on whether a given method is more effective in concentrating or in fractionating.

The essence of preparative fractionations, as distinct from the analytical fractionations to be discussed in the following chapters, is that they are non-destructive and the product is an active protein. Also, preparative fractionations are usually done on a larger scale than analytical fractionations, but the scale is very dependent upon the particular problem being addressed.

After concentration of the extract by one of the methods discussed in Chapter 3, it is assayed for activity and analysed, for example by polyacrylamide gel electrophoresis (see Chapter 5). As mentioned in Chapter 1, in the absence of any other information regarding the protein, experience suggests that ion-exchange chromatography is the best method to use for the first preparative chromatography step, since ion-exchange columns have a large sample capacity and a good resolving power. Molecular exclusion chromatography is usually best reserved for later in the procedure since its sample capacity and resolving power are both relatively limited.

4.1 Principles of chromatography

The word "chromatography" means "writing with colour" and refers to the early observations on the separation of dyes by paper chromatography. All chromatographic separations depend upon the differential partition of solutes between two phases, a mobile phase and a stationary phaseí. Such partition between two phases is described by the so-called partition coefficient or distribution coefficient.

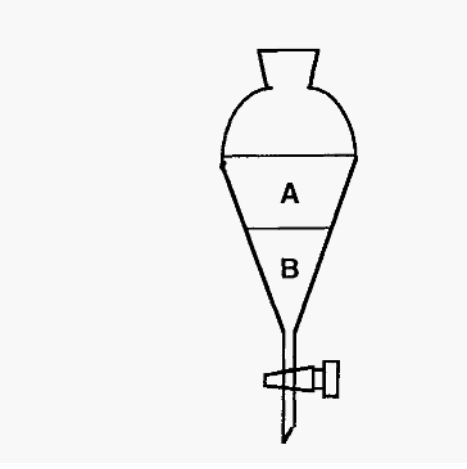


Figure 49. Distribution of a solute between phases in a separating funnel.

Students may recall from Chemistry classes how a dyestuff, for example, will distribute itself between two non-miscible liquid phases in a separating funnel. For any two solvents at a constant temperature, the distribution coefficient (K_d) is a constant and can be defined as:-

$$K_d = \frac{\text{concentration of solute in A}}{\text{concentration of solute in B}}$$

The distribution of a solute is not limited to two liquid phases and the distribution coefficient may describe the distribution between any two phases, such as liquid/solid or gas/liquid phases. In chromatography there is always a distribution between two such phases, one kept stationary while the other - the mobile phase - flows over or through it. The stationary phase can therefore be a solid, a liquid, or a solid coated with a liquid. Since it must be fluid, the mobile phase must be either a gas or a liquid. The mechanism of distribution may not always be simple partition, as in a separating funnel. Examples of the different forms of chromatography are shown in Table 2.

Table 2. Forms of chromatography

Stationary phase	Mobile phase	Distribution mechanism	Name
solid	liquid	adsorption	Adsorption chromatog.
liquid	liquid	partition	Paper chromatography, Counter-current distribution
solid	liquid	ion-exchange	Ion-exchange chromatog.
liquid (in gel)	liquid	molecular exclusion	MEC
immobilised biomolecule	liquid	bio-affinity	Affinity chromatography
liquid	gas	partition	GLC

In the case of chromatography, the distribution coefficient is defined as:-

$$K_d = \frac{\text{concentration of solute in stationary phase}}{\text{concentration of solute in mobile phase}}$$

$$= \frac{\text{wt solute in stationary phase/volume of stationary phase}}{\text{wt solute in mobile phase/volume of mobile phase}}$$

$$= \frac{\text{wt in stationary phase}}{\text{wt in mobile phase}} \; x \; \frac{\text{volume of mobile phase}}{\text{volume of stationary phase}}$$

$$= kfl$$

Where k is called the partition ratio (or capacity ratio) and fl is the phase ratio.

In chromatography the stationary phase is typically packed into a tubular column and the mobile phase flows through the packed column. There is continual equilibration of solutes between the mobile and stationary phases, and that length of column where there is effectively one equilibration - such as would occur in a separating funnel - is called a "theoretical plate". This terminology is derived from fractional distillation of volatile solvents. Since chromatography columns are usually vertically orientated, the length of column in which one equilibration effectively occurs is called the "height equivalent to a theoretical plate", abbreviated HETP. The HETP is more of a concept than a reality, however, because equilibration is actually continuous, not discrete.

The principle of chromatography may be considered by imagining the column to consist of a stack of theoretical plates and, for clarity, the mobile phase may be considered on one side and the stationary phase on the other (Fig. 50). A column packed with a bead-form stationary phase (A) may be considered as consisting of a vertical stack of theoretical plates (B) in each of which an equilibration between the mobile phase and the stationary phase takes place. Subsequent movement of the mobile phase will displace the mobile "half" of each equilibrated pair downwards, forming new pairs, initially not in equilibrium, but which will equilibrate before, in turn, being displaced.

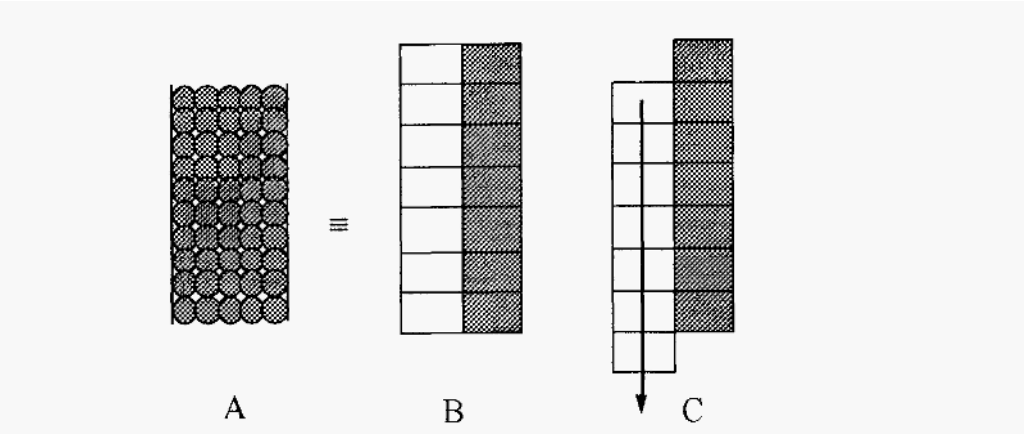


Figure 50. A representation of the mechanism of chromatography.

This representation can be used to illustrate the principle of chromatography, as in the tutorial exercise shown in Fig. 51.

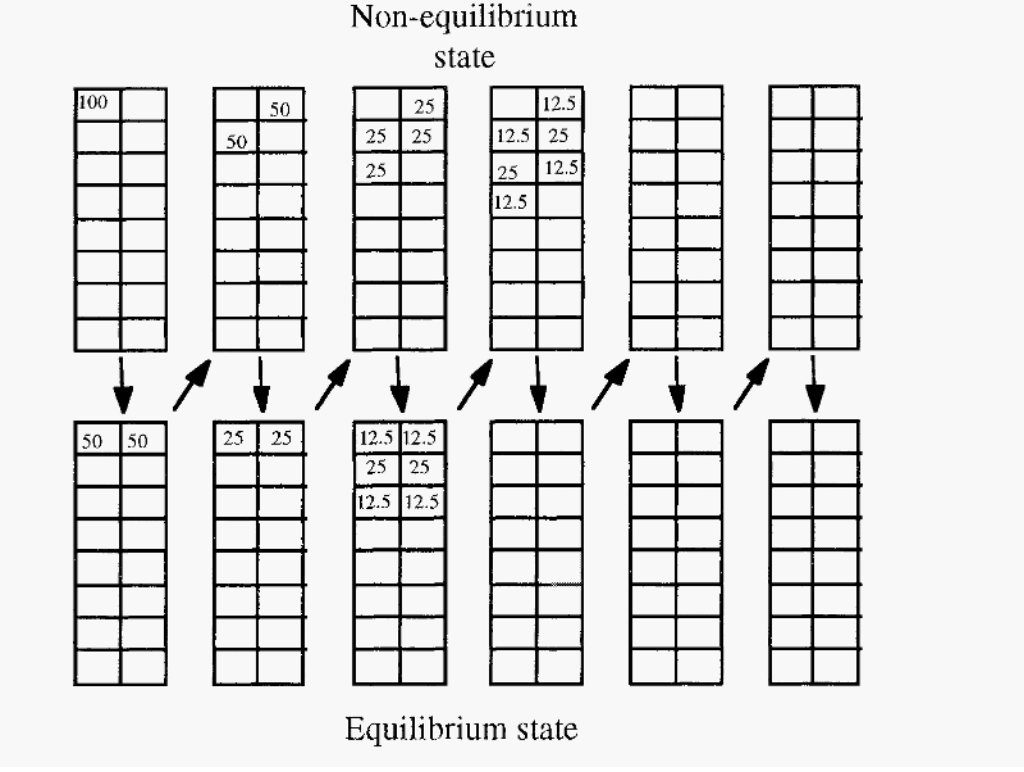


Figure 51. A tutorial illustrating the principle of chromatography.

One hundred units of solute arc injected into the mobile phase of the column (Fig. 51, top left). This then equilibrates with the stationary phase (bottom left) - assume a partition ratio of 1 in this case. Movement of the mobile phase carries the solute downwards to a new area of the column (top, second from left), where equilibrium again occurs (bottom, second from left). To see if you have grasped the concept. try to fill in the remainder of the numbers, until the right hand columns are filled in. Note the movement of the "peak", relative to the mobile phase, and note how the peak spreads out.

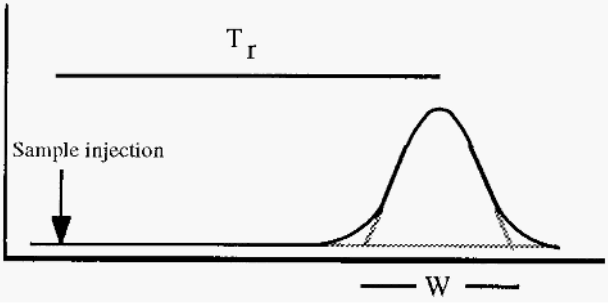


Figure 52. Illustration of retention time (T,) and peak width (W).

The number of theoretical plates (N) in a column is given by the equation:-

$$N = a\left(\frac{T_r}{W}\right)^2 \qquad 4.1$$

Where Tr = retention time
W = peak width, measured as shown in Fig. 52.
a = a method-dependent constant.

Dividing the length of the packed column bed by the number of theoretical plates gives the HETP. Note that the larger the value of N, the smaller the HETP value and the more efficient the column. For a given retention time, equation 4.1 indicates that an efficient column (where N is large) will give peaks of smaller width than an inefficient column.

Resolution of peaks.

The resolution (R), which describes how well any two peaks are separated, is described by the equation:-

$$R = \frac{T_{r2} - T_{r1}}{\left(\frac{w_2 + w_1}{2}\right)}$$

From this it will be seen that the narrower the peak (i.e. the higher the value of N), the better the resolution will be.

The magnitude of the HETP, which should be as small as possible, is influenced by:

- the particle size of the stationary phase, and,
- the flow rate of the mobile phase.

4.1.1 The effect of particle size

Reconsider the equilibration of a solute between two phases in a separating funnel. How quickly equilibrium is achieved will depend upon:-

- diffusion across the boundary between A and B, which is proportional to the surface area of the boundary, and,
- diffusion within A and B to the boundary; the time taken depending upon the distance from the boundary.

For a minimal time to equilibrium, therefore, the boundary surface area should be maximised and the distance of any part of the solutions, A and B, from the boundary should be minimised. This could be achieved by using a separating funnel of unusual design as shown in Fig. 53.

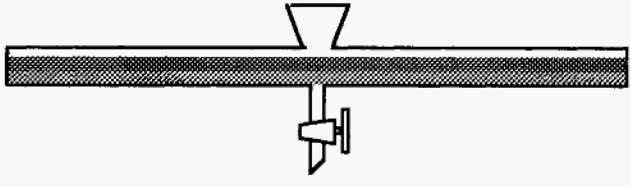


Figure 53. A hypothetical separating funnel for rapid equilibration.

However, the more conventional way of speeding up the attainment of equilibrium is by shaking the separating funnel, so that the solutions

are well mixed. The two phases remain separate but one solution will be dispersed in the other, usually in the form of small spheres.

The volume of a single sphere is given by:-

$$\text{Volume} = \frac{4}{3}\pi r^3 \qquad 4.2$$

If the total volume of the dispersed phase is "V", then V will be dispersed into "N" spheres, where:-

$$N = \frac{3}{4\pi} \cdot \frac{V}{r^3} \qquad 4.3$$

i.e. the number of spheres is inversely proportional to r^3.

The surface area of a single sphere is given by:-

$$\text{Surface area} = 4\pi r^{\hat{2}} \qquad 4.4$$

Therefore, surface area of ìNî spheres

$$= 4\pi r^2 \cdot \frac{3}{4\pi} \cdot \frac{V}{r^3}$$

$$= \frac{3V}{r} \qquad 4.5$$

i.e. as the radius of the spheres "r" gets smaller, the total surface area gets larger.

Since the surface area constitutes the interface between the phases, a small value of "r" will ensure a maximal interface area and rapid equilibration. Also, in a sphere, the greatest distance that a solute molecule can be from the surface is "r", the radius of the sphere. Therefore, to minimise the diffusion distance and the time to equilibrium, "r" should be minimal. The largest possible distance to the surface for a molecule outside of the packing material also decreases as ìrî decreases.

Shaking a separating funnel vigorously is an effective way of making small spheres and hence of rapidly equilibrating the phases. Similarly, for rapid equilibration, the best size for the spherical particles of a chromatography resin is "as small as possible". For even packing and good flow characteristics, the resin particles should also be of uniform size (see equation 2.20).

As "r" decreases, however, the total surface area increases and so the resistance to the flow of the mobile phase also increases. Very small particles, therefore, dictate the use of high pressure pumps - hence HPLC (high pressure liquid chromatography).

4.1.2 The effect of the mobile phase flow rate

The effect of the flow rate of the mobile phase is expressed by the so-called Van Deemter equation[2]:-

$$HETP = A + \frac{B}{F} + CF \qquad 4.6$$

Where, HETP = height equivalent to a theoretical plate
F = mobile phase flow rate
A = eddy diffusion (independent of F)
B = molecular diffusion (increases as F decreases)
C = resistance to mass transfer (i.e. smearing) (increases as F increases)

An "eddy" is a swirl in a liquid. "Eddy" diffusion refers to the fact that the mobile phase has to follow a tortuous path around the resin particles, inevitably resulting in some mixing and consequent dilution of a solute peak.

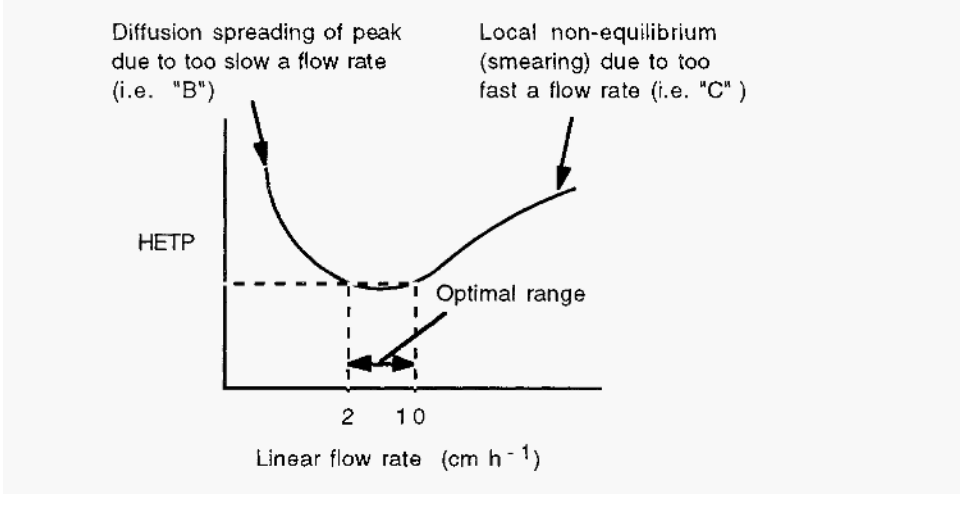


Figure 54. A schematic plot of the van Deemter equation.

If there was no flow in the column, the solute peak would spread with time due to diffusion of the solute molecules, from an area where they are in high concentration to an area where their concentration is less. Similarly, when the flow rate is too slow, the peak will have ample time to spread due to diffusion. On the other hand, if the flow is too fast, solute molecules in the mobile phase will pass the stationary phase without properly equilibrating with it, resulting in peak broadening due to "smearing". The optimal linear flow rate for typical low-pressure, molecular exclusion chromatography lies in the range of 2-10 cm h^{-1}.

4.1.2.1 The relationship between linear and volumetric flow rates.

The easiest way of measuring the mobile phase flow rate is to collect the effluent stream in a measuring cylinder and measure the amount collected in a given time interval. The result will be the volumetric flow rate, which can be expressed in ml h^{-1}. For the chromatographic process, however, the important point is not the volumetric flow rate *perse* but how fast the mobile phase flows past the stationary phase.

A momentís reflection will reveal that a given volumetric flow rate will give very different chromatographic conditions in a thin column compared to a fat column as the mobile phase will flow past the stationary phase faster in the thin column than in the fat column. To make the chromatographic conditions the same in columns of any diameter, the flow rate can be expressed as the linear flow rate, with units of cm h^{-1}.

The relationship between the volumetric and linear flow rates is given by the equation:-

$$\text{Volumetric flow rate} = \pi r^2 \times \text{linear flow rate} \qquad 4.7$$

As an example of the utility of the concept of linear flow rate, imagine that you have developed a successful chromatographic separation, using a column of 2.5 cm i.d. and a volumetric flow rate of 50ml h^{-1}. You then move to another lab to apply your separation method, as a temporary visitor, but you find that the new lab only has columns of 2.0 cm i.d. What can you do about this?

First calculate the length "ℓ" that 50 ml would occupy in the 2.5 cm column:-

$$50 = \pi r^2 \ell$$

Therefore, $\ell = \frac{50}{\pi r^2}$

Hence, $\ell = \frac{50}{\pi \times 1.25^2}$

$= 10.186 \text{ cm}$

The linear flow rate in the *2.5* cm i.d. column is thus 10.186 cm h^{-1}. From this, calculate the Volumetric flow ("x") in the 2.0 cm i.d. column that would give the same linear flow rate, i.e.;-

$x = \pi r^2 \times 10.186$

And for $r = 1 \text{ cm},$

$x = 32 \text{ ml } h^{-1}$

Thus the required volumetric flow rate in the 2.0 cm column is *32* cm h^{-1}. The 2.0 cm i.d. column could be operated at the same length as the 2.5 cm i.d. column, but at the reduced volumetric flow rate of 32 cm h^{-1}.

4.2 Equipment required for low pressure liquid chromatography

4.2.1 The column

A *sine qua non* for column chromatography is the column. Basically this consists of a glass tube with adapters - preferably at either end - to spread the liquid flow from the thin bore input tubing out to the relatively large bore of the column and back in to the thin bore of the output tubing (Fig. 55). The column packing is supported on a sieve of some sort and a key element in the efficiency of the column is the "dead" volume between this sieve and the output tubing, which should be as small as possible. The purpose of the column is to effect a separation of different types of solute molecules and the whole purpose is defeated if the separated molecules arc allowed to remix, due to the dead volume being too large.

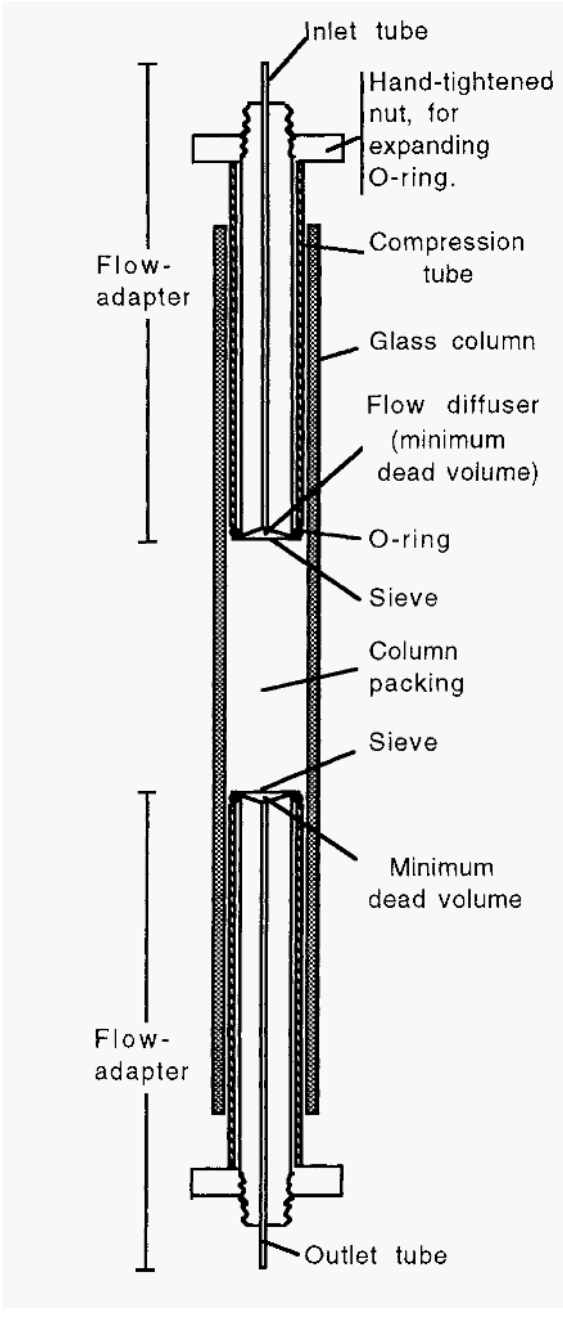


Figure 55. Schematic cross-section of a chromatography column.

Moveable flow-adapters enable different column bed volumes to beused. It is useful to have such flow adapters on both ends of the column. After packing and equilibration of the column bed, the adapter on the inlet side can be adjusted to be in contact with the upper surface of the packed resin bed. This is advantageous in that it facilitates sample application, as the sample can be simply introduced through the inlet tubing. It is also necessary if any form of gradient elution is to be used. In the absence of an upper flow adapter, the incoming buffer will mix in the dead volume above the packed resin bed and it will be impossible to get smooth, reproducible, gradient conditions.

The ratio between the internal diameter (i.d.) of the column and its length, the so-called "aspect ratio", differs depending upon the application ofthe column. Generally, where adsorption occurs, such as in ion-exchange or affinity chromatography, columns with an aspect ratio of *ca.* 1:10 or less are used, whereas for molecular exclusion chromatography aspect ratios of about 1:50 are used.

Low pressure liquid chromatography columns typically consist of a uniform bore, thick walled, glass tube, with plastic adapters etc. Glass is favoured because it is chemically stable, transparent, and has good

thermal conductivity. Obviously, all materials used to make columns must be chemically stable to buffers etc. and must not react with sample proteins. Proteins do adsorb to glass to some extent and this can be prevented by silanising the column before use, though this is only warranted for the most critical work.

4.2.2 Moving the mobile phase

The chromatographic process requires movement of the mobile phase and the simplest way of effecting this is by siphoning the buffer from a reservoir which is elevated above the end of the outlet tube from the column (Fig. 56).

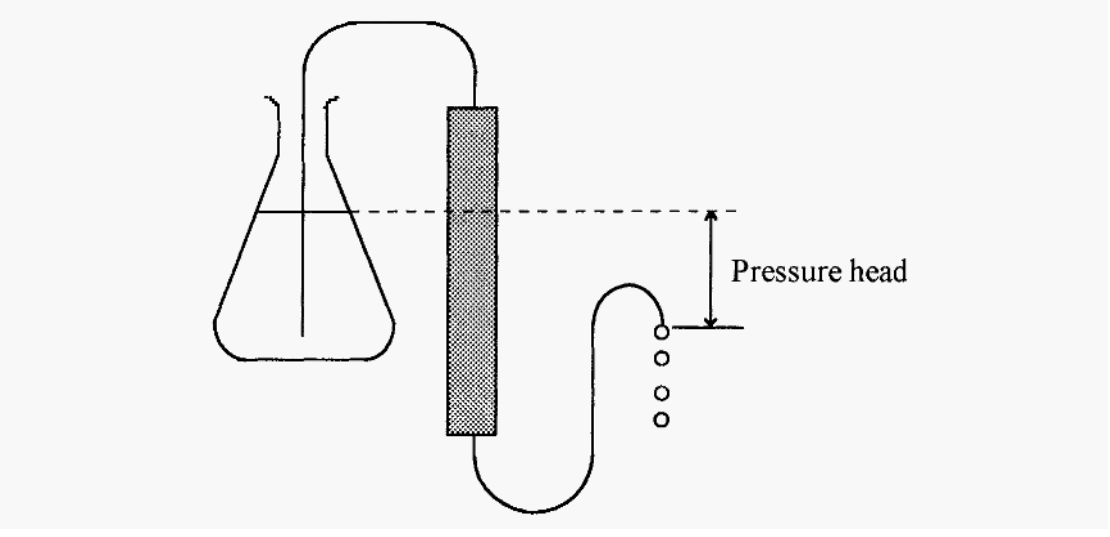


Figure 56. Simple chromatography, using a siphon to generate the mobile-phase flow.

The difference in potential energy (height) between the surface of the liquid in the buffer reservoir and the end of the outlet tube constitutes the "pressure head" which will cause the mobile phase to flow. A problem with this simple set-up is that, as the level of liquid in the reservoir drops, the pressure head will get smaller and the flow rate will decline. To keep the pressure head constant a so-called ìMarriotte flaskî may be used (Fig. 57).

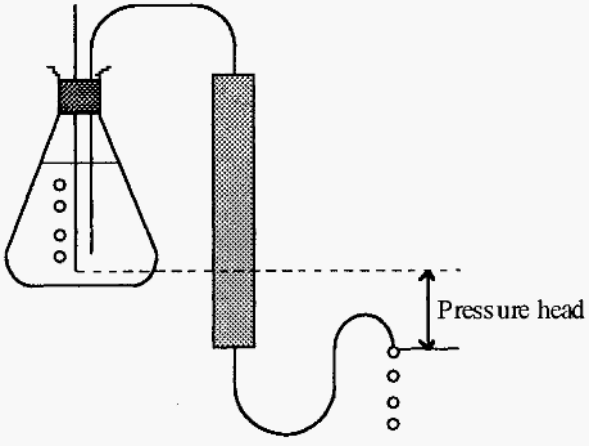


Figure 57. Use of a Mariotte flask to maintain a constant pressure head.

Low-pressure column chromatography occurs at a somewhat leisurely pace and it is too tedious to attend to the column the whole time it is running. On the other hand, using the simple set-ups shown in Figs 56 and 57, there is a danger that an unattended column might exhaust the buffer supply and run dry. If this happens it becomes necessary to remove the resin and repack the column, which is tedious. Gravity-flow columns can be protected against running dry by arranging the inlet tubing to loop down below the outlet from the outlet tubing (Fig. 58).

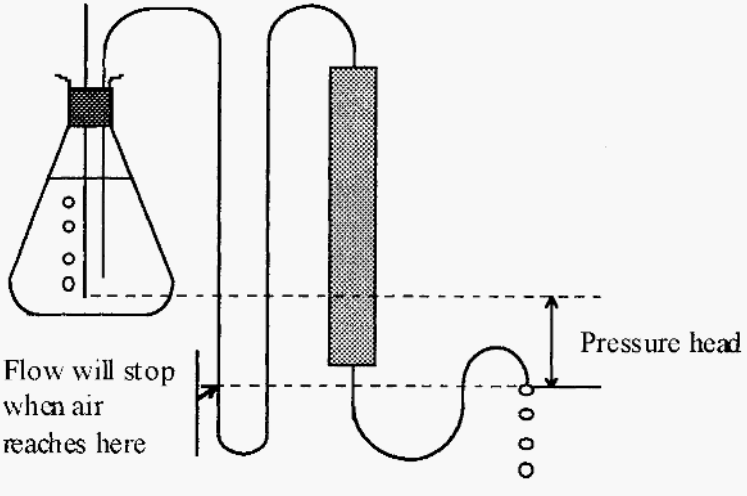


Figure 58. A run-dry protection loop on a gravity-flow column.

Note that gravity-flow columns can be operated with the flow going either downwards (Figs 56→58) or upwards through the column (Fig 59). An advantage of upward flow is that it is easier to arrange the system so that it will not run dry. Upwards flow is recommended with very soft gels, such as Sephadex G-200, which otherwise tend to be crushed by the combined effects of gravity and the buffer flow. With ascending flow, the flow supports some of the weight of the gel.

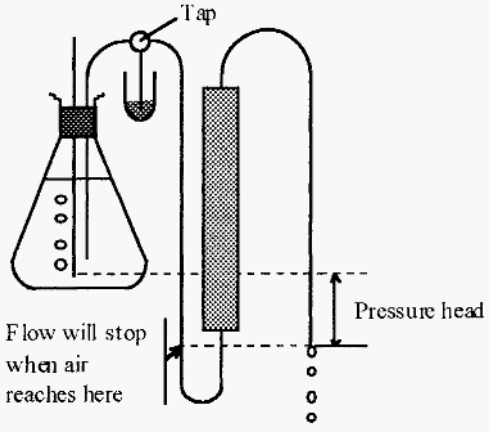


Figure 59. Ascending flow, with run-dry protection, and a tap for sample application.

With a gravity flow system the sample is most easily applied using a three-way tap on the inlet side of the column, as shown in Fig. 59.

Better than a Marriotte flask, if the budget allows, is a peristaltic pump. "Peristalsis" refers to the rhythmic, wave-like, contractions that pump the gut contents along the digestive tract. By analogy, a peristaltic pump is one in which a flexible silicone tube is pinched by a roller which runs along the tube, pumping the tube contents in the same direction as it does so (Fig. 60). A peristaltic pump gives a smooth, almost pulse-free, flow.

The advantage in using a pump is that it gives more precise flow control and greater freedom in the chromatography lay-out, i.e. the buffer reservoir does not have to be higher than the column outlet. To prevent the column running dry during unattended operation when using a peristaltic pump, a timer switch is required. The timer can be arranged to switch off the pump - and any other associated apparatus - after a pre-set time. Note that because the peristaltic pump rollers pinch the silicone tube, there can be no flow of liquid through the pump when it is switched off.

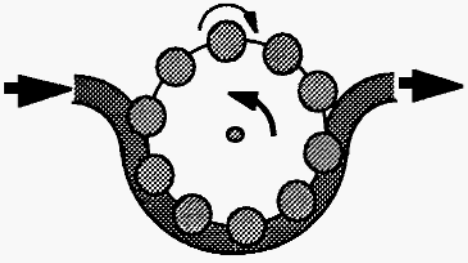


Figure 60. Schematic drawing of a peristaltic pump.

When using a peristaltic pump, the sample can be applied simply by stopping the pump, transferring the inlet tubing from the buffer reservoir to the sample container, restarting the pump until all of the sample is sucked up, wiping the tubing and returning it to the buffer reservoir.

4.2.3 Monitoring the effluent and collecting fractions.

The purpose of column chromatography is to separate solute molecules and it follows that some means is required of monitoring the separation achieved, and of separately collecting the resolved solute molecules. The separated fractions are most easily collected using an automatic fraction collector. Fraction collectors are available in different versions that collect the effluent stream in fractions on the basis of time, volume, or number of drops. Time-based fraction collectors are the simplest and most economical and, if the mobile phase flow rate is accurately controlled with a pump, the fractions collected will be of equal volume.

The simplest means of monitoring the separation of proteins is to collect the column effluent for the whole run in a convenient number of fractions - say, 100 - and to measure the A^{280} of each fraction in a spectrophotometer. The results can be used to construct a so-called ìelution profileî, in which A_{280} is plotted against the elution volume.

Such manual reading of the elution profile is inexpensive in capital terms, but it consumes operator time and, perhaps more importantly, some detailed information is lost. A better way, again if the budget can afford it, is to use a flow-through UV-monitor, that continuously reads the absorbance of the effluent stream at 280 nm. Such a monitor is plumbed into the effluent line, between the column and the fraction collector. Besides a power source, it requires two other electrical connections; an output to a recorder and an event-marker connection between the fraction collector and the recorder. Each time the faction

collector changes tubes, it sends a pulse to the recorder so that the event - the tube change - is recorded. In this way it becomes possible to subsequently correlate the recorder trace of A_{280} with the collected fractions, so that fractions corresponding to the required peaks can be harvested. A flow-through UV-monitor constitutes a time-saving automatic system which also captures the fine detail of the elution profile.

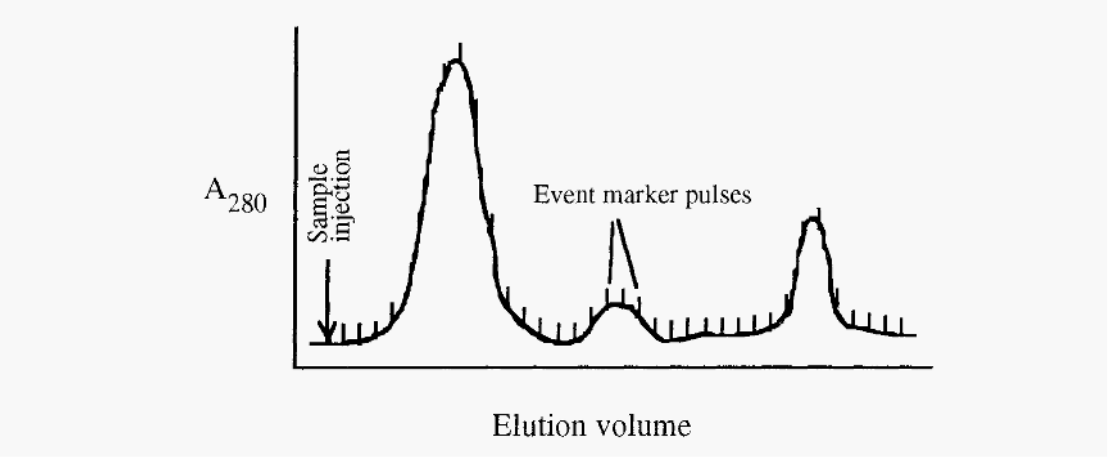


Figure 61. A typical elution profile of A_{280} vs elution volume with event marker pulses.

4.2.4 Refrigeration

Proteins are structurally labile and are susceptible to microbial degradation. For these reasons, wherever possible, protein solutions are maintained at low temperature and preservatives are added to the buffers. Denaturation and degradation are both minimised by keeping the proteins cold and protein separations are therefore usually carried out at about 4 C.

Chromatography is usually done in coldrooms, but working in coldrooms is miserable and unhealthy. A better and more versatile system is to have a refrigerated cabinet with some components of the chromatography set-up being kept at 4 C and others at room temperature (Fig. 62). In Fig. 62, items labelled on the left are within the cabinet at 4 C and those labelled on the right are at room temperature. Electrical apparatus kept in a coldroom or fridge can be damaged by condensation of moisture onto its circuits. For this reason it is best to keep as much as possible at room temperature. The fraction collector must, however, be kept in the fridge.

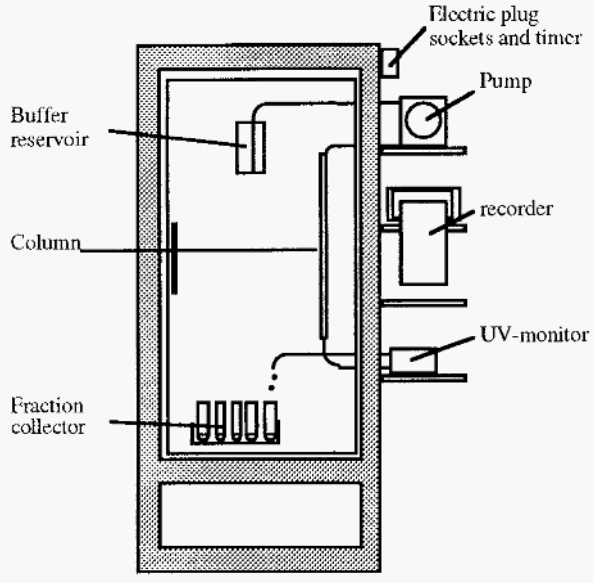


Figure 62. A protein chromatography system in a glass-fronted, refrigerated cabinet.

Remember if any electrical item is ever removed from the fridge for servicing, it must be allowed to warm up to room temperature and all moisture must be allowed to dry off before it is switched on. **If** this is not done, short-circuits caused by condensed moisture may burn out the electronics.

4.3 Ion-exchange chromatography (IEC)

Consider the situation where a single anion type, say CH_3COO-, and a number of cation types, say Na+, K+ and Protein+, exist together in an aqueous solution, where the aqueous component will additionally contribute OH- and H+ ions. These ions will establish a dynamic equilibrium, comprised of a number of sub-equilibria, e.g.

$$CH_3COO^- + Na^+ \leftrightarrow CH_3COONa$$

$$CH_3COO^- + K^+ \leftrightarrow CH_3COOK$$

$$CH_3COO^- + Protein^+ \leftrightarrow CH_3COOProtein$$

$$CH_3COO^- + H^+ \leftrightarrow CH_3COOH$$

The overall equilibrium condition will be determined by the values of the respective dissociation constants:-

$$K_{(CH_3COONa)} = \frac{[CH_3COO^-].[Na^+]}{[CH_3COOK]}$$

$$K_{(CH_3COOK)} = \frac{[CH_3COO^-].[K^+]}{[CH_3COOK]}$$

$$K_{(CH_3COOProtein)} = \frac{[CH_3COO^-].[Protein^+]}{[CH_3COOProtein]}$$

$$K_{(CH_3COOH)} = \frac{[CH_3COO^-].[H^+]}{[CH_3COOH]}$$

The overall equilibrium state is a result of-

- intrinsic affinities between ions (expressed in the dissociation constants), and,
- competition between ions (a function of the relative concentrations of the ions).

To develop a chromatography system, the CH_3COO^- ion, in the form of a carboxymethyl group ($-CH_2COO^-$), could be covalently attached to the stationary phase and the dissociated cations allowed to move with the mobile phase: net electrical neutrality being maintained, however. With an immobilised anion, this would constitute a cation exchange system Conversely, immobilisation of a cation would constitute an anion exchange system.

If the above ions were applied to the chromatography system as a sample "plug" and the system was subsequently eluted with a buffer, say lithium citrate, the differential affinities of the ions for the stationary anion would be manifest as differential rates of migration through the column. The ions would migrate at relative rates proportional to their respective dissociation constants. Their manifest affinity, and thus their absolute rates of migration, would depend upon the *competition* that they encountered from the buffer cation, Li+ in this example. With increasing Li+ concentration, the sample cations would face increasing competition

in their association with the immobilised anion and so would be increasingly dissociated, resulting in an increase in their rate of migration through the column. In the case of proteins, the dissociation constant is affected by pH, so elution can also be effected by a change in pH.

4.3.1 Ion-exchange "resins"

The term "resin" comes from early polystyrene-based ion-exchangers which had a translucent yellow appearance, like the resin exudates from pine trees. The term has stuck, although modern ion-exchangers used for protein separations are generally opaque and white.

All ion-exchange resins are comprised of a matrix to which are attached ionic substituent groups. For low pressure chromatography of proteins, the matrix is often comprised of a hydrophilic biopolymer, such as cellulose, Sephadex™, or agarose. These materials cannot withstand high pressures and for medium to high pressure liquid chromatography, the trend is towards silica-based resins, or synthetics such as Trisacryl™.

Cellulose is a polymer of ß-D-glucose units, linked with $1\rightarrow4$ bonds. It is relatively inexpensive and provides good flow properties, but large interstitial spaces lead to relatively poor resolution. Sephadex consists of dextran chains, comprised of $1\rightarrow6$ linked dextrose (glucose) residues, cross-linked with epichlorhydrin (sec Fig. 73). The name "Sephadex" is a contraction of the words "separating", "Pharmacia" and "dextran". It is sold in the form of dry xerogels which absorb water and swell into hydrated spherical particles. Substituted Sephadex ion-exchangers give good resolution but they are subject to marked volume changes with changes in buffer ionic strength. This is a disadvantage as it is difficult to apply an accurate salt gradient to a shrinking gel, and it may become necessary to re-pack the column after only a few runs.

Agarose is the neutral polysaccharide component of agar, an extract of kelp, which is a type of seaweed. It is a linear polysaccharide composed of alternating residues of D-galactose and 3,6-anhydro-L galactose, linked by $1\rightarrow4$ and $1\rightarrow3$ bonds.

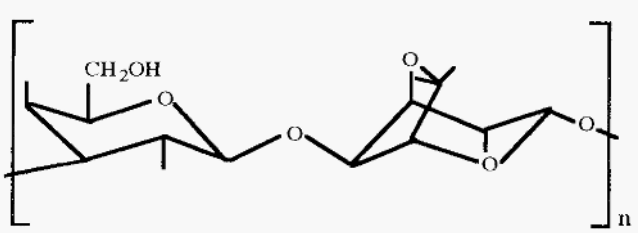


Figure 63. The structure of agarose.

Agarose is freely soluble in water at 100°C and upon cooling forms an exceptionally strong, so-called macroreticular gel, with large pores (Fig. 64).

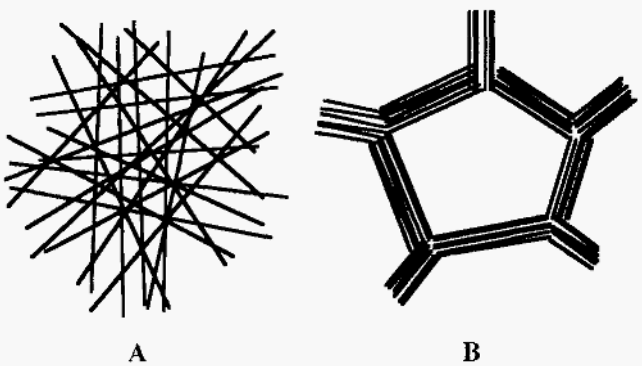


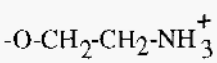
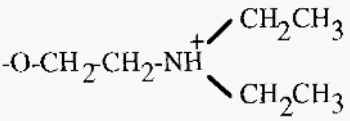
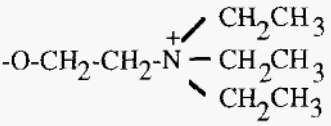
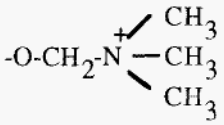
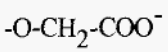
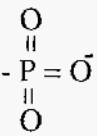
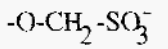
Figure 64. Comparison of micro- and macroreticular gels.

Sephadex (Fig. 64A) is an example of a microreticular gel. In a macroreticular gel (Fig. 64B), e.g. agarose, the gel fibres align into bundles resulting in a much stronger gel and a larger pore size at a given gel concentration. The sketch in Fig. 64 is a 2-D representation, but gels actually form 3-D labyrinths.

The macroreticular structure of agarose makes it very suitable as a matrix for ion-exchangers as proteins have easy access to the gel interior, so that in effect the gel has a very large surface area to which ionic substituent groups may be attached. The macroreticular structure is also mechanically strong, so that substituted agarose ion-exchangers do not shrink or swell with changes in buffer ionic strength. The gel structure of agarose is maintained by non-covalent bonds and agarose gels cannot be dried and reconstituted. They are consequently supplied in the form of a slurry. They also cannot be boiled or autoclaved, as they simply melt to a sol at high temperatures.

Common substituent groups are shown in Table 3. The common weak base anion exchanger group is DEAE- and the common weak acid cation exchanger group is CM-. The pH range over which these groups are ionised is shown in Fig. 65.

Table 3. Some common ion-exchange substituent groups.

Designation	Ionisable group	Exchanger
Aminoethyl- (AE-)		Anion
Diethylaminoethyl- (DEAE-) (Weakly basic)		Anion
Triethylaminoethyl- (TEAE-)		Anion
Trimethylaminomethyl- (Q-) (Strongly basic)		Anion
Carboxymethyl- (CM-) (Weakly acidic)		Cation
Phospho- (P-)		Cation
Sulfomethyl- (S-) (Strongly acidic)		Cation

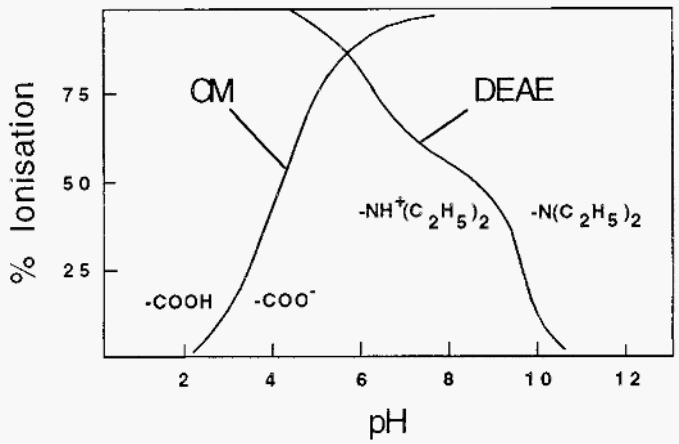


Figure 65. Ionisation characteristics of CM- and DEAE- substituent groups.

It can be seen from Fig. 65 that exchangers comprised of weak acid or weak base substituent groups are not completely ionised at most pH values of interest and, perhaps a greater drawback, the degree of ionisation changes with pH in the useful pH range. Exchangers based on strong acids or bases, by contrast, are Completely ionised over a much larger pH range, so their degree of ionisation is less subject to change with pH (Fig. 66).

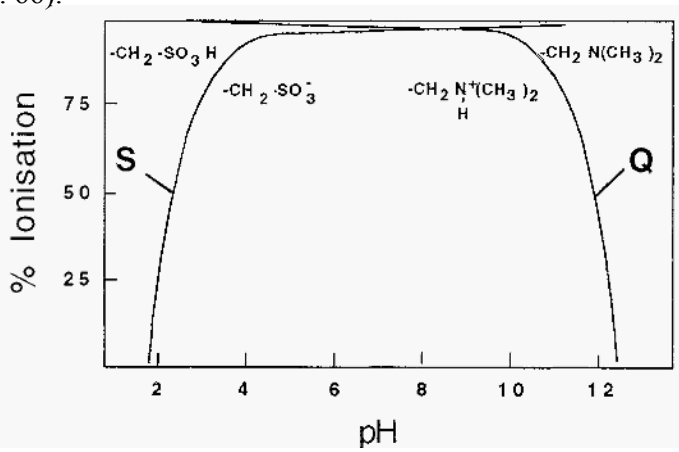


Figure 66. Ionisation of strong acid and strong base ion-exchange substituent groups.

4.3.2 Gradient generators

Ion-exchange chromatography often requires elution of the bound proteins by a change in either ionic strength, pH, or both. If the system

being separated is well characterised, then appropriate stepwise changes can be made, An example is in the ion-exchange separation of amino acids in an amino acid analyser. More commonly, in protein isolation the exact characteristics of the components being separated are unknown and it is then necessary to use a gradient generator to effect an ionic strength or pH gradient.

Gradients are commonly generated in one of two ways. A two-chamber device, with a magnetic stirrer stirring one chamber, may be used. Fig. 22 (p38) illustrates one such device, but an even simpler arrangement is to have two conical flasks with a siphon arranged between them (Fig. 67).

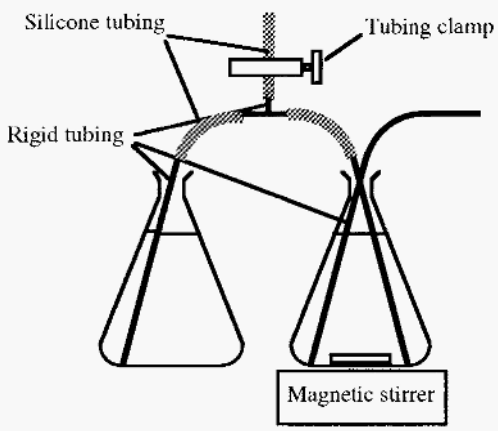


Figure 67. A simple gradient generator set-up.

The starting solution is placed in the right hand vessel and the finishing solution in the left hand vessel. A siphon is established between the vessels by sucking solution up and clamping the T-piece side tubing. The rigid tubing can consist of flexible tubing inserted inside of, for e.g., plastic disposable pipettes.

A more sophisticated, but more expensive, method of generating a gradient is to use a micro-processor controlled proportioning valve which draws liquid alternately from one vessel and then the other, in small amounts which gradually change in proportion with time. A mixer is placed in the line, downstream of the proportioning valve, to change the small stepwise changes in buffer composition into a gradual and continuous change. Gradient generators based on proportioning valves are common components of complete chromatography systems, commercially available from a number of manufacturers.

4.3.3 Choosing the pH

One of the decisions that has to be made before conducting ion-exchange chromatography is what pH to use. The selection of pH and of the type of ion-exchanger to use may be facilitated by establishing the so-called titration curves of the proteins in the mixture to be separated. An electrophoretic titration curve can be determined by establishing a pH gradient in a gel (see Section 5.10) and conducting an electrophoretic separation (see Section 5.8) at right-angles to the pH gradient (Fig. 68).

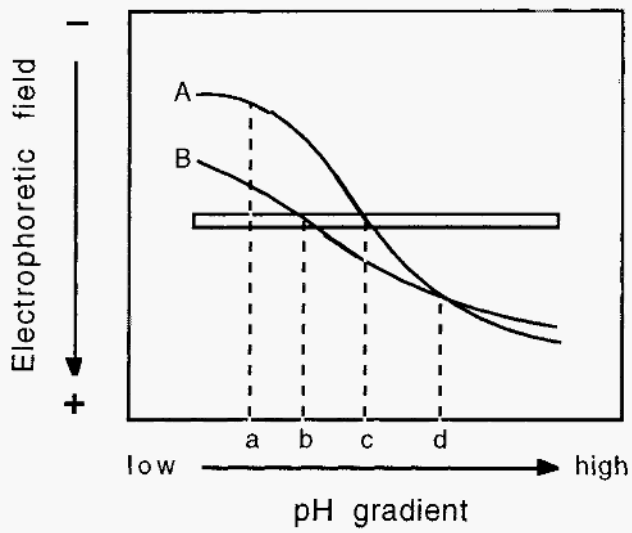


Figure 68. Electrophoretic titration curves of proteins.

In Fig. 68, pH "a" is the pH of maximal charge difference between proteins A and B. At this relatively low pH, both proteins have a positive charge and a cathodic migration. This information suggests that cation-exchange chromatography, conducted at pH "a", would most likely effect the best separation between proteins A and B. By contrast, at pH "d" the proteins have no apparent charge difference. This implies that anion-exchange chromatography would be less successful at resolving A and B, especially at pH "d". Points "b" and "c" represent the pI values of proteins B and A, respectively. The difference in these pI values gives an indication of the separation which may be expected from isoelectric focusing (Section 5.10) or chromatofocusing (Section 4.4).

4.3.4 An ion-exchange chromatography run

The choice of the type of exchanger, cation- or anion-exchanger, may be arbitrary, in the absence of any knowledge of the characteristics of the protein of interest, e.g. its pI and/or its pH stability range. As a first approach, it is generally best to choose a pH, within the protein's stability range, where it will adsorb to the ion-exchanger. Usually, this means that an anion-exchanger should be used at pH values above the pI of the protein and a cation-exchanger at a pI below the pI. (It must be realised, however, that the pI refers to an overall property of the protein, whereas binding to an ion-exchanger is a function of the charge on one surface of the protein. It is therefore possible to have a protein bind to an anion-exchanger at a pH below its pI or to a cation-exchanger at a pH above its pI. It becomes a matter of experimentally exploring the behaviour of each new unknown protein.)

With the resin chosen and the column packed, it is necessary to equilibrate the column with several column volumes ("colvols") of starting buffer, a buffer of low ionic strength and of a pH which will promote binding of the protein of interest to the resin. The sample protein mixture must contain a low salt concentration, achieved by equilibrating it with the starting buffer; either by dialysis, ultrafiltration, molecular exclusion chromatography or, following TPP, by simply re-dissolving the precipitate in the starting buffer.

The sample solution is applied to the column and chased with at least 2 colvols of starting buffer in order to elute the unbound fraction. The A_{280} may be monitored during this process and elution with starting buffer stopped once the A_{280} returns to the baseline. At this point a buffer gradient may be applied. As a first approach, a gradient of increasing ionic strength is the best choice, and is applicable to both cation- and anion-exchange.

The resolution of peaks is a function of the steepness of the eluting gradient. A shallow gradient gives better resolution, but takes more time, so a trade-off must be made. With an unknown system, the best first approach is to use a steep gradient, as this gives a quick assessment of the number of peaks to be expected, and the separation can subsequently be optimised. A suitably steep gradient for a first approach is $0 \rightarrow 1$ M NaCl in starting buffer, in 3 colvols. For subsequent optimisation the gradient limits or the number of colvols can be altered, to change the gradient slope.

It must be appreciated that the gradient is applied to the inlet side of the column, whereas monitoring of the effluent is done on the outlet side. There is thus a colvol difference between the influent and effluent

streams, Consequently, after application of the gradient, it is necessary to elute with at least one colvol of finishing buffer to ensure that the whole gradient itself is eluted and that all peaks which would be eluted by the gradient are, in fact, washed from the column. An example of the reporting of an ion-exchange chromatography run is presented in Fig. 69.

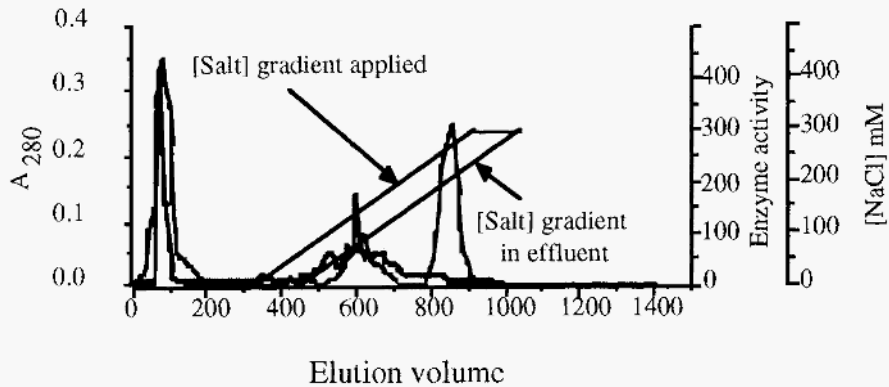


Figure 69. An example of ion-exchange chromatography: Purification of cathepsin S, from a TPP fraction from bovine spleen, by chromatography on S-Sepharose.
Column, 2.5 an i.d. x 17 cm; Starting buffer, 20 inM Na-acetate containing 1 mM EDTA and 0.02% NaN3, pH 5.0; Gradient, 0→300 mM NaCl in 600 ml. (Note that the column is eluted with 1 column volume of finishing buffer after the gradient is applied, in order to elute proteins which may have been displaced by the buffer but not yet eluted from the column.) The thick line indicates cathepsin S activity and the thin line is A_{280}. (The apparent activity in the break-through peak reflects the fact that the assay is not absolutely specific for cathepsin S).

S-Sepharose is a cation exchanger. If an anion exchanger were used, elution with an ionic strength gradient could be effected in exactly the same way. A different strategy is needed for cation and anion exchangers, however, if a pH gradient is used to elute the bound proteins. In the case of a cation exchanger, a gradient of rising pH is required, whereas with an anion exchanger a gradient of descending pH is required.

After completion of the chromatography run, with either a cation or anion exchanger, firmly bound sample components may be eluted with a high salt concentration, such as 1 M NaCl. In the case of cation exchangers, high salt concentration may be combined with a high pH, and with anion exchangers the high salt may be combined with a low pH. The extreme pH values used must, of course, be within the stability limits of the ion-exchanger resin. Finally, the column is re-equilibrated with several colvols of starting buffer, in preparation for the next run.

Ion-exchange chromatography thus requires cycling through large changes in ionic strength and possibly also of pH. As mentioned previously (Section 4.1.3.1) it is an advantage if the resin can withstand the required ionic strength and pH changes, without shrinking or swelling, as this makes it possible to cycle through the changes in buffer composition without having to repack the column.

4.4 Chromatofocusing

A technique which is related to ion-exchange chromatography, but which separates on a different principle, is chromatofocusing[3-6]. In chromatofocusing, use is made of the buffering capacity of the ion-exchange substituent groups, themselves. The column is equilibrated with starting buffer, the sample applied, and immediately the finishing buffer is applied. Displacement of the starting buffer by the finishing buffer generates a moving pH gradient in the column. Proteins which fall behind their pI on this gradient will no longer bind to the column and will be swept along faster than the pH gradient. Proteins which move ahead of the pH gradient, by contrast, will bind strongly to the column and will be immobilised until overtaken by the pH gradient. The net result is that proteins will be eluted from the column at their respective pI values.

In practice it is found that simple displacement of one buffer with another in a conventional exchanger causes too sharp a change in pH. A shallower pH gradient, more suitable for chromatofocusing separations, can be generated by using ampholytes as the eluting buffer, and a substituent group, such as polyethyleneimine, which titrates over a larger pH range. Ampholytes are mixtures of randomly substituted poly amino-poly carboxylic acids. They are also used in isoelectric focusing and in isotachophoresis. This requirement for ampholytes makes chromato-focusing more expensive than normal ion-exchange chromatography.

4.5 Molecular exclusion chromatography (MEC)

As shown in Fig. 64, gels are comprised of a large volume of water immobilised by a relatively small volume of hydrophilic polymer fibres, arranged in a randomly ramified 3-D network. Covalent or non-covalent cross-links between the fibres make the gel insoluble.

In molecular exclusion chromatography, the gel is arranged in the form of small, uniformly-sized spheres ("beads") which are suspended in buffer and packed into a column. In this situation the aqueous solvent may be considered in two parts; that within the gel spheres, which is held stationary, and that between the gel spheres which is free to move.

Fig. 70 is a 2-D representation of part of a gel structure and shows that a smaller particle "A" has access to a larger volume of the immobilised water. as indicated by the shaded area in the left hand figure, compared to that in the right hand figure. The shaded area indicates the accessible water while the inaccessible water is the unshaded area bounded by the lines representing the gel fibres. The proportion of the stationary phase to which a solute molecule has access is thus inversely proportional to its size, i.e. smaller solute molecules (A) can "get at" more of the water within the gel beads, whereas larger particles (B) are able to "get at" less of the immobilised water. Expressed the other way around, we may say that the larger molecules are "excluded" from a larger proportion of the immobilised solvent, and this gives the technique its name.

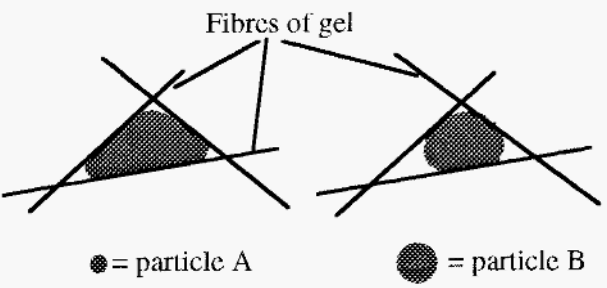


Figure 70. Gel volumes accessible to proteins of different sizes.

The differential access of differently-sized solute molecules to the stationary phase is reflected in differences in their distribution coefficients, and consequently in their chromatographic behaviour. Large molecules, which have little access to the stationary phase (reflected in a small value of the distribution coefficient), will elute before smaller molecules which have greater access to the stationary phase (reflected in larger distribution coefficient values), and which will be relatively retarded. The order of elution from a molecular exclusion column is therefore in decreasing order of molecular weight.

Fig. 71 shows a representation of the different volumes which contribute to the total bed volume of a molecular exclusion column. The total volume (V_t) is made up of the void volume (V_o), which is the space between the gel beads, plus the volume of the stationary phase (V_s), which is the immobilised water within the gel beads, plus the volume of the gel-forming polymer strands (V_g).

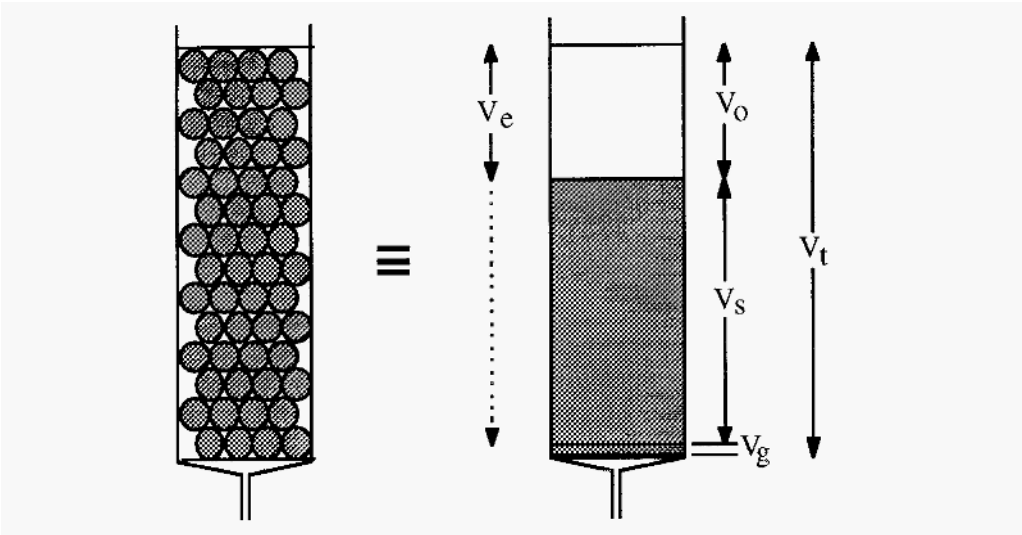


Figure 71. The volumes comprising the total volume of a molecular exclusion column. V_t is the total column volume, V_s is the volume of the water immobilised within the gel beads, V_o is the volume of the mobile water between the gel beads, V_g is the volume of the gel polymer strands and V_e is the elution volume of a solute. Depending upon the size of the solute, V_e may vary between V_o and $V_o + V_s$.

The distribution coefficient (K_d) is given by the equation:-

$$K_d = \frac{V_e - V_o}{V_s}$$

Where, V_e = elution volume
V_o = void volume (volume of the mobile phase, which is the liquid between the gel spheres)
V_s = volume of the stationary phase.

K_d has the limits of 0 (when $V_e = V_o$), and 1 (when $V_e = V_o + V_s$). A practical problem with the use of K_d, is that it is difficult to measure V_s values and so an alternative, the "availability constant", K_{av}, is more commonlyused. K_{av} is an approximation of K_d, because in defining K_{av} it is assumed that V_g. the volume of the gel-forming polymer strands, is negligible. K_{av} is defined as the fraction of the stationary phase which is available to a given solute, and is described by the equation:-

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

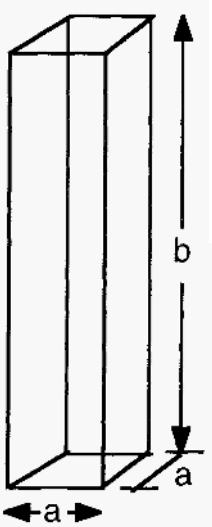
where, V_e = elution volume
V_o = void volume
V_t = total column volume.

For all practical purposes, however, K_{av} may be considered the same as K_d: it has the same limits of 0 (when $V_e = V_o$, i.e. for large molecules) and 1 (when $V_e = V_t$, i.e. for small molecules).

Note that all molecules should elute between the limits of V_o and V_t. This is a limitation of MEC which distinguishes it from IEF, for example, where the elution volume can be varied greatly and can extend many fold greater than the column volume.

Since V_t is fixed by the dimensions of the packed column bed, it may be imagined that, in MEC, a greater volume for separation of peaks could be created by reducing V_o. Could this be achieved by reducing the size of the gel beads?

4.5.1 The effect of gel sphere size on V_o



Consider a column of square cross-section with a side "a" and length "b", filled with spheres of radius "r", closely packed in uniform layers. On average, ignoring edge effects, which are small if "r" is small compared to the column dimensions, the number of spheres (N_S) in one layer is given by:-

$$N_S = \left(\frac{a}{2r}\right)^2 \qquad 4.8$$

The vertical separation between layers (d) is:-

$$d = \sqrt{\frac{2}{3}}.2r \qquad 4.9$$

Therefore, the number of layers (N_l) is;-

$$N_l = \frac{b}{\sqrt{\frac{2}{3}}.2r} \qquad 4.10$$

From equations 4.8 and 4.10, the number of spheres in the column (N_{tot}) is given by:-

$$N_{tot} = \frac{b}{\sqrt{\frac{2}{3}}.2r}.\left(\frac{a}{2r}\right)^2$$

$$= \frac{ba^2}{8r^3\sqrt{\frac{2}{3}}} \qquad 4.11$$

The volume occupied by N_{tot} spheres (V_{sp}) is:-

$$V_{sp} = \frac{ba^2}{8r^3\sqrt{\frac{2}{3}}}.\frac{4}{3}\pi r^3$$

$$= ba^2.\left(\frac{4\pi}{8.3.\sqrt{\frac{2}{3}}}\right)$$

$$= 0.6413\ ba^2$$

Now, $V_t = V_o + V_{sp}$

Therefore, $V_o = V_t - V_{sp}$

i.e. $V_o = ba^2 - 0.6413\ ba^2$

Hence, $\mathbf{V_o = 0.36V_t}$ 4.12

Equation 4.12 implies that the void volume is independent of the size of the gel beads, if these are of uniform size, and is proportional only to the total column volume, being just over one-third of V_t. This may strike one as a surprising, and perhaps counter-intuitive, result!

It follows from Equation 4.12 that in MEC the phase ratio (see p73), which is the volume of the mobile phase (V_o) divided by the volume of the stationary phase ($V_t - V_o$), is:-

$$\frac{V_o}{V_t - V_o} = \frac{0.36\,V_t}{0.64\,V_t} \approx 0.56$$

Uniformly sized spheres are desirable, because these pack evenly under gravity (see Equation 2.20, p36), and small spheres are desirable because these give the fastest equilibrations between the mobile and stationary phases (see Equation 4.5, p77). This raises the question of how the required small, uniformly sized, gel beads may be made.

4.5.2 The manufacture of small, uniform, gel spheres

Certain non-polar liquids, such as petroleum ether, are not miscible with water but are less dense than water and will tend to float as a separate phase on top of an aqueous phase. Other non-polar liquids, such as chloroform, are also not miscible with water but are more dense and will separate out as a phase beneath the aqueous phase. It follows that there must exist some mixture of petroleum ether and chloroform which has a density exactly equal to that of the aqueous phase (containing gel-forming polymers). If an aqueous solution is dispersed into such an organic solvent mixture, it would form spherical "droplets" of aqueous phase, suspended in the organic phase'. The suspended droplets can be made smaller by vigorous agitation of the solution, and by the addition of an emulsifying agent, which will stabilise the individual droplets and prevent them from coalescing into larger spheres. If a gelling reaction is induced while the aqueous phase is dispersed as small, spherical, droplets this will result in the formation of small, spherical gel particles. The beads formed may be subsequently size-fractionated by sedimentation through a column of water.

4.5.3 Determination of MW by MEC

The factor having the greatest influence upon the V_e and K_{av} of a protein subjected to MEC is the size (i.e. the radius, r) of the molecule which, in the case of globular (roughly spherical) molecules, is related to the molecular weight as follows:-

$$MW = \frac{4}{3}\pi . r^3 \rho$$

Therefore,

$$r \propto \sqrt[3]{MW}.$$

Many attempts have been made to establish relationships between elution volume and molecular weight and to construct calibration curves relating these two factors. Some of these are based upon theoretical models and some are empirical.

An early attempt was that of Laurent and Killander[8], who used a model in which a gel is visualised as being comprised of a random network of rigid fibres. The space between the rigid gel fibres, available to spherical particles, was calculated as a function of the sphere radius. A plot of;

$$\sqrt{-\ln K_{av}} \quad vs \quad \sqrt[3]{MW}$$

gave a sigmoidally-shaped curve. The middle part of this curve was approximately linear and a curve constructed with proteins of known molecular weight could be used to estimate the molecular weights of unknown proteins. Hjerten[9] has used a thermodynamic model to arrive at a plot not too dissimilar to that of Laurent and Killander. Hjerten's plot is;

$$-\ln K_{av} \quad vs \quad (MW)^{\frac{2}{3}}.$$

Andrews[10] has proposed an empirical plot of;

$$V_e \quad vs \quad \log MW.$$

Again, this gives a sigmoidal curve, with an approximately linear centre section. Although it lacks a theoretical basis, the Andrews plot has the over-riding merit of simplicity. A limitation, however, is that by plotting V, values it is the particular column that is standardised. If that column runs dry or is re-packed, it becomes necessary to construct a new standard curve. This limitation of the Andrews plot has been addressed by Fischer11 who has proposed plotting;

$$K_{av} \quad vs \quad \text{Log MW}$$

Using the Fischer plot it is the particular *gel* that is standardised, not the column, so the standard curve established with one column can be

applied to another column packed with the same gel. In view of its simplicity and versatility, the Fischer plot (Fig. 72) has become the most commonly used method of estimating MWs from MEC data.

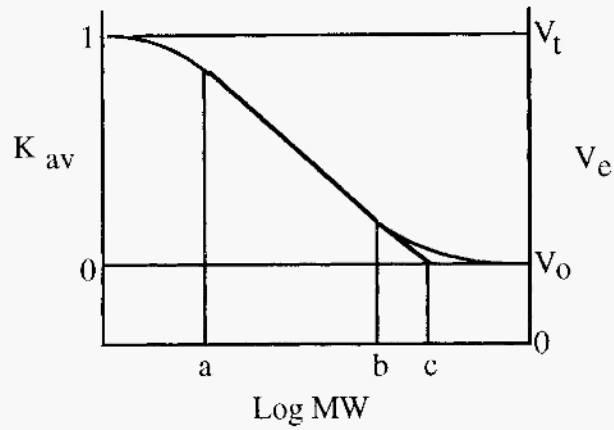


Figure 72. A Fischer plot, relating K_{av} to Log MW

In Fig. 72, the range "a" to "b" represents the effective separating range of the gel. Point "c" gives the MW corresponding to the exclusion limit of the gel.

4.5.4 Gels used in MEC

A number of different types of gels, made by different manufacturers, are commercially available. In each case the gel is comprised of a network of cross-linked hydrophilic polymers. The nature of the polymer and the type of the cross-link affect the properties of the gel, especially the non-sieving interactions with the sample proteins. These interactions may or may not improve a particular separation and the only way to find out is to try the different gels with the proteins one is trying to separate.

A type of gel which has been popular almost since the advent of MEC is Sephadex, a product of Pharmacia Biotech, which consists of dextran (1→6 linked polymer of glucose), crosslinked with epichlorhydrin (Fig. 73).

Different porosities of Sephadex gels are prepared by varying the degree of cross-linking. The different gels are designated by G-numbers, viz. G-10→G-200. Sephadex G-10 has the smallest pore size and the smallest water regain value, i.e. the ml water taken up per gram of xerogel, and Sephadex G-200 has the largest pores and the largest water

regain. Because it consists of a large volume of water immobilised by a relatively small amount of gel, Sephadex G-200 is relatively soft and is best used with ascending elution.

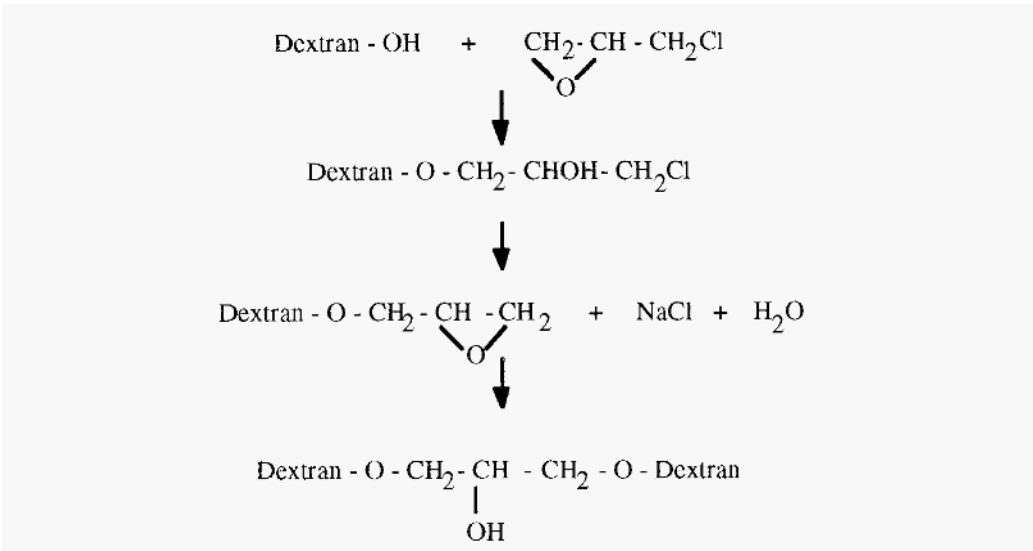


Figure 73. The cross-liking of dextran chains to form Sephadex gel.

The cross-links appear to have some hydrophobic character because Sephadex gels will bind small, hydrophobic, molecules with an affinity that is proportional to the degree of cross-linking, i.e. hydrophobic binding is more marked with Sephadex gels with a small G-number. Tryptophan, for example, emerges from Sephadex G-100 at a volume greater than V_t, and dyes generally bind tightly to Sephadex G-25.

Advantages of Sephadex are that it is stable over the pH range 2-12, it is biologically inert and, being a permanent gel it is autoclavable, i.e. it does not melt to a sol upon heating. **Disadvantages** are the hydrophobic interaction mentioned above and the fact that, being constituted of biopolymers, Sephadex is biodegradable and is thus subject to microbial degradation. It is thus necessary to include preservatives in the buffers used with Sephadex gels.

PDX, a product of Polydex Biologicals Inc., is also comprised of cross-linked dextran polymers, in bead form. PDX GF 25 separates in the range 1→5 kDa and PDX GF *50* in the range 1.5→30 kDa.

Bio-Gel® is the trade name of Bio-Rad Laboratories of California, for a range of gels for molecular exclusion chromatography. Bio-Gels are produced by co-polymerizing acrylamide (I) with N,N'methylenc bisacrylamide (II) to form a cross-linked polyacrylamide.

I $CH_2 = CH - CONH_2$

II $CH_2 = CH - \underset{\underset{O}{\|}}{C} - NH - CH_2 - NH - \underset{\underset{O}{\|}}{C} - CH = CH_2$

Again, the porosity is varied by varying the proportion of cross-linking agent (N, N', methylene bisacrylamide), resulting in a range of gels denoted Bio-Gel P-2, P-10, P-20, P-30, P-100, etc. Here the numbers refer approximately to the exclusion limit x 10^{-3}; thus Bio-Gel P-20 has an exclusion limit of about 20 kDa. The "exclusion limit" refers to the molecular weight of a globular protein which, theoretically, is just completely excluded from the gel (see Fig. 72). This is, of course-conceptually equivalent to the molecular weight of a globular protein that is just **not** excluded from the gel.

Bio-Gel is chemically inert and is a permanent gel and so can be autoclaved. It also binds some compounds by a hydrophobic mechanism. Bio-Gel, however, is not comprised of biopolymers and so is less subject to microbial degradation.

Agarose is a linear polysaccharide comprised of alternating residues of D–galactose and 3,6-anhydro-L-galactose. Agarose itself forms reversible gels, which melt to the sol at high temperature. The melting and gelling temperatures are different - an example of hysteresis - and commercial grades of agarose are available with high or low melting and gelling temperatures. Agarose in bead form, suitable for MEC, is available under the trade names of Sepharose (Phamiacia Biotech) and Bio-Gel A (Bio-Rad Laboratories), among others, Because of its macroreticular gel structure, agarose is suitable for fractionating molecules or complexes of very large molecular weight (>500 kDa), e.g. virus particles. Also because of their macroreticular structure, agarose gels have exceptional mechanical strength and are resistant to compaction. The pore size of agarose gels can be varied by varying the gel concentration.

A cross-linked form of agarose, with markedly increased thermal and chemical stability, has been described[12] and is commercially available. Although the chemistry involved in the cross-linking reactions is different, the final cross-link structure is the same as that in epichlorhydrin cross-linked Sephadex (see Fig. 73).

The mechanical properties of agarose make it especially suitable as a medium for chromatography. However, its fractionating range is too high for most purposes. A logical development, then, would be to make a

composite of agarose, which forms a macroreticular structure, with another gel which forms a microreticular structure. Such a composite should have the strength of agarose, and the separating properties of the microreticulargel. This concept has been realised in the Superdex range ofgels from Pharmacia Biotech. Superdex consists ofhighly cross-linked agarose beads, to which dextran is covalently bonded. The dextran chains determine the separating properties of the composite, while the agarose provides excellent strength.

Table 4 Some commercially available media for MEC

Gel	Polymer	Cross-linking	Fractionation range(kDa)
Sephadex	dextran	epichlorhydrin	
G-25			1→5
G-50			1.5→30
G-75			3→70
G-100			4→150
G-150			5→300
G-200			5→500
PDX	dextran		
G.F.			1→5
G.F.			1.5→30
Bio-Gel	polyacylamide	bis-acylamide	
P-2			0.1→1.8
P-4			0.8→4
P-6			1→6
P-10			1.5→20
P-30			2.5→40
P-60			3→60
P-100			5→100
Sephacryl HR	dextran	bis-acylamide	
S-100			1→100
S-200			5→250
S-300			0→1,500
S-400			0→8,000
S-500			)→20,000
Trisacryl Plus	N-tris[hydroxymethyl] methyl methacrylamide		
GF2-M			1→15
GF4-M			5→25

Sephacryl High Resolution (HR), a product of Pharmacia Biotech, is a composite gel of a different sort. It consists of allyl dextran (the constituent polymer of Sephadex) cross-linked with N, N'-methylene bisacrylamide (the cross-linking agent of acrylamide gels) to form a gel with high mechanical strength. The porosity of the gel is determined by the concentration of the dextran. Gels having five different porosities are available, denoted Sephacryl S-100 HR $\rightarrow$ S-500 HR. The "HR' refers to the high resolving power of the resins, which is a consequence of their small and uniform particle size (wet bead diameter = $25 \rightarrow 75$ μm). An S-1000 resin is available; this is not denoted "HR" as the particles are bigger and more variable (wet bead diameter = $40 \rightarrow 105$ μm). S-1000 separates in the range $500 \rightarrow 100{,}000$ kDa.

Trisacryl Plus, a product of Sepracor Inc., consists of poly-(N-tris [hydroxymethyl] methacrylamide) in bead form with a narrow size distribution (40-80 μm) for high resolution. It is available in two pore sizes, GF2-M, which fractionates in the range $1 \rightarrow 15$ kDa, and GF4-M, which fractionates in the range $5 \rightarrow 25$ kDa.

4.5.5 An MEC run

The MEC column should be packed and equilibrated with at least one colvol of buffer, but preferably more. The buffer should ideally contain at least 0.3 M NaCl, to minimise ion-exchange effects[13], but it must be borne in mind that increasing salt concentration increases hydrophobic interactions. After equilibration, the upper column flow-adapter is adjusted down onto the gel bed.

For best resolution, the sample should be about $2 \rightarrow 5\%$ of the column volume but for simple desalting it can be up to 20%. The sample may be applied, and the column subsequently run, at a flow rate of $2 \rightarrow 10$ cm h^{-1} Theoretically, provided there are no marked non-sieving effects, the next sample could be applied as soon as V, has eluted but, ideally, at least one colvol of buffer should be run through the column before application of the next sample.

4.6 Hydroxyapatite chromatography

Crystalline calcium hydroxyphosphate, $[Ca_5(PO_4)_3(OH)]_2$, the major component of tooth enamel, is known as hydroxyapatite (or hydroxylapatite)[14,15]. and its particular usefulness in protein isolation is that it binds proteins by a unique mechanism, different from MEC and simple ion-exchange, and it can therefore separate proteins which may not be separable by other means[14,15]. Hydroxyapatite forms blade-like

crystals and because the protein binds to the surface of the crystals, rather than within a gel lattice, the protein binding capacity is relatively low. For this reason, hydroxyapatite is best suited for use as one of the final steps in a purification.

Blade-shaped crystals are not optimal for chromatography and they tend to be brittle, thus generating "fines" which block the column and limit its life to three or four runs. Several manufacturers have attempted to overcome this by making spherical forms of hydroxyapatite, e.g. macro-prep ceramic hydroxyapatite from Bio-Rad and HA ultragel from Pharmacia. However, if the hydroxyapatite is used at the end of a purification, the fact that the classical crystals have a limited life is of lesserconsequence.

4.6.1 The mechanism of hydroxyapatite chromatography

The separating mechanism of hydroxyapatite is summarised in the review by Gorbunoff[14]. Hydroxyapatite crystals have positive surface charges, due to their constituent calcium ions, and negative charges due to their phosphate groups. The net charge can be varied by the buffer - it is negative in phosphate buffer, neutral in NaCl and positive in CaCl, or $MgCl_2$.

Positive amino groups of proteins bind electrostatically to negative charges on the hydroxyapatite, and are thus influenced by its net charge.

Hydroxyapatite - $P0_4^-$.....H_3^+N - Protein

Negative carboxyl groups, on the other hand, bind by complexing with calcium in the hydroxyapatite.

Hydroxyapatite - Ca - OOC - Protein

The retention of acidic (negatively charged) proteins is thus affected by the net charge on the hydroxyapatite in a manner opposite to that of basic (positively charged) proteins. CaCl, and MgCl, increase the binding of acidic proteins by formation of salt bridges between protein carboxyl groups and hydroxyapatite phosphate sites.

Hydroxyapatite - PO_4^- .....Ca^{2+} .....OOC - Protein

Basic proteins may be eluted from hydroxyapatite by negative ions such as F^-, Cl^-, and HPO_4^{2-}, which compete with its negative phosphate sites, or by Ca^{2+} or Mg^{2+} ions, which specifically complex with its phosphate sites and neutralise their charges. Acidic (negative) proteins

may be eluted by displacement of their carboxyl groups from hydroxyapatite complexing sites by ions, such as phosphate or F^-, which form stronger complexes with calcium.

Most proteins contain both amino and carboxyl groups and it will be noticed that phosphate is effective in eluting both types. Consequently, a common means of eluting proteins from hydroxyapatite is by the application of a phosphate gradient - often K-phosphate, because Na-phosphate has a limited solubility at low temperature. Gorbunoff[13] discusses alternative approaches, where the effects of CaCl, and MgCl2, and of NaCl or KCl, can additionally be exploited in elution schemes. As previously mentioned, using these devices, separations may be achieved which are not possible using other chromatographic systems and hydroxyapatite is thus a valuable technique in the biochemist's portfolio.

4.7 Affinity chromatography

The chromatographic methods discussed above are all dependent upon the gross physicochemical properties of the protein. However, the biological activity of the protein is generally more subtle and depends upon the very specific, complementary, steric relationship between the active site and a substrate (or inhibitor), or a binding site and a ligand, as the case may be. Affinity chromatography[16,17] exploits this biospecific relationship between a protein and a ligand, to specifically select out a desired protein from a crude mixture, essentially in a single step.

The specific ligand, which in the case of an enzyme may be a substrate or an inhibitor, is immobilised by conjugation to an insoluble matrix, in a manner which does not interfere with its interaction with the protein. This may require the use of a spacer arm, which typically consists of a chain of about 6-10 carbon atoms. An affinity chromatography resin is thus comprised of three parts, i) the matrix, which is similar to the matrices used for ion-exchange chromatography, ii) a spacer arm, and iii) the ligand. Matrix/spacer arm combinations are commercially available, since these are universal reagents, and simply require the addition of an appropriate ligand.

The sample solution is passed through the column and by its specific interaction with the immobilised ligand the protein of interest is retained, while all other proteins pass straight through the column. Subsequently, the protein can be eluted by a change in either the pH or ionic strength of the buffer or by addition of a free competing ligand, or of a chaotrope.

Because the protein is immobilised in a small volume of resin, affinity columns are generally quite small. Also, the volume of solution in which the protein occurs may be large and to pass this volume through the

column in a reasonable time, while maintaining the linear flow rate within limits, the column is usually relatively wide (e.g. 1.5x 1.5 mm i.d.). To overcome the problem of excessive volumes, there may be some advantage in preceding affinity chromatography by a quick concentrating method, such as TPP.

4.8 Hydrophobic interaction (HI) chromatography

HI-chromatography[18] was discovered serendipitously when, in control experiments, ligands were omitted from the matrix/spacer arm combination. It was found that the resulting resins were nevertheless effective at separating proteins, due to hydrophobic interactions between the sample proteins and the aliphatic spacer arms. Following this discovery, HI-resins were purposefully designed to optimise the hydrophobic interaction.

Hydrophobic "bonds" are increased in strength by an increase in buffer ionic strength. HI-chromatography therefore conveniently fits into an isolation scheme, immediately after a salting out step, as the high salt levels will promote binding to the HI-resin. Proteins can subsequently be eluted by decreasing the buffer ionic strength, either in a stepwise manner or in a gradient.

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4.9 Chapter 4 study questions

1. Define the term "distribution coefficient" as applicable to chromatography,
2. Define "HETP".
3. What value of HETP is best?
4, What factors influence HETP?
5. What is the optimum value for each of the factors that affect the HETP?
6. The distribution coefficient of substance A is 0.4 and of substance B is 0.6. Which will move more slowly through a chromatography column'?
7. Why is it necessary to have a minimum dead volume on the outlet side of a chromatography column?
8. How can a gravity-fed column be protected against running dry?
9. What are some desirable properties of the matrix of an ion-exchanger?
10. Give the structure of a DEAE group.
11. Is DEAE generally suitable for binding a protein, a) below the pI of the protein, b) at its pI, or, c) above its pI?
12. Two proteins have pI values of 7.6 and 8.1. Briefly describe an ion-exchange procedure that may be used to separate these.

13. With regard to the substituent on a cation exchanger, is a strong acidic or basic group generally better or worse than a weak group? Explain
14. Why, after eluting an ion-exchange column with a gradient from a gradient mixer, it is necessary to elute with a further column volume of finishing buffer?
15. For eluting an ion-exchange column, is an ionic strength gradient usually better/worse than a pH gradient? Explain.
16. A linear gradient generator has two vessels of identical size. Draw the shape of the gradient which would be obtained if the second vessel (with the finishing concentration) were larger.
17. A chromatographic column (25 mm i.d. x 95 cm) is run at a volumetric flow rate of 50 ml h^{-1}. (a) At what volumetric flow rate should an 18 mm i.d. column be run to give equivalent chromatographic conditions? (b) If the sample volume applied to the 25 mm column was 30 ml, what volume of sample should be applied tu the 18 mm column? (c) Assuming the 2.5 x 95 cm column was filled with a molecular exclusion gel, what time period should be set on an automatic shut-off timer to ensure that all peaks would be completely eluted? (d) If a fraction collector with 90 tubes was available, what time per tube should be set to collect the entire run? (e) What would be the volume in each tube? (f) How long after application of the sample would one expect to see the peak elution of a sample component which is larger than the exclusion limit of the gel? (g) How long after application of the sample would one expect to see elution of a peak having a K_{av} of 0.5?
18. Describe the difference between a microreticular and a macroreticular gel and say which of the following gels is which:- Sephadex, agarose, polyacrylamide
19. A molecular exclusion column was standardised with the following standard globular proteins:-

Protein	MW (kDa)	K_{av}
Cytochrome C	13	0.62
Myoglobin	17	0.55
Chymotrypsinogen	25	0.45
Ovalbumin	45	0.32
BSA	67	0.20

i) Determine the MW of a protein having a K_{av} of 0.40.
ii) It is known that this unknown protein consists of two subunits of equal size, which dissociate in 8 M urea. If the column size was 1.5 x 50 cm, in what volume would you expect the unknown protein to elute in a buffer containing 8 M urea?

20 A molecule has a Kav of 0,6. What % of the stationary phase is available to it?

21 The calculated Kav value ofa glucosidase enzyme on Sephadex G-100 was>1.

i) What does this tell you?

ii) Can you offer a possible explanation for this phenomenon?

22 When in a protein isolation would the use of HI chromatography be most appropriate'?

23 Assuming evenly sized spherical gel particles, how is V_o affected by the resin particle size?

24 Calculate the phase ratio for a molecular exclusion gel. (Clue: remember that $V_o = 0.36\ V_t$).

25 Calculate the partition ratio for a solute having a K_{av} of 0.75 on an MECgel.

Chapter 5

Principles of Electrophoresis

Active fractions isolated by a preparative fractionation procedure may be subjected to a number of analytical fractionation procedures to determine their purity. Analytical fractionations are distinguished from preparative fractionations by the criteria shown in Table 5.

Table 5. The difference between preparative and analytical fractionations.

	Analytical	Preparative
Scale	Small	Large
Fate of sample	Destroyed	Preserved
Product	Information	Active fraction

In an analytical fractionation, therefore, a small amount of sample is sacrificed in order to gain information about the state of purity of the material being analysed. Of the many physico-chemical techniques which have contributed to our knowledge of proteins (and nucleic acids), electrophoretic techniques occupy a position of primary importance. Electrophoresis finds its greatest usefulness in the analysis of mixtures and in the determination of purity, although certain forms of electrophoresis may be applied on a preparative scale.

5.1 Principles of electrophoresis

Electrophoresis may be defined as the migration of charged ions in an electric field. In metal conductors, electric current is carried by the movement of electrons, largely along the surface of the metal. In solutions, the electric current flows between electrodes and is carried by ions. The negative electrode - the cathode - donates electrons and the positive electrode - the anode - takes up electrons to complete the circuit. The ions that result from the take up of electrons from the cathode will be negatively charged and will thus migrate towards the positive anode. Because of their anodic migration, negative ions are called “anions”.

“PA **NI**C” - Positive Anode
Negative Cathode
(NI) — Not the Ions

Ions which result from the donation of an electron to the electron-deficient (i.e. positively charged) anode will themselves be electron deficient, and thus positively charged. These will migrate to the cathode and are thus called cations.

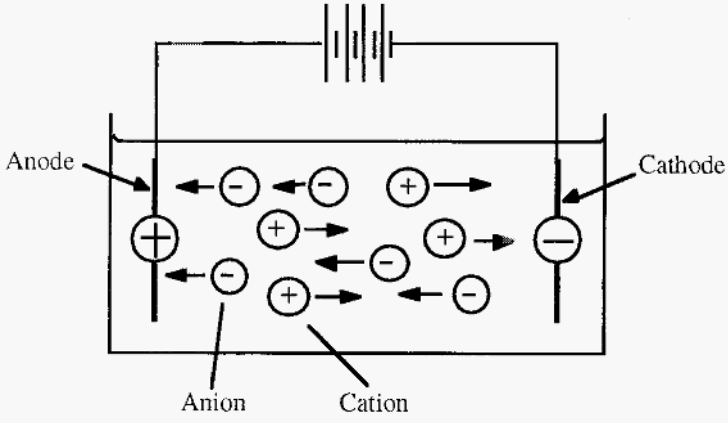


Figure 71. Electrophoresis: the movement of ions in an electric field.

There is a potential difference (voltage) between the anode and the cathode and if the solution between these is of constant composition and constant cross-section (i.e. constant resistance), the voltage gradient between them *(dV/dx)* will be linear, with units of volts cm^{-1}. (In Section 5.11 the effects of non-linear voltage gradients will be explored.)

An ion placed in such an electric field will experience a force:-

$$F = K.q\frac{dV}{dx} \qquad 5.1$$

Where, F = electrophoretic force
K = a constant (embodying the Faraday constant and Avogadro's number)
q = net charge on the protein (atomic charges/protein molecule)

$\frac{dV}{dx}$ = the voltage gradient (volts cm^{-1}).

This force will cause the protein to accelerate towards either the cathode or the anode, depending on the sign of its charge.

As the protein moves it will experience a retarding frictional force (hydrodynamic drag), which at the speeds involved is proportional to the speed of movement.

$$F_{fric} = f.\frac{dx}{dt} \qquad 5.2$$

Where, F_{fric} = frictional force

$\frac{dx}{dt}$ = velocity of movement (cm sec^{-1})

f = frictional coefficient

It will be recalled that this situation is very similar to that obtaining during centrifugation (Section 2.5), and the frictional coefficient can be determined in the same way.

$$D = \frac{RT}{f}$$

Hence, $$f = \frac{RT}{D}$$

The proteins very soon reach terminal velocity, at which point the electrophoretic (propelling) force equals the frictional (retarding) force, i.e. from equations 5.1 and 5.2:

$$K.q\frac{dV}{dx} = f.\frac{dx}{dt} \qquad 5.3$$

The free electrophoretic mobility, (μ), with units of (cm^2 $volt^{-1}$ sec^{-1}) can be defined as the velocity per unit of voltage gradient, i.e.:

$$\mu = \text{velocity (voltage gradient)}^{-1}$$

$$= \frac{dx/dt}{dV/dx}$$

Hence, from equation 5.3,

$$\mu = K\frac{q}{f} \qquad 5.4$$

The electrophoretic mobility is thus a function of the charge on the protein ion and the medium through which it is travelling. Electrophoretic techniques exploit the fact that different ions have different mobilities in an electric field and so can be separated by electrophoresis.

The flow of electricity in electrophoresis is subject to the same physical laws as other forms of electricity. For example, Ohm's law applies:

$$I = \frac{V}{R} \qquad 5.5$$

Where I = current (amps)
V = potential difference (volts)
R = resistance (ohms).

The unit of electrical charge is the coulomb and the unit of current [the ampere (amp)] may be defined as coulombs sec^{-1}, i.e.,

$$I = \frac{\text{coulombs}}{t} \qquad 5.6$$

$$\therefore \quad \text{coulombs} = I.t$$

The flow of electricity involves work, which generates heat, and the work (W, in joules) done in transferring a charge of q coulombs between a potential difference of V volts is:-

$$W = qV = (I.t)V = IVt \qquad 5.7$$

And, since, from eqn 5.5,

$$V = IR,$$

Then, $$IVt = I(IR)t = I^2Rt$$

$\therefore$ from eqn 5.7,

$$W = I^2Rt \qquad \text{(Jouleís law of heating)}$$

Which means that I^2Rt joules of heat will be developed in the conductor.

The power (in watts) (defined as the rate of work) gives the rate of heating (joules sec^{-1}).

Thus, $$\text{Watts} = \frac{I^2Rt}{t} = I^2R \qquad 5.8$$

$$= \frac{V^2}{R} \left(\text{since } I = \frac{V}{R}\right)$$

5.1.1 The effect of the buffer

The buffer in which electrophoresis is conducted, has a large influence on the migration of proteins. Firstly, the buffer *pH* will influence the charge (q) on the protein and hence the direction and speed of its migration. Secondly, the buffer *ionic strength* influences the proportion of the current carried by the proteins - at low ionic strength the proteins will carry a relatively large proportion of the current and so will have a relatively fast migration. At high ionic strength, most of the current will be carried by the buffer ions and so the proteins will migrate relatively slowly.

An analogy might be useful in visualising this effect of ionic strength. Imagine a bank where there are two counters - one for deposits (≡ the anode) and one for withdrawals (= the cathode), with electrons being the money. The ions may be considered as customers waiting to be served at either counter, which one can visualise as being at opposite ends of the banking hall.

In Fig. 75, the circles represent customers queuing for service. In electrophoresis, these queues would be along the so-called *field lines,* which are usually (but not necessarily) straight lines. The lighter coloured circles represent buffer ion "customers" and the dark circles represent protein "customers". When the "customer" at the counter is served, they move away, creating a "hole". This "hole" is filled by the next customer in line, and so on, and so the "hole" moves backwards along the

line. No matter how far away from a counter any customer is, they will be drawn towards the counter by the periodic appearance of a "hole" in the queue, immediately in front of them. If the counter assistants were very energetic (giving a high current) these "holes" would appear frequently and the customers would all progress quickly. On the other hand, if the counter assistants were lethargic (giving a low current) the "holes" would appear infrequently and progress of the customers would be slow.

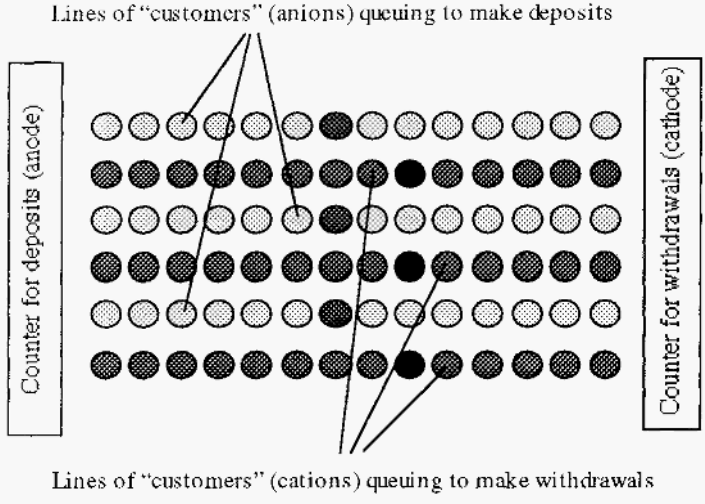


Figure 75. A banking hall analogy of electrophoresis.

In Fig. 75, the relative proportions of protein ions to buffer ions shown is such that there is one protein ion in each queue. However, if we have the counter assistants working at the same rate (i.e. with the same current) but increase the number of customers (i.e. increase the ionic strength), then we will get the situation shown in Fig. 76.

With more buffer ions present, they will get most of the service (carry most of the current) and the progress of all ions in their respective queues, including the protein ions, will be slower.

In electrophoresis, therefore, a low ionic strength is preferred as it increases the rate of migration of proteins. A low ionic strength is also preferred as it gives a lower heat generation. Assuming a constant voltage, if the ionic strength is increased, the electrical resistance decreases but the current will increase. According to Eqn 5.8, heating is proportional to I^2, but is only linearly affected by changes in resistance.

A high ionic strength buffer will therefore lead to greater heat generation, and so a low ionic strength is preferred.

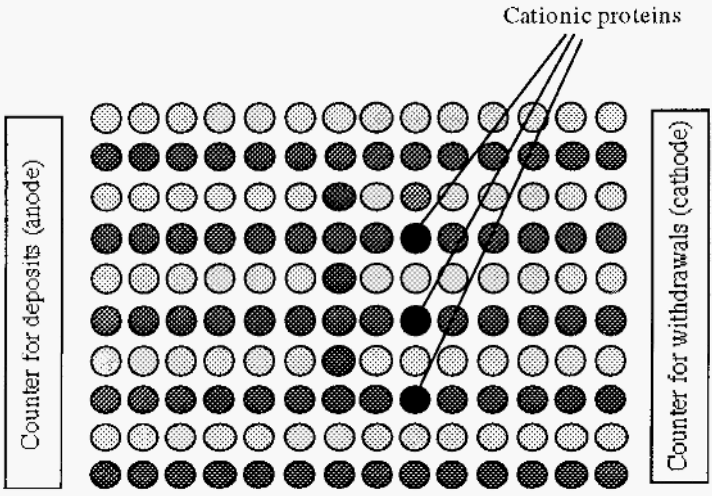
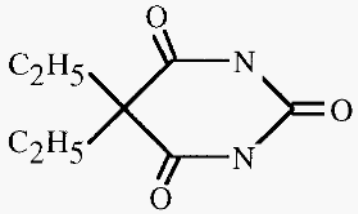
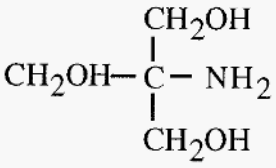


Figure 76. Illustration of the effect of ionic strength in electrophoresis.

Strictly speaking, it is not the ionic strength *per se* which is the important factor in electrophoresis, but the mobility of the buffer ions. Thus at equivalent ionic strengths (i.e. at comparable buffering capacities), large buffer ions will migrate more slowly than small buffer ions (because of their greater frictional coefficient, f). Large buffer ions will thus lead to less heat generation and a faster migration of the proteins. For example, barbitone (I) has a mobility about one quarter of that of Tris (II), and can therefore be used at four times the concentration of Tris - at which concentration it will be roughly four times more effective as a buffer.



I



II

The effect of ionic strength is actually more complex than indicated in the simplistic model given above. The reason is that ionic strength also has an effect on the electrical double layer which surrounds proteins in solution. The ions in the electrical double layer have the effect of decreasing the apparent charge on the protein. As the protein moves under electrophoresis, it takes with it a part of the electrical double layer. As the ionic strength increases, the thickness of the electrical double layer decreases and more of the counterions are drawn along with the migrating protein, effectively reducing its charge. The mobility of the protein thus decreases with increasing ionic strength. A more complete discussion of this effect is given by Kyte[1].

5.2 Boundary (Tiselius) electrophoresis

One of the earliest forms of electrophoresis was the so-called moving boundary method, in which a protein mixture was introduced into a U-tube and subjected to an electric field (Fig. 77).

In this method, the proteins are not completely separated, but, theoretically, the number of proteins in a mixture can be determined by analysis of the number of boundaries formed after a period of electrophoresis. The boundaries can be detected by schlieren optics, which detects changes in refractive index.

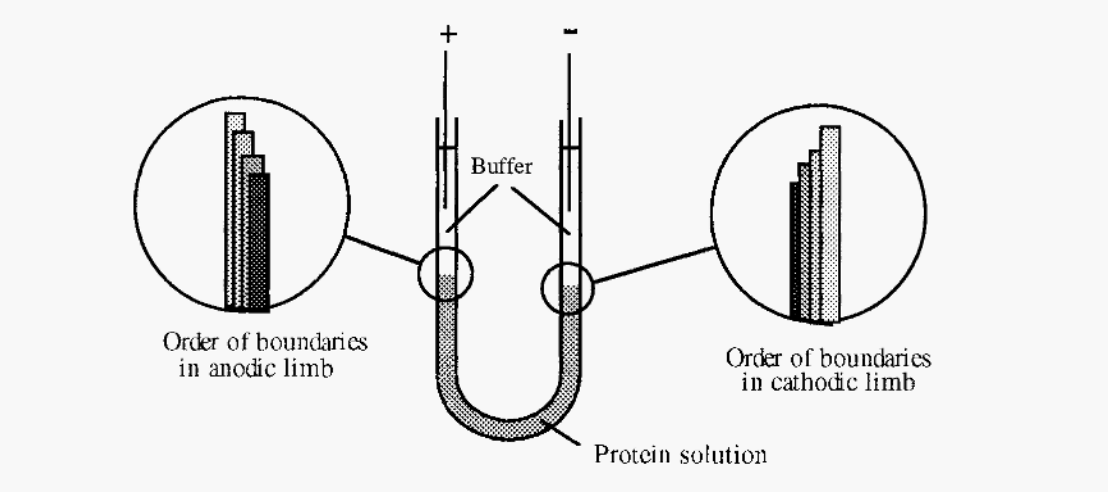


Figure 77. Moving boundary (Tiselius) electrophoresis.

In practice, the sensitivity of moving boundary electrophoresis is very low and so it is not much used today for protein analysis. However, it is

an interesting example of free electrophoresis, i.e. where there is no supporting medium, and it is conceptually different from all modern forms of electrophoresis which are all so-called ìzone electrophoresisî methods, in which zones of different proteins can become completely separated from one another.

5.3 Paper electrophoresis

One of the earliest forms of zone electrophoresis for the separation of proteins was paper electrophoresis. In this a strip of filter paper was used as a medium to support a thin layer of buffer. Since the paper served only to support the buffer, paper electrophoresis can be considered as a form of free electrophoresis (as opposed to electrophoresis in a sieving gel, which will be discussed in Section 5.1.5) The experimental set-up for paper electrophoresis is shown in Fig. 78.

A strip of filter paper, typically 20 x 150 mm, is marked with pencil to indicate the anodic and cathodic ends and a line is lightly drawn transversely in the middle, where the sample is to be applied. The strip is soaked in buffer, blotted briefly and suspended between supports in the apparatus. Buffer is added to both the anode and the cathode compartments: it is important that the levels in the two compartments are the same to prevent siphoning through the filter paper. The filter paper is connected to the buffer by filter paper wicks, which must be the same width as the filter paper strip, but can be made of several layers of filter paper.

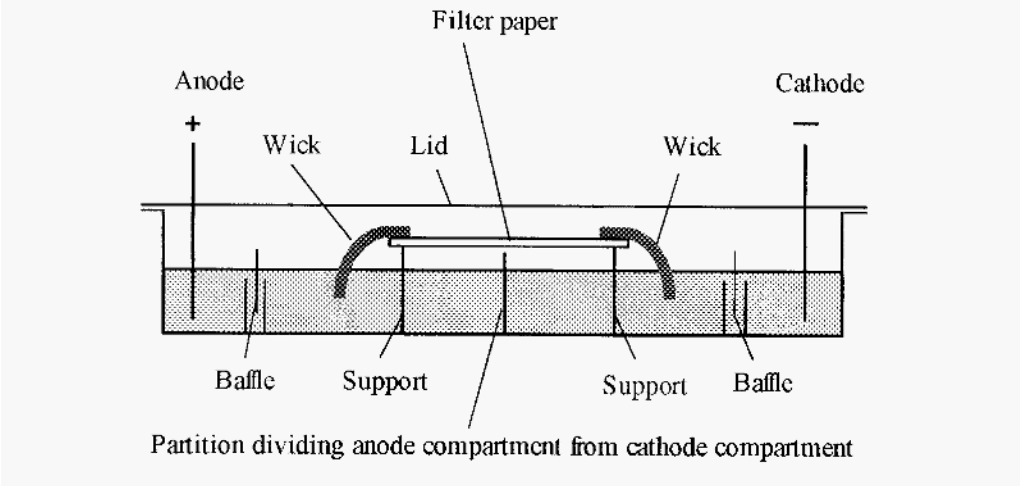


Figure 78. Diagrammatic cross-section of an apparatus for paper electrophoresis.

Sample can be applied as a thin line across the middle of the paper strip, but not within *ca.* 5 mm of the edges. There are different ways of applying the sample: a simple way, but which requires some manual dexterity, is to use a Pasteur pipette, drawn down to a thin capillary. After application of the sample, the apparatus is sealed with a lid This enables the atmosphere within the apparatus to become saturated with water vapour, thereby preventing evaporation of water from the buffer on the strip. The buffers have a maximal exposed surface area to encourage rapid equilibration of the water vapour. As a safety precaution. the apparatus lid is usually coupled with the electrode connections, so that removal of the lid breaks the electric circuit. Without this precaution, fatal shocks might result from inadvertent contact with the electrode solutions. Electrophoresis is run, usually for a number of hours, typically using a voltage gradient of *ca.* 10 volts cm^{-1}. To keep electrolysis products away from the protein samples being separated. the buffers in the electrode compartments are separated from the wicks by a baffle system.

After separation, the protein bands are fixed in position and stained with a protein-specific stain, such as Ponceau S or Amido Black, and destained. Paper electrophoresis was used in medical diagnostics and a typical result for the separation of serum is shown in Fig. 79.

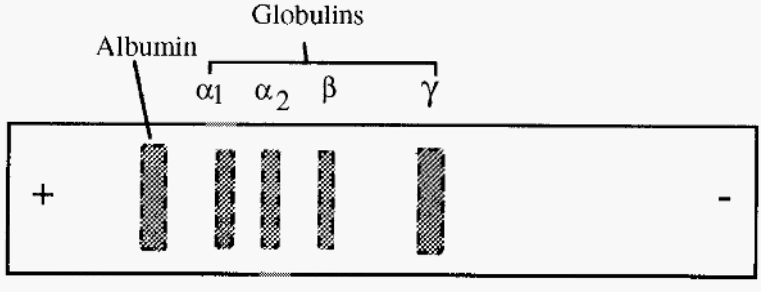
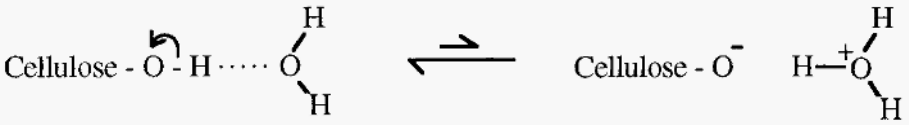


Figure 79. A typical separation of human serum by paper electrophoresis at pH 7.2.

5.3.1 Electroendosmosis

Paper is comprised largely of cellulose, a $\beta 1 \rightarrow 4$ linked polymer of glucose. Glucose is hydrophilic due to the polarity of its many -OH groups. which readily form hydrogen bonds with water. Cellulose as a whole is not water soluble, however, because of its extensive interchain hydrogen bonds. In forming hydrogen bonds with water, the H of the -OH groups of glucose is shared with the oxygen of water, giving the water a $\delta+$ charge and the cellulose oxygen a $\delta-$ charge.

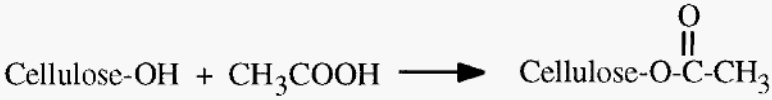


Cellulose thus acquires an overall negative charge, called the "zeta potential". When placed in an electrical field, a strip of paper (cellulose) tends to move towards the anode, but cannot do so as it is fixed in place. The hydroxonium ions, H^3O^+, however, are free to move towards the cathode and do so, resulting in a net drift of the buffer towards the cathode. This drift of the buffer towards the cathode, known as electroendosmosis, increases the apparent mobility of cations and decreases that of anions. For example, in Fig. 79, γ-globulin is seen to have an apparent migration to the cathode. However, the pI of γ-globulin is 6.8, and so at pH 7.2 it would be expected to have a net negative charge and a consequent anodic migration. In fact it does have a slight anodic migration but the electroendosmotic flow is faster than this, resulting in the apparent cathodic migration.

5.4 Cellulose acetate membrane electrophoresis (CAM-E)

Cellulose has a rather open, porous structure and a negative charge which, besides causing marked electroendosmosis, can result in the binding of positively charged proteins. These properties of cellulose result in band spreading and consequent poor resolution of bands.

Cellulose acetate is a finer-grained derivative of cellulose made by the esterification of a proportion of the free -OH groups of cellulose to the acetate ester.



Cellulose acetate membranes give sharper protein bands and better resolution than paper, bind proteins less and have less endosmosis. As a result, CAM-E replaced paper electrophoresis and remained in use, mainly in medical diagnostic laboratories, for many years. The apparatus required for CAM-E is the same as for paper electrophoresis.

Cellulose acetate membranes are white and opaque but can be readily clarified and rendered transparent by immersion in "liquid paraffin". The clear strips, with bands of stained protein, can be scanned to give an objective and quantitative assessment of the separation obtained

(Fig. 80). A more modern way of achieving the same end would be to capture a digital image of the CAM strip which could then be subjected to appropriate image analysis.

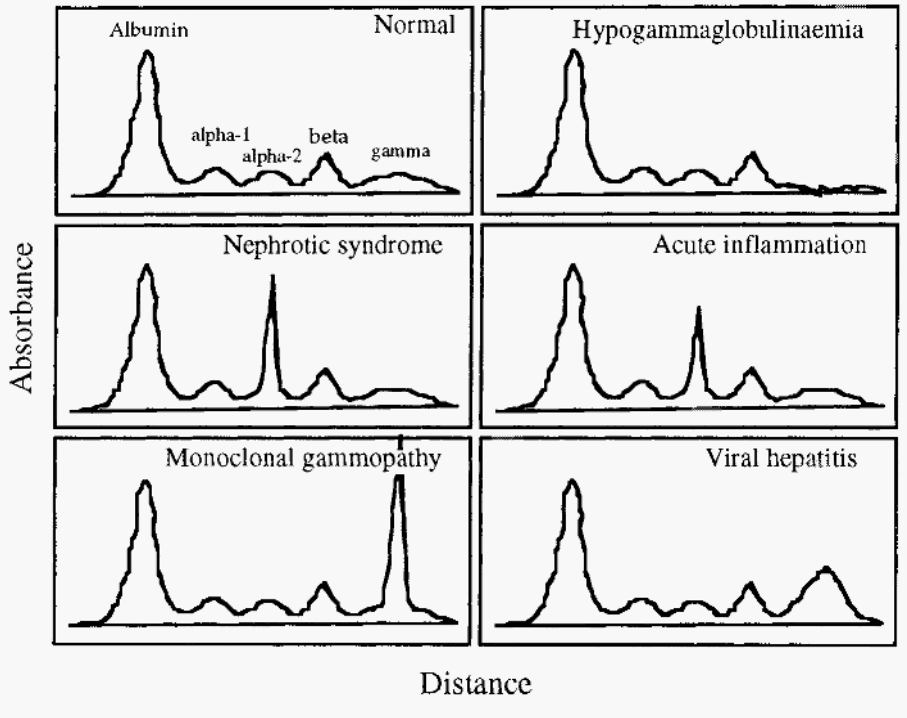


Figure 80. Densitometric scans of CAM-E results from different pathological sera.

5.5 Agarose gel electrophoresis

CAM, in turn, has largely been replaced by agarose as a support medium for free electrophoresis in medical diagnosis[2]. Although agarose is a gel, it has a macroreticular structure (see Fig 4.14) and thus does not impede the electrophoretic migration of molecules of less than *ca.* 500 kDa. Proteins are thus not usually retarded but nucleic acids can be separated on the basis of their size by gel sieving (see Section 5.6). As with all gels, water cannot flow through agarose. There is therefore no necessity to maintain the two buffer reservoirs at the same height, since the buffer is unable to siphon through an agarose gel.

Agarose has other advantages: it can be obtained in a form with zero electroendosmosis, it can be cast onto a sheet of flexible plastic Gel-Bond® and, after staining and destaining, it can be dried onto the Gel-Bond to provide a durable record which is easily filed.

5.6 Starch gel electrophoresis

Historically, starch gel electrophoresis preceded agarose electrophoresis but here the order of discussion is turned about to group mechanistically related techniques. Starch gel electrophoresis was introduced by Smithies[3] in 1955. Starch forms microreticular, thermosetting gels comprised of interlocking starch helices, cross-linked by H-bonds. The microreticular nature of starch gels introduced the phenomenon of *gel sieving* which revolutionised electrophoresis by greatly increasing its resolution and sensitivity.

In a microreticular gel, a protein migrating under electrophoresis faces a greatly increased frictional resistance, due to the fact that the proteins have to migrate through the 3-D gel network. This resistance is an inverse function of the size of the protein, so that small proteins will migrate with less friction than larger proteins, while proteins larger than the exclusion limit of the gel (see p106) will not be able to enter into the gel at all.

Ferguson[4] has determined that the mobility of a protein in a starch gel, as a function of the gel concentration, is described by the equation:-

$$u_i = u_i^0 e^{-\,{}^iK_i T_S} \qquad 5.9$$

Where u_i = the electrophoretic mobility of protein i in a starch gel of concentration T_S percent

u_i^0 = the free electrophoretic mobility of protein i.

${}^iK_i$ = a constant unique to protein i.

i.e. the mobility decreases logarithmically as the gel concentration increases. A plot of In u_i vs T_S gives a straight line, of slope $-{}^iK_i$ known as a Ferguson plot.

The same apparatus as used for paper electrophoresis and CAM-E can be used for starch gel electrophoresis. The gel is cast as a horizontal slab, which is connected to the buffer reservoirs using filter paper wicks. The slab must not be too thick to prevent excessive heat build-up. For extra cooling, the slab may be supported on a block internally cooled by circulating cold water. To accommodate the samples to be separated, small slit-like wells are cast in the gel slab, using a purpose-made mould, or cut with a scalpel. In the latter case the sample can be introduced into the slit by inserting a small strip of filter paper impregnated with sample.

After sample is introduced into the sample wells, the electric field is applied across the length of the gel. Under the influence of the electric

field, the sample proteins will move either towards the cathode or the anode, depending upon their charge at the buffer pH. Initially, they will migrate through the buffer in the sample wells by free electrophoresis. When they strike the well wall, on either the anodic or cathodic side. the resistance to their migration will increase and the sample will be concentrated into a narrow band.

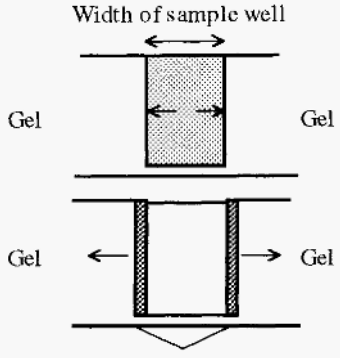


Figure 81. Sharpening of starting zones in starch gel electrophoresis.

The resolution (see p76) in electrophoresis is a function of the starting bandwidth and ofthe band spreading during electrophoresis. The latter is largely due to diffusion of the protein from its zone of highest concentration. The structure of a microreticular gel, however, not only impedes the progress of proteins undergoing electrophoresis, brit also limits diffusion. The combination of narrow starting bands and reduced diffusion results in the marked improvements in resolution and sensitivity of gel electrophoresis. The sensitivity is increased since proteins are more easily detected the higher their concentration and, by the initial concentration of the bands and subsequent minimisation of diffusion, proteins present at low levels can be detected.

A down-side of SGE is that, due to the variability of starch which is a natural product, results tend to vary from lab to lab and in the same lab at different times. This motivated a search for a more uniform, synthetic gel. Nevertheless, starch gel electrophoresis is still widely used today, mainly by biologists exploring the taxonomic relationships of organisms or in plant breeding. An advantage of starch gels is that they are non-toxic and biodegradable and are thus suitable for large-scale screening.

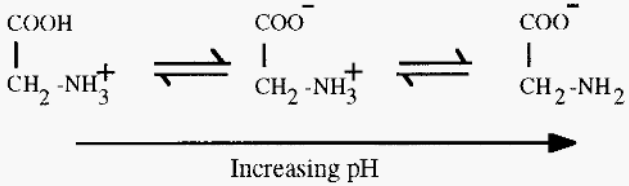
5.7 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide, as a medium for gel electrophoresis was introduced by Ornstein[4]. It has the advantage of being a synthetic gel, which is highly reproducible. Moreover, the pore size can be controlled by varying the proportions of acrylamide and the crosslinking agent, bisacrylamide (see p106). Polyacrylamide can be used as a direct replacement for starch, though it is less conveniently used than starch in the horizontal slab format because polymerisation of polyacrylamide is inhibited by oxygen, so it must be cast into a sealed mould.

5.7.1 Disc electrophoresis

Polyacrylamide was first introduced concurrently with a new electrophoretic method called "disc electrophoresis". This was first conducted in glass tubes, in which the separated protein bands constituted a series of discs. The method also embodied discontinuities in the buffer and gels used and so it may be described as discontinuous electrophoresis, abbreviated to disc. electrophoresis. Today, because of the better cooling and the fact that comparison of different samples is facilitated, disc. electrophoresis is generally conducted in vertical gel slabs. With this lay-out, the sample is applied to one end of the gel slab and so, unlike with a horizontal slab gel lay-out, only anions or cations, but not both, can be analysed at one time. The original method of Ornstein[5], is an anionic system (i.e. anions are analysed).

In an anionic system, there is usually a common buffer cation, e.g. Trisí throughout. In the buffer compartments, a buffer with an anionic component having a pH-dependent electrophoretic mobility is used, e.g. glycine-. Above its pI, the anodic mobility of glycine increases with pH as the proportion of glycine- ions increases.



In the gels, an anionic component having a high, pH-independent mobility, e.g. Cl^-, is used.

Two gels are used; a large pore stacking gel and a smaller pore running gel. At the outset, both gels contain Tris-HCl buffer, but at different pH values. The buffer in the stacking gel is of lower pH than that in the

gel. A schematic view of the apparatus at the start of an electrophoresis run is shown in Fig. 82. The samples are mixed with sucrose or glycerol to increase their density and are layered directly under the electrode buffer.

Upon application of the electrical field, a sharp interface develops between the high mobility so-called "leading ion", i.e. Cl, and the less mobile so-called "trailing ion", i.e. glycine, according to Kohlrausch's regulating functions[6]. In order to visualise the interface between the leading and trailing ions a small amount of a dye, such as bromophenol blue, may be added to the samples or to the upper electrode solution.

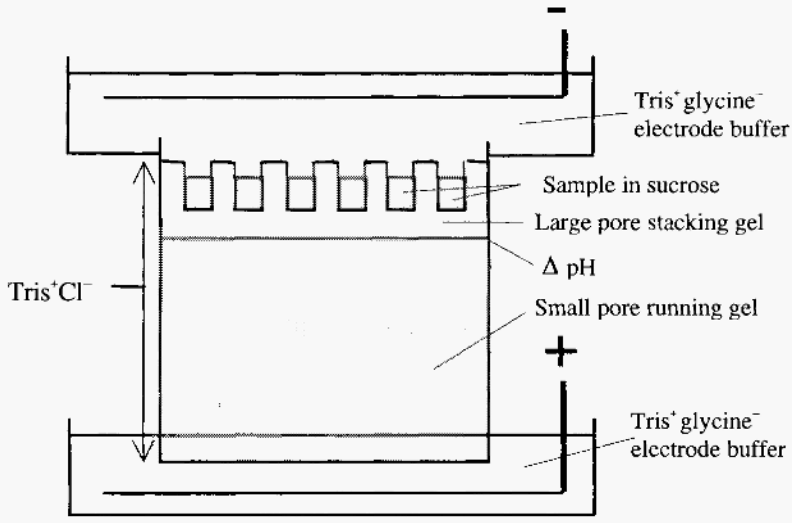


Figure82. Experimental set-up for discontinuous PAGE.

As the interface moves downward, the protein molecules with mobilities intermediate between Cl^- and glycine, will be swept up and concentrated into a very thin band. Within this band, individual proteins will become stacked in order of their mobilities, with those of highest mobility immediately next to the Cl^- ions. During the later parts of this so-called stacking phase, therefore, all of the proteins will move downwards at the same speed, i.e. this could be called an isotachophoresis stage (iso = "the same", "tach" = speed). The concentrated band of protein has a higher density than the surrounding buffer and the system would be convectively unstable were it not stabilised by the stacking gel.

The voltage gradient *(-dV/dx)* within the separating part of the apparatus will not be uniform but will have a stepwise decrement at the

interface between the leading and trailing ions. If proteins are additionally present, the step will be comprised of a number of smaller sub-steps, corresponding to interfaces between the different proteins.

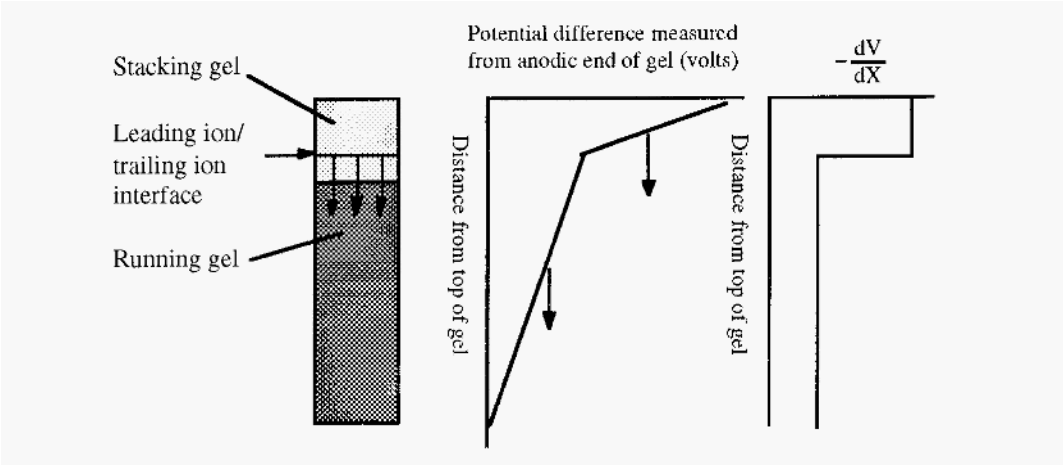


Figure 83. The voltage profile during the stacking phase of disc gel electrophoresis.

Arrows in Fig. 83 indicate the direction of movement of the interface and its associated voltage discontinuity. The voltage gradient is steeper behind the interface than in front. This follows from the fact that all the ions are moving at the same speed. For ions of a low mobility to move as fast as ions of a high mobility, they must be in a steeper voltage gradient. Any protein which falls behind the interface, say by diffusion. will find itself in an area with a steep voltage gradient and will thus be accelerated towards the interface, Conversely any protein which diffuses ahead of the interface will enter an area with a shallow gradient and will slow down and be overtaken by the moving interface. In this way the proteins are focused into thin layers in the interface.

When the interface reaches the junction between the stacking and running gels, two things happen. Firstly the pH increases, resulting in an increase in the mobility of the glycine trailing ion as a larger proportion will exist in the glycine- form at the higher pH. Secondly, the proteins encounter the sieving effect of the small pore running gel. Together these result in the leading ion/trailing ion interface overtaking the stack of proteins, which are left to separate in a linear voltage gradient according to their respective mobilities in the running gel.

The bromophenol blue dye remains with the interface, making its progress easily visible. When the interface reaches near the end of the gel the electric field can be switched off, in the sure knowledge that no proteins will have migrated further than the interface and that all will still

be in the gel. The separated proteins can be visualised by staining, for example with Coomassie blue, and destaining.

A number of disc PAGE systems have been described by Jovin[7].

5.7.1.1 Isotachophoresis

Although it is not a PAGE system, it is convenient to discus isotachophoresis at this point because it is conceptually related to the stacking phase of disc electrophoresis.

In the later stages of the stacking phase of disc electrophoresis, the proteins are stacked on top of one another in very thin layers (Fig. 84). If the electrophoresis is conducted in a tube, then the thin layers will be in the form of discs.

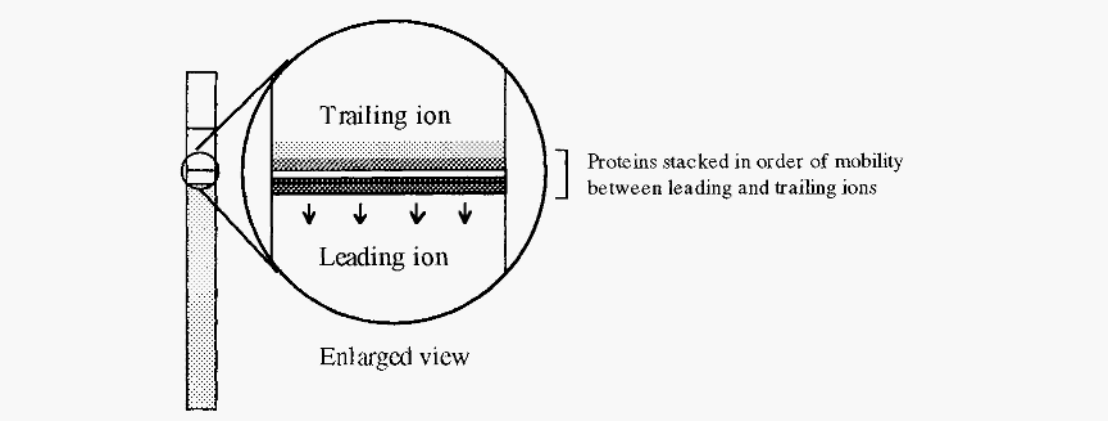


Figure 84. Proteins stacked as thin layers in the stacking phase of disc gel electrophoresis.

The different proteins are in fact separated from one another, although adjacent bands are touching, but this separation is not useful as the bands are so thin that it is impossible to distinguish between them. However, some improvement in the situation could be achieved by making the tube much thinner, so that a given amount of protein would occupy a greater length.

Ultimately, if the separation was carried out in a capillary tube, the bands would occupy a greater length in the capillary tube. In this situation, it is possible to distinguish the different bands. At each interface there is a step change in the voltage gradient, which corresponds to a change in resistance due to the fact that the proteins have different mobilities. This change in resistance at each interface corresponds to a change in heat production and this can be detected with

a thermocouple detector. The output is a sharp peak as each interface passes the detector.

In this way the number of proteins present can be determined, but they cannot be identified. If one protein was missing, the others would simply close up and one fewer interface would be detected but it would be difficult to determine which protein was missing. This problem can be overcome by adding a mixture of "ampholytes" to the protein sample. Ampholytes are synthetic polyamino-polycarboxylic acids, in which the amino and carboxyl groups are randomly added to a carbon chain backbone - they are sold under a number of trade names, e.g. Ampholine, Servalyte, Bio-lyte. The result is a mixture of molecules with a range of pI values, resulting in a range of electrophoretic mobilities.

Because of their range of mobilities, there will always be some ampholyte molecules with mobilities intermediate between those of the different proteins and in isotachophoresis these will serve to space the protein molecules a distance apart from one another, and so are known as "spacers". The separated protein molecules can be detected by UV absorption. Ampholytes will be revisited later in a discussion of isoelectric focusing (Section 5.9).

Isotachophoresis is discussed here largely as a conceptual development of the stacking phase of disc electrophoresis. In fact it is a technique which is not much used. However, the concept of conducting electrophoresis in a capillary tube has been highly successful and capillary electrophoresis has become a versatile and popular analytical technique, which can be applied to proteins and other ions. An advantage of the capillary system is that a capillary tube at once controls convection and gives excellent cooling so that high voltages can be used, giving rapid separations. A down-side to capillary electrophoresis is that the apparatus tends to be expensive.

5.8 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was introduced in 1967 by Shapiro *et al.*[8] and has since become one of the most popular PAGE methods. The method is dependent in the first instance upon the interaction of the protein molecule with SDS. This is a detergent having a 12 carbon hydrophobic tail and a hydrophilic, sulfonic acid head group (I).

I

Proteins are denatured by boiling in the presence of SDS. The SDS molecules interact with the proteins to give rod-like complexes, containing a constant ratio of *ca.* 1.4 mg of SDS per mg of protein[9]. At this level the negative charge on the SDS is sufficient to mask the charge on the protein and all proteins consequently have essentially the same charge/mass ratio and an anodic migration[10].

The lack of charge differences between different protein/SDS complexes means that the stacking phase will not be as effective as in conventional disc electrophoresis. However, Laemmli[11] has devised a convenient method whereby disc PAGE and SDS-PAGE can be conducted with the same set of reagents, simply with or without SDS. The Laemmli method has become one of the most popular PAGE methods in use today. However, the Laemmli method does not separate small proteins very well and an alternative SDS-PAGE method, using Tricine buffer, has been described by Shagger and Von Jagow[12]. The Tricine method gives uniquely good separation of proteins under 20 kDa.

As the intrinsic charge differences between proteins is masked by the SDS, separation of proteins is due solely to differences in size and hence the method can be used to determine molecular sizesî. A linear relationship between mobility and log MW obtains over a molecular weight range dependent upon the gel pore size. The gel can thus be standardised with proteins of known molecular weight and subsequently used to estimate the molecular weights of unknowns. Mobility is conveniently expressed as relative mobility (R_m), i.e. mobility relative to the bromophenol blue tracker dye.

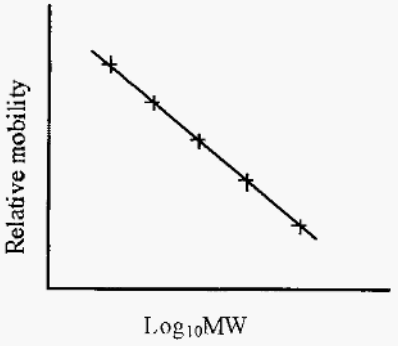


Figure 85. Standard curve for estimation of protein MWs by SDS-PAGE.

Some caveats apply to the estimation of molecular weights. If proteins are not reduced, then disulfide bridges may constrain the structure and prevent formation of the rod-like complexes. This will

result in an incorrect apparent MW. On the other hand, this provides a way of detecting the existence of disulfide bridges. The glyco moiety of glycoproteins will also not bind SDS, and yet will contribute to the steric resistance of the molecule. In consequence, the molecular weights of glycoproteins tend to be overestimated. Finally, boiling in SDS not only denatures the protein but will dissociate the subunits of oligomeric proteins. The MWs obtained by SDS-PAGE will therefore be of the subunits and not of the intact protein.

5.8.1 An SDS-PAGE zymogram for proteinases

Zymography is a method for the detection of a specific enzyme among the bands separated by electrophoresis. The method usually relies on an enzyme specific reaction to generate a colour to reveal the position of the enzyme. Usually zymogram methods cannot be applied to SDS-PAGE as proteins are normally denatured in this technique. However, Heussen and Dowdle[13] have devised an ingenious SDS-PAGE zymogram method for proteinases. The method depends upon combining the proteinase-containing mixture with SDS, but without boiling the solution. In this form the SDS can apparently combine only partially with the protein, perhaps "tweaking" its conformation slightly and suppressing the activity of proteinases.

The SDS/protein mixture is separated by electrophoresis in a polyacrylamide gel containing a low concentration of gelatin (less than 1%). The proteinase/SDS complex does not bind to the gelatin, as would a free proteinase, and migrates as a narrow band. Subsequent incubation in a non-ionic detergent, such as Triton X-100, removes the SDS from the proteinase and reconstitutes its activity. The reactivated proteinase digests away part of the gelatin and its position can be detected by subsequently staining the gel. The proteinase-digested gelatin appears as a clear band on the blue stained gel.

5.9 Pore gradient gel electrophoresis

In pore gradient electrophoresis[14,15], the separation is carried out in a direction in the gel in which the gel concentration increases and its pore size decreases. Proteins migrating along the gel concentration gradient will encounter an increasing frictional force, due to gel sieving, which will increasingly impede their progress. Eventually the proteins will cease migrating at a position where the gel pore size becomes smaller than their diameter. As different proteins will have different diameters, they will

each reach a different position on the gel. By reference to standards, MWs can be calculated.

5.10 Isoelectric focusing

Of the separation methods based upon gross physical properties such as charge or size etc., isoelectric focusing[16,17] is one of the most discriminating. Only methods based upon some biological property, which requires a subtle stereospecific relationship, are more discriminating. Sometimes IEF can almost be **too** discriminating because multiple bands can be obtained from what is essentially the same glycoprotein species, with only small differences in glycosylation a phenomenon known as "micro-heterogeneity". A downside of IEF is that it is a relatively expensive, because of the cost of the ampholytes required.

Proteins are ampholytes having a pH-dependent net charge, which is positive at pH values below the proteinís PI and negative at pH values above the PI.

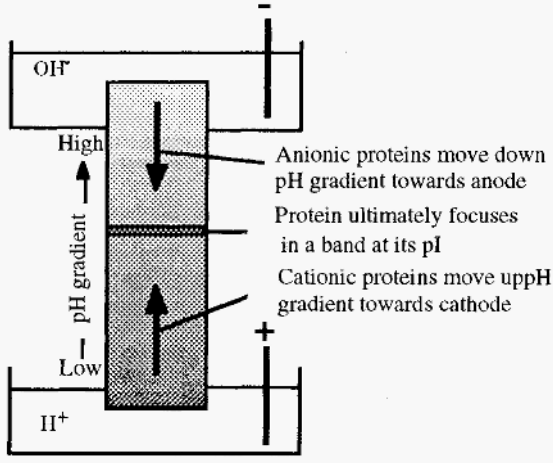


Figure 86. Schematic view of isoelectric focusing.

If proteins are distributed throughout a solution in a pH gradient, then upon application of an electrical potential across the gradient, with the anode at the low pH end, molecules in the low pH zone (which will be positively charged) will migrate to the cathode. Their migration will take them through zones of increasing pH, as a consequence of which they will gradually lose their positive charge and their rate of migration will slow

down. Conversely, molecules in the high pH zone will be negatively charged and will consequently migrate, through zones of decreasing pH, towards the anode. When each protein reaches a position where the pH is equal to its pI, it will lose all of its charge and its migration will cease. After a sufficient time, therefore, the respective molecules will in consequence become "focused" at their isoelectric points. In this way, a mixture of proteins can be separated, as each will focus at a characteristic pI, Furthermore, the pI values can readily be measured in this way. In practice, three difficulties must be overcome:

- A stable, uniform pH gradient must be established and maintained.
- The system must be stabilised against disturbances due to convection.
- A system must be devised for the measurement of the pH gradient and for determination of the positions of the focused bands.

5.10.1 Establishing a pH gradient

If a pure ampholyte, such as a protein, is added to pure water, the water will acquire a pH equal to the isoionic point of the ampholyte, which for most practical purposes is the same as the pI of the ampholyte[18]. So, a stack of ampholytes of increasing pI, arranged one on top of the other, would constitute a pH gradient.

Electrophoretic mobility is also a function of pI and, as has been outlined in the discussion of isotachophoresis (Section 5.7.1.1), it is possible to electrophoretically stack ampholytes in order of their mobilities. However, in isotachophoresis a buffer is present to control the pH. If there were no buffer present, except the ampholytes, then in arranging themselves in order of mobility they would simultaneously generate a pH gradient, the pH at each point corresponding to the pI of the ampholyte at that point. Since each ampholyte would finally be at its pI, where it has no net charge, there should theoretically be no net movement of the pH gradient.

If the ampholytes making up the pH gradient were proteins, the gradient would have a few steps (as many as there are proteins), but these steps would tend to be quite large (Fig. 87). However, if synthetic, randomly substituted, polyamino-polycarboxylic acid ampholytes were used (see p133), then there would be a very large number of very small steps, which in effect gives a smooth pH gradient. A protein introduced into such a gradient will cause a plateau to be formed at its pI, the length being proportional to the amount of protein (Fig. 88).

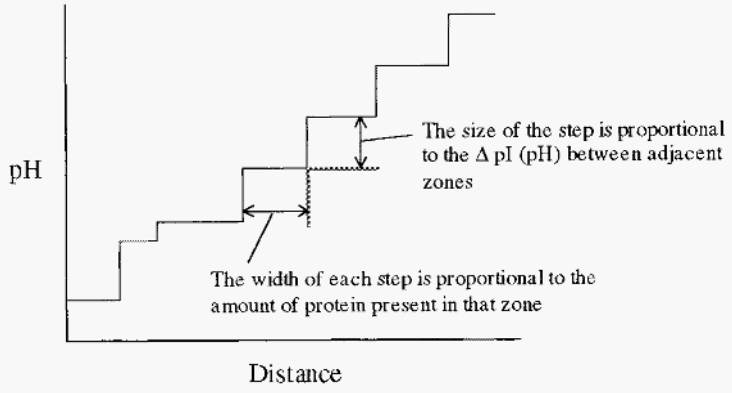


Figure 87. A pH gradient constructed from a stack of seven proteins.

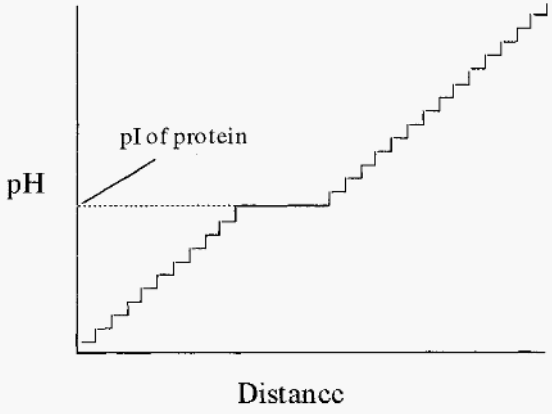


Figure 88. A pH gradient constructed from randomly synthesised polyamino-polycarboxylic acid ampholytes (and containing one protein).

The anode solution is acidic and the cathode solution is basic. Consequently, ampholyte molecules immediately in contact with the anode solution will be positively charged and those in contact with the cathode solution will be negatively charged. At zero time the pH distribution across the apparatus will be low at the anode end and high at the cathode end, but with a central plateau, corresponding to the pH of the sample plus mixed ampholytes (Fig. 89). When the electrical potential is applied across the electrodes, ampholytes will move to the anode or cathode, depending upon their charge, until they reach a pH at which they will have zero charge. Each ampholyte species will establish

the pH at its pI and so with time the ampholytes will arrange themselves into an order of charge and in doing so will establish a smooth pH gradient (Fig. 89).

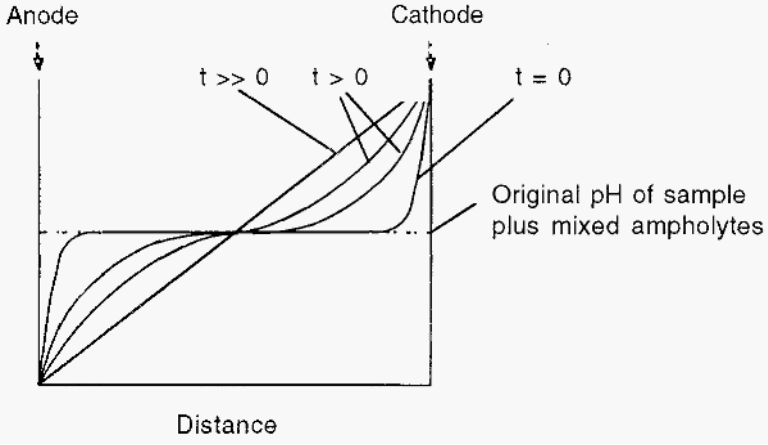


Figure 89. Time course of the establishment of a pH gradient in IEF.

Sample proteins added into this gradient will participate as ampholyte species and each will focus at its particular pI, introducing a small plateau in the gradient. Generally, it is desirable that samples are added in a way that avoids their exposure to the extreme pH values near the electrode solutions. This consideration has made open, flat bed systems popular as the pH gradient can be established and the samples can subsequently be applied at a position away from the electrodes.

One of the reasons for the discriminating power of IEF is that the principle of its operation intrinsically counteracts the effects of diffusion, which in other methods is responsible for the broadening of bands with time. Any protein which diffuses out of a focused band will enter a region of different pH where it will acquire a charge. In consequence it will immediately experience an electrophoretic force tending to move it back into the focused band. In this way the band is kept focused.

The steepness of the pH gradient, and its consequent resolving power, can be altered by using ampholytes covering different pH ranges. The range pH 3-10 is used first to get an overview of where the proteins focus and an appropriate choice of ampholytes can be made for a second round of focusing over a smaller range, say pH 5 to 7. The smaller the pH range covered, the greater will be the resolution.

5.10.2 Control of convection

Convective disturbances are currents induced in fluids by the effects of gravity upon fluids which differ in density in different parts. In IEF they are caused by heating effects, which reduces the density of the heated solution, and from the fact that focused protein bands are more dense than the surrounding ampholyte solutions. Practical ssstems consequently require some way of obviating these convective disturbances.

Since convection depends upon differences in density, the effect of gravity and a consequent movement of fluid, to avoid convection any one or more of these three elements could be targeted. Thus, an early approach was to conduct IEF in a sucrose gradient, thereby pre-imposing a density gradient which would damp out any convective disturbances. To eliminate the effects of gravity, IEF may be conducted in a rotating tube, so that the gravity vectors cancel out with time, or, at somewhat greater expense, the experiment may be conducted under microgravity conditions in outer space. Finally, because convective disturbances are a consequence of fluid movement, another approach is to have a system where the liquid cannot move, such as in a gel, and a popular modern method is to conduct IEF in a gel slab.

5.10.3 Applying the sample and measuring the pH gradient

In any practical system, all of the attendant problems must be solved simultaneously. Thus, in addition to controlling convection, the system must allow for sample application - avoiding the pH extremes - and for measurement of the pH gradient after the separation. Different systems are suitable for analytical and preparative purposes and one of each will be briefly described, by way of illustration.

5.10.3.1 An analytical IEF system

The most common analytical system in use at present is the thin slab gel system. In this a thin layer of gel - either large pore polyacrylamide or agarose - containing an appropriate ampholyte mixture, is cast onto a backing sheet of Gel-Bond®, a product of FMC Inc. The sheet is positioned on top of a template on a cooling block. The template marks sample tracks, and serves as a guide to the application of the sample Wicks, made of several layers of filter paper, are impregnated with the appropriate acid or base electrode solution and placed on top of the gel at each end (Fig. 90). When the apparatus is sealed, electrodes contact the

filter paper wicks, thereby closing the electrical circuit (this description is based on the Pharmacia Biotech Multiphor apparatus).

The pH gradient is allowed to become established before the sample is applied. Sample is applied to the gel by carefully laying small rectangles of filter paper, impregnated with sample solution, on the gel over a track mark on the template. If necessary, the same sample solution can be applied at different positions, i.e. at different pH values, on the gel.

Samples are drawn out of the filter paper and into the gel by electrophoresis and by diffusion. When this has happened the potential is switched off and the sample applicator papers are removed, so that they do not subsequently distort the electrical field.

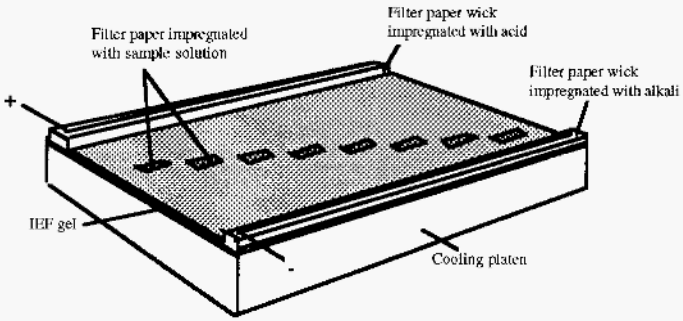


Figure 90. Sketch of apparatus used for analytical flat bed IEF.

After focusing is complete, the gel is removed and stained to reveal the position of the protein bands. Ampholytes are polyamino compounds and at most pH values they will react with and precipitate dyes such as Coomassie blue. To obviate this, the ampholytes can be removed by washing the gel in trichloracetic acid before staining. A simpler method, however, is to use the principle of the Bradford assay for protein (see Section 2.2.4), i.e. to stain with Coomassie blue G-250 in acid solution[19]. Using this approach only the proteins are stained and the ampholytes do not interfere.

In order to determine the pI values of the separated proteins, it is necessary to measure the pH gradient. This can be done, after focusing but before staining, by using a surface-probing pH electrode. However, as with other techniques, once the values for a few proteins have been established, these can be used as standards, and the values of unknowns can be determined by interpolation. Standard proteins, of known pI values can thus be run in parallel with unknowns - on the same gel but in different tracks - and a standard curve of pH vs distance can be constructed from the standards and used to determine the pI values of the

unknowns. A table of pI values of standard proteins has been published by Chambers and Rickwood[20], and pI calibration kits are commercially available.

5.10.3.2 Preparative IEF

Preparative IEF[21] differs from analytical IEF in that it is done on a much larger scale, i.e. with much more sample and, as the products sought are active protein fractions, provision must be made for recovery of the separated fractions after IEF. The central problem with preparative IEF is stabilisation of the system against convection during the focusing process while still being able to elute the separated components at the end of the process, Different approaches to the solution of this problem have been adopted by different authors and by the makers of commercially available apparatus. The most common approach is to conduct the focusing in a convection-stabilised liquid phase. With the proteins and ampholytes being recovered in solution, measurement of the protein concentration and pH of each fraction is relatively straightforward. An alternative approach, suited to smaller-scale preparative uses is to conduct the focusing in a slab gel, to cut out the focused band and electro-elute this[22,23]. In this case the pH gradient would be measured as described for analytical IEF.

The first approach tried, and commercialised by Pharmacia, was to conduct the focusing in a sucrose gradient, which could be eluted from the apparatus at the end of the experiment. An ingenious, but expensive, apparatus had provisions for cooling the annular focusing column on both its inner and outer surfaces, and valves to isolate the electrode solutions during the elution phase.

An approach used by LKB (which has since merged with Pharmacia) was to use a flat bed ofgranulated gel. After focusing, the gel bed could be divided into a number of segments, the gel from each segment being scooped out and packed into a mini-column from which the focused protein and ampholytes could be eluted.

In the Bio-Rad Rotoforô apparatus, convection is controlled by conducting the focusing in a horizontal column which is rolled at 1 rpm about its central axis. Rolling serves to negate the effects of gravity and enables the proteins to be focused in free solution. A central ceramic cold finger serves to remove heat. The column is divided into 20 segments by polyester membranes. After focusing, solution in the segments can be rapidly eluted from the side of the column into 20 test-tubes, without mixing. The Rotofor is currently a favoured apparatus for preparative IEF[21]

Although good results can be obtained by preparative IEF, and for many separations it may be the only practicable method, the technique is constrained by the high initial cost of the apparatus and the cost of the ampholytes consumed in each experiment.

5.11 2-D Electrophoresis

In 2-D electrophoresis, proteins are separated in the first dimension, according to their isoelectric points, by IEF, and in the second dimension, according to their molecular weights, by SDS-PAGE[24,25]. The first, IEF, stage is conducted in a long, thin, spaghetti-like gel. This gel must be of large pore-size so that focusing is possible, but this makes it very soft and fragile. For the second stage the long, thin, gel is transferred to the origin of a slab gel, sealed in position and an SDS-PAGE separation is carried out. The result, after staining, is a number of spots distributed in two dimensions over the slab gel. Because the first-stage gel is of a small diameter, only a small amount of ampholytes is consumed per run and so 2-D electrophoresis is relatively inexpensive.

Although conceptually simple, it took a number of years for the method to be developed to its present stage of practicability. One of the technical difficulties to be overcome was the handling and transferral of the first-stage gel soft spaghetti is not the easiest type of material to handle!

5.12 Non-linear electrophoresis

Electrophoresis can be visualised by an analogy. Imagine spherical glass marbles rolling down a slope in a tank of syrup. The profile of the slope could be described by plotting contour lines. The marbles would move very slowly because of the viscosity of the syrup and so they would essentially acquire no momentum. As a result they would always move in the direction of the slope, even if this was to change its direction, i.e. the locus of any one marble would always be at right angles to the contour lines. The locus would reflect the direction of the force vector acting upon each marble and the magnitude of the force would be inversely proportional to the spacing between the contour lines.

In electrophoresis, the equivalent of contour lines would be *isovoltage contours* and the loci of ions undergoing electrophoresis would correspond to *field lines,* which are always at right angles to the isovoltage contour lines.

In most forms of electrophoresis, the field lines are straight and the isovoltage contours are evenly spaced. In the marbles-in-syrup analogy,

this is equivalent to the marbles moving down a straight plane surface, inclined at an angle to the horizontal. However, deviating from this ìstraight and narrowî approach can be instructive and one can pose a number of "what if?" questions to probe oneís own insight into electrophoresis, e.g.

What if the gel was of increasing cross-section, i.e. either conical or wedge-shaped?

What if the gel was not straight, but went through a 90 bend?

What if the gel had a hole cut in it (say if there was a pillar passing through the gel)?

These questions have been explored theoretically and empirically by Dennison *et al.*[26,27].

If the gel were conical or wedge-shaped, the voltage gradient *(dV/dx)* would be steeper at the narrow end and shallower at the wider end. The net result is that the migration of slower ions would be increased, as they would spend more time in the steep part of the gradient, whereas that of faster ions would be slowed as they would reach the shallower parts the gradient more quickly. Normally, in gel electrophoresis the mobility of ions is a logarithmic function of their molecular weight, so that small ions are separated better than larger ions. In a wedge-shaped gel the relationship is made more linear[26]. Although this result has not found much utility in the separation of proteins, wedge gels have proved useful in the separation of nucleic acids. In DNA sequencing gels, a greater number of bases can be sequenced in a wedge gel compared to a straight-sided slab gel[28-30], and the separation of plasmids is also improved in wedge-shaped gels[31]. The wedge-gel concept has been extended to preparative electrophoresis by Rolchigo and Graves[32], and to IEF by Pflug[33].

An empirical analysis of electrophoresis around corners was done by Dennison *et al.*[27] (Fig. 91). Around a comer, the isovoltage contours remain as straight lines but become closer together on the inside of the corner than on the outside, rather like the steps in a spiral staircase. As a result, the voltage gradient is steeper on the inside of the corner and the proteins on this side will accelerate when negotiating the corner. By contrast, the isovoltage contours are further apart on the outside of the corner and the proteins will slow down. The net result is that the protein band will become skewed as it rounds the comer (Fig. 5.19a). Negotiating a second comer in the opposite direction does not ìundoî the distortion[27].

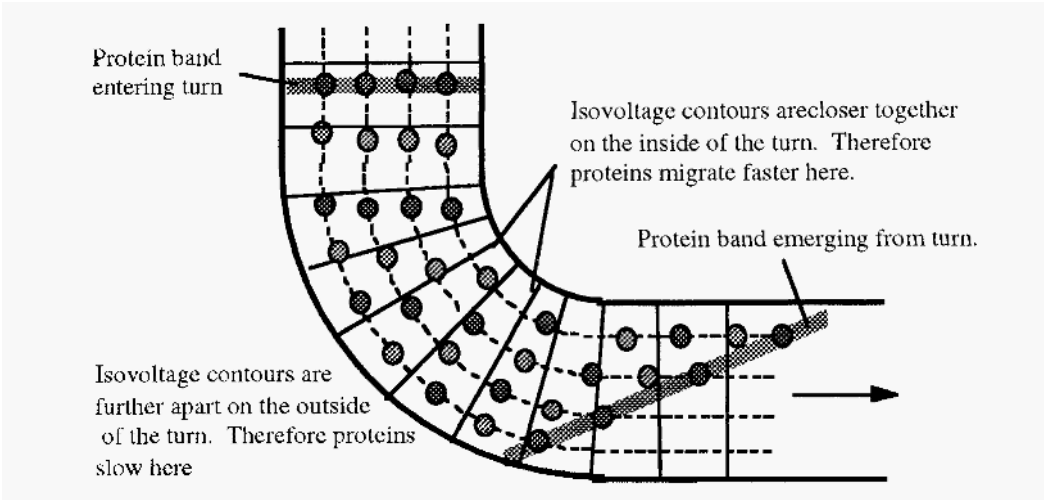


Figure 91. The effect of a bend in the gel upon elcctrophoretic behaviour in PAGE. From Dennison *et al.*[27],

Electrophoresis around a circular obstruction in the gel is shown in Fig. 92. The isovoltage contour lines (shown as solid lines) can easily be visualised, remembering the fact that they are always at right angles to the field lines (shown as dotted lines). An interesting conclusion was drawn from these experiments, i.e. that the equations describing the behaviour of ions undergoing electrophoresis are identical to those describing the irrotational flow of an ideal incompressible fluid. Ideal fluid behaviour was previously considered to be only an abstract concept; in an ideal fluid the molecules have no momentum.

If one has a playful nature, the shapes of the voltage gradient surfaces can be visualised on a visit to the beach. Pouring loose beach-sand down a slope, in which a circular obstruction has been placed, will form a contoured surface around the obstruction. In oneís mindís eye one could imagine this surface in the tank of syrup and visualise the movement of marbles down the surface it will be found to be identical to the behaviour of ions around the circular obstruction shown in Fig. 92!

To summarise, the following points can be noted about non-linear electrophoresis:

- Field lines pass smoothly around comers or obstructions and resemble "streamlines".
- Ions will migrate along field lines, i.e. the field lines will represent the loci of migrating ions.
- Isovoltage contour lines are always at right angles to the field lines.
- Ions will migrate quickly where the isovoltage contour lines are close together (i.e. where the voltage gradient is steep) and slowly where the

isovoltage contour lines are far apart (i.e. where the voltage gradient is shallow).

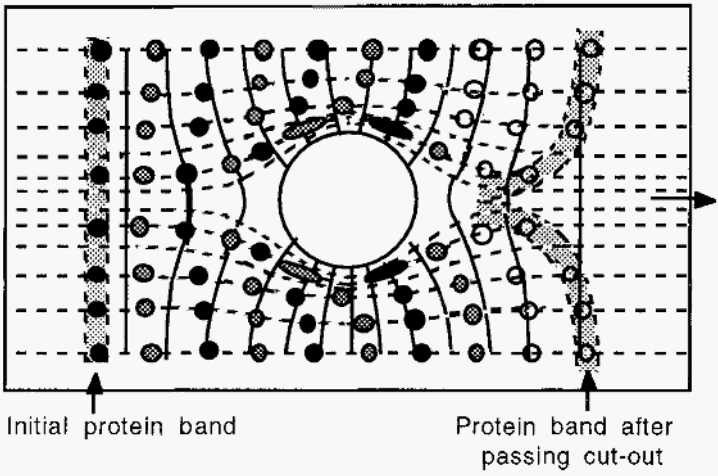


Figure 92. The effect of a circular hole in an electrophoresis gel.

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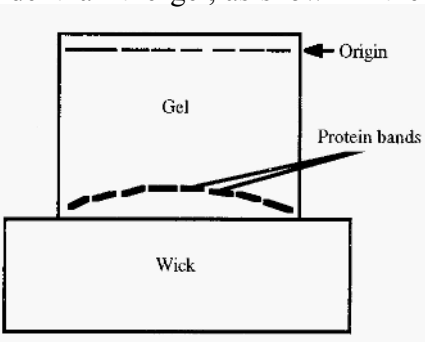
5.13 Chapter 5 study questions

1. Express in words the effect in the electrophoresis of a protein of, a) increasing the steepness of the voltage gradient, b) increasing the charge on the protein.
2. A protein is moving by electrophoresis through a buffer which does not contain sucrose. Explain what would happen if the protein migrated into a region of buffer containing, say, 20% sucrose.
3. If the cross-sectional area of a gel is decreased, what will be the effect on its electrical resistance?
4. In an electrophoresis system, at equal ionic strength, the conductivity of a smaller ion is greater/smaller than that of a smaller ion and this will cause the protein ions to have a lower/higher mobility (select the correct word in each case).
5. Towards which electrode does the buffer tend to flow, in most forms of electrophoresis, and what is this due to?
6. Answer True or False. i) Water can siphon through agarose gel, ii) SDS-PAGE is a useful preparative technique, iii) In disc-PAGE the anode must be in the lower vessel.
7. Name one advantage of polyacrylamide over starch, as a medium for electrophoresis.
8. What will be the pH of a solution of a pure protein dissolved in pure water?
9. In disc-PAGE, a) What is the function of the stacking b) What is the function of the running gel?
10. An appropriate standard curve applicable to SDS-PAGE, is _____ vs _____ ?
11. In relation to that measured by MEC, the *MW* measured by SDS-PAGE will always be smaller/smaller-or-equal/larger/larger-or-equal?
12. What are "ampholytes"?
13. What function do ampholytes play in isotachophoresis?
14. What is the object of using discontinuous gel and buffer systems in disc-PAGE?
15. List three problems which must be addressed in the design of a practical IEF system.

16. In a PAGE experiment in which the gel slab is 10 cm long, a potential of 300 volts is applied, giving a current of 30 mA. a) What is the voltage gradient? b) If a further, identical, gel slab is run in parallel with the first and the current is kept constant at 30 mA, what will the new voltage gradient be? c) Qualitatively, what would happen to the voltage gradient if a smaller buffer ion was used?
17. A standard mixture, comprised of six proteins of known MW (12.9, 16.6, 21.6, 31.2, 40.5, 59.4 kDa), plus bromophenol blue, was subjected to SDS-PAGE, in parallel with a purified unknown protein. Under non-reducing conditions the standard mixture gave six stained bands, 1.1, 1.8, 2.6, 3.8, 4.4 and 5.0 cm from the origin, while the unknown yielded one band 1.5 cm from the origin. The bromophenol blue, in each gel, migrated 6.1 cm. Under reducing conditions, the standard mixture gave seven stained bands, 1.2, 2.7, 3.5, 4.0, 4.6, 4.8 and 5.2 cm from the origin, while the unknown yielded two bands, 3.1 and 3.7 cm from the origin, respectively. The bromophenol blue, in this case had migrated 6.4 cm in each gel. a) Interpret these results, in quantitative terms, b) Explain what you understand by, "reducing" and, "non -reducing" conditions.
18. Can water move through a gel under the influence of gravity?
19. Can water move through a gel under the influence of an electric field? Explain.
20. How can the pore size of a gel be varied?
21. What is meant by a, "macroreticular" gel?
22. You are running an SDS-PAGE experiment. In a previous experiment you observed that, at a potential of 90 volts, the bromophenol blue marker migrated 35 mm in 1 h. In your present experiment, you are using a potential of 150 volts and the bromophenol blue has 50 mm to go, before reaching the end of the gel. You wish to leave the lab, to go for supper: how long can you afford to be away?
23. Joe was conducting an electrophoresis experiment in a flat bed system, with the ends of the gel connected to the electrode buffers with filter paper wicks. Because he thought it wouldnít matter, Joe had made the filter paper wicks wider than the gel, as shown in the sketch below (only one end of the gel is shown). By an analysis of the field lines and the isovoltage contours, explain why this arrangement would lead to the protein bands becoming curved as shown in the sketch.



Chapter 6

Immunological methods

Antibodies are proteins made by the immune system of animals as part of a defence system against infection by foreign organisms. The immune system must be able to distinguish between "self" and "non-self", and to eliminate the latter, and antibodies play a role in this process. Because of their specificity and versatility, antibodies are also very useful reagents in the identification and analysis of proteins and it is largely in this light - as useful reagents - that antibodies will be considered in this chapter.

6.1 The structure of antibodies

Antibodies are a class of blood proteins known as γ-globulins. The most common γ-globulin is the IgG type, which consists of four peptide chains, two heavy and two light, held together by disulfide bonds. A schematic sketch of the structure is shown in Fig. 93.

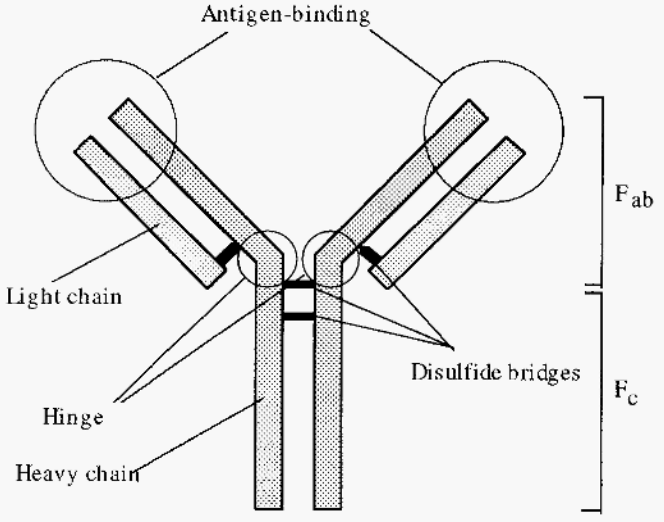


Figure 93. A simplified schematic representation of the structure of an IgG antibody.

The IgG molecule can be cleaved in the hinge region by papain, to yield three fragments, two F_{ab} fragments and one F_c fragment. "F_{ab}" stands for "fragment, antigen binding" and this reflects the fact that the outer aspect of the heavy and light chains, in the F_{ab} fragment, contain so-called hypervariable regions which constitute the antigen binding site. The hypervariable regions are different in the IgG molecules synthesised by different plasma cell clones, but will be identical in every molecule originating from a single clone of plasma cells (See Section 6.2). The hypervariable regions are constituted of loops at one end of a fl-barrel structureí, Such loops can vary without disturbing the underlying stability of the barrel structure. "F_c" stands for "fragment, crystallisable" and this reflects the fact that the F_c fragment is invariant and therefore can be crystallised, even when it is derived from a polyclonal antiserum.

In mammals, antibodies are transferred to the neonate in the form of colostrum, which is the first milk produced in the early post-partum stage. In birds the transfer of antibodies occurs via the egg yolk and egg yolk thus provides a convenient source from which antibodies may be isolated. The antibodies from egg yolk have a structure similar, but not identical, to IgG and are known as IgY antibodies.

6.2 Antibody production

The immune system is designed to ward off "foreign" invaders. Injection of a foreign molecule, usually a protein, into an animal elicits an immune response, which includes the production of antibodies which react with the foreign protein and target it for removal from the system. The foreign protein in this context used to be called an antigen, from antibody generator, but the terminology has changed and now the molecule which elicits antibody production is called an immunogen and the molecule with which the antibody reacts is called an antigen (Fig. 94).

Immunogen	elicits antibody production
Antigen	reacts with antibody

The terminology changed when it was realised that although the immunogen and the antigen are often the same molecule, sometimes they are not. Also, some molecules are antigenic (i.e. they react with antibodies) but are not immunogenic (i.e. they will not elicit antibody production when injected into an animal). The current hypothesis takes account of the fact that the immunogen and the antigen may or may not be the same molecule.

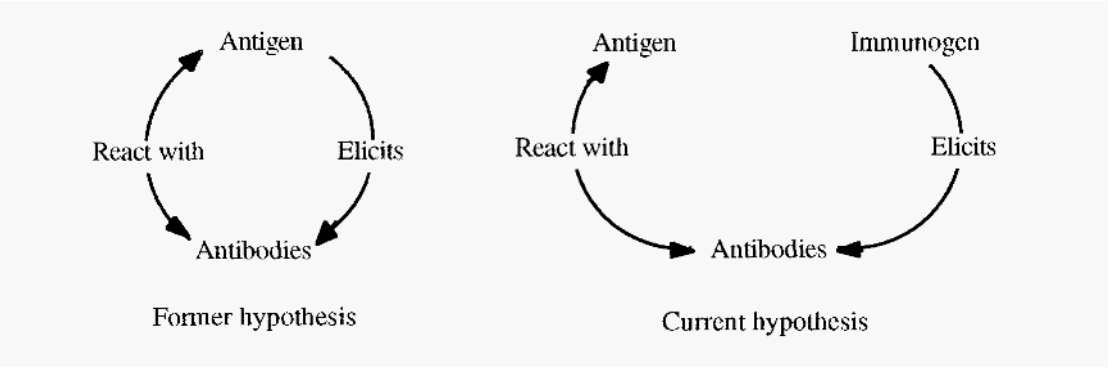


Figure 94. The relationship between immunogens, antibodies and antigen.

Many small molecules will not by themselves elicit antibody production but when conjugated to a larger molecule they will elicit antibodies, which will react with the unconjugated small molecule. Such small molecules are known as *haptens.* The cut-off in size is not absolute and experience with peptide antibodies (antibodies elicited by peptide immunogens) suggests that it may be that smaller molecules simply take *longer* to elicit antibodies. For example, free peptides are often able to elicit antibodies, but the antibody titre takes longer to rise than when a peptide conjugated to a carrier protein is used as the immunogen.

Haptens are small molecules which can react with specific antibodies but which, by themselves, cannot elicit antibody production.

A single injection of an immunogen is not optimally effective in eliciting antibody production. In a natural infection, molecules from the infecting agent will leak from the site of infection to become exposed to the immune system in small amounts, but over an extended time. To elicit antibodies, this natural process must be mimicked. One way would be to inject a small amount of immunogen at a time and to repeat the injection many times over a time period. This would work, but it would subject the animal to unneccessary trauma, which is ethically unacceptable. Another way would be to make an emulsion of the aqueous antibody solution with an adjuvant oil and inject this subcutaneously or intramuscularly. The emulsion is made by a process known as *trituration.* The injected emulsion would exist at a focal site, mimicking a natural infection, and would break down slowly over time, thereby slowly releasing the immunogen and exposing it to the immune system over a

An *adjuvant* slowly leaks the immunogen for exposure to the immune system in small amounts over a period of time.

period. This is the principle of Freund's incomplete adjuvant. However, the immune system is particularly geared to combating microbial infection and its response is stimulated by the presence of components of the bacterial cell wall. Freundís complete adjuvant thus contains bacterial cell wall components, in addition to an emulsifying oil.

The first injection of an immunogen gives a relatively small response and the antibodies produced are of the high molecular weight IgM type (IgM antibodies are comprised of five subunits, each equivalent to a single IgG molecule, joined together). This is known as the primary response. Further inoculations with the same immunogen gives a much greater secondary response, in which mainly IgG-type antibodies are produced (Fig. 95).

Antibodies arc made by a class of white blood cells (leukocytes) known as *B-cells.* In any one animal there are a large number of different B-cells, each capable of making a single type of antibody molecule. Each B-cell carries an "advertisement" γ-globulin on its surface and interaction of this molecule with an antigen molecule causes that B-cell to divide into a clone of similar B-cells, a process known as *clonal expansion.* Some of these B-cells mature into antibody-producing *plasma cells* and some remain as memory cells. The existence of an expanded clone of memory cells accounts for the faster and more extensive secondary response.

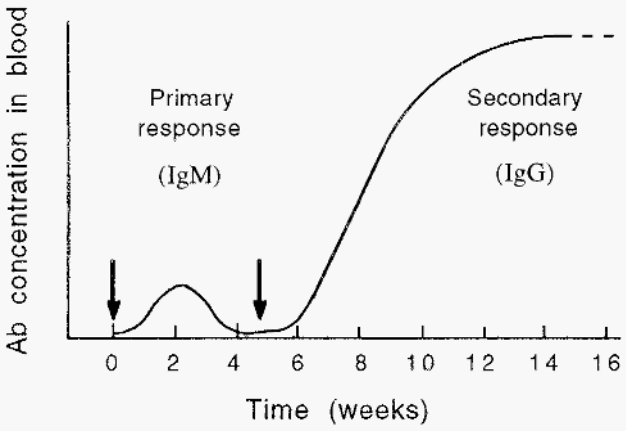


Figure 95. Time-course of the immune response.
Arrows indicate inoculations with immunogen.

6.2.1 Making an antiserum

An antiserum, or an antibody isolated from it, constitutes a useful reagent in protein biochemistry. However, it is a reagent which is specific to the immunogen/antigen couple and often it must be prepared in-house, especially when a novel protein is under investigation Preparation of an antiserum starts with the immunogen, which is usually a protein isolated by one or more of the methods described in the previous chapters. The more pure the immunogen, the more specific will be the antibodies which it elicits. For most purposes, therefore, it is best to use as pure an immunogen as possible.

Alternatively, for the production of so-called peptide antibodies, the immunogen might be a synthetic peptide of ten or more residues. The peptide is chosen from the amino acid sequence of the Ag of interest, i.e. the complete protein which it is hoped the Abs will recognise. For peptide antibodies to recognise the whole protein, it is necessary that the peptide sequence chosen be accessible, i.e. it must be on the surface of the protein. This can be readily determined if the 3-D structure of the protein is known. If the 3-D structure is not known, then the peptide can be chosen by analysis of the amino acid sequence of the Ag of interest for hydrophobicity[2,3] (since hydrophilic residues will tend to be on the surface) or mobility[4] (since residues on the surface, and especially at the N- or C-terminus are likely to be more mobile).

For inoculation into an animal, the immunogen must be emulsified with Freundís complete adjuvant and this can conveniently be done by trituration in an apparatus such as shown in Fig. 96. In this device, the solutions are emulsified by passage back and forth between two syringes, through a fine stainless steel mesh.

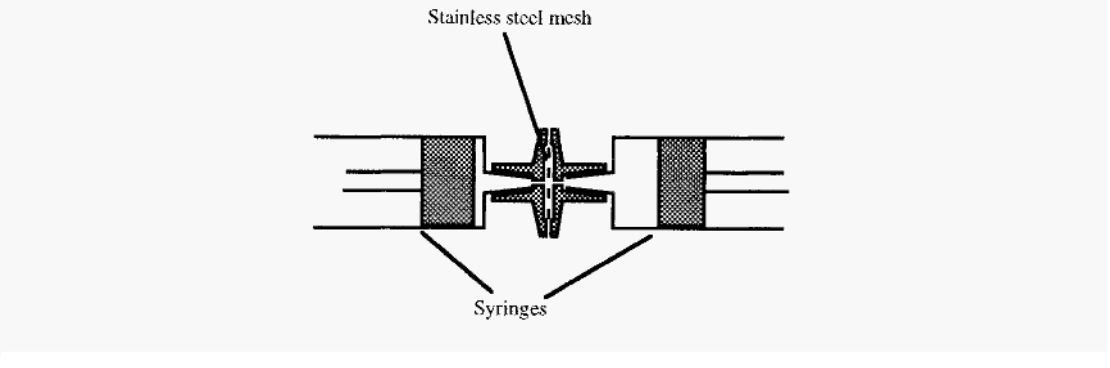


Figure 96. A device for emulsifying antibody solution with adjuvant oil.

The triturated immunogen/adjuvant emulsion may be injected subcutaneously in a rabbit, or into the breast muscle of a laying hen. Animals have an idiosyncratic response to immunogens and so it is best to use at least two animals, in case the one is a poor responder this is provided sufficient immunogen is available, of course. A typical inoculation schedule would involve injection of $50 \rightarrow 100$ μg of protein per time, first in Freund's complete adjuvant, followed at one, two, four and six weeks thereafter by further inoculations in Freundís incomplete adjuvant. If necessary, further booster inoculations can be given at monthly intervals. In the case of rabbits, blood samples are taken immediately before each inoculation, so that the increase in antibody titre with time can be followed. An illustrative example of the increase in antibody titre with time is shown in Fig. 97.

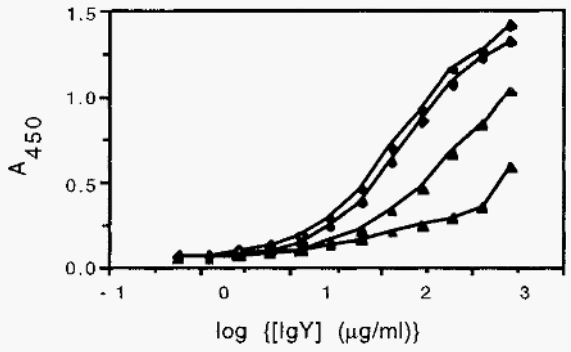


Figure 97. A typical ELISA of the progress of an immunisation. The open triangles represent preimmune serum and the other three symbols represent serum at 2,4 and 6 weeks.

An advantage of using hens for antibody production is that it is not necessary to bleed them in order to harvest the antibodies. It is, of course, necessary to bleed rabbits and this may be done by warming one of their ears with a hot, wet towel, in order to dilate the blood vessels, and nicking the peripheral ear vein with a sharp scalpel blade. About 25 ml can be collected from a rabbit at one time. The blood is best collected into a clean, dry 25 ml conical flask as this has an almost optimal ratio of volume to surface area and the exposed area is also minimal. With a high volume to surface area ratio, the clot can more easily contract away from the vessel walls, thereby obviating tearing of the clot and lysis of the red blood cells. Optimal clot formation is promoted by incubation overnight at 4 C. Ideally, no haemolysis should occur and the serum

should be a pale straw colour. It may be harvested by careful aspiration with a Pasteur pipette.

An IgG preparation may be isolated from the antiserum, or IgY isolated from egg yolks, by precipitation with polyethylene glycol[4,5] (See Section 3.5).

6.3 Immunoprecipitation

Immunoprecipitation is the basis of a number of analytical techniques, which will be discussed below. Many of these techniques are very ingenious, but they are now mostly of historical interest only. The reason is that immunoprecipitation requires relatively large amounts of antibody and antigen in order to form a visible immunoprecipitate, i.e. it is not a very sensitive technique, and so it has largely been replaced by techniques which involve an amplification step to make the Ab/Ag reaction more easily detected.

The paratope of an antibody and the complementary epitope on an antigen have a very specific stereo relationship with one another. If an antigen contains at least two epitopes, it may form a precipitate upon reaction with its specific (polyclonal) antibodies at optimal proportions. Monoclonal or peptide antibodies, which target only a single epitope, will not form an immunoprecipitate, as they will not be able to form the extended network necessary. The mechanism of formation of an immunoprecipitate is shown in Fig. 98. Formation of the matrix required for immunoprecipitation requires at least two epitopes, each targeted by a different antibody.

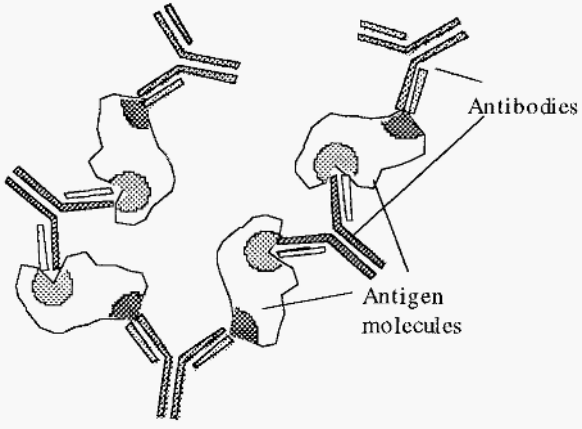


Figure 98. The formation of an immunoprecipitate.

Formation of an immunoprecipitate requires optimal proportions of antibodies and antigen and if either the antibody or the antigen is present in excess, then an immunoprecipitate will not form (Fig. 6.7).

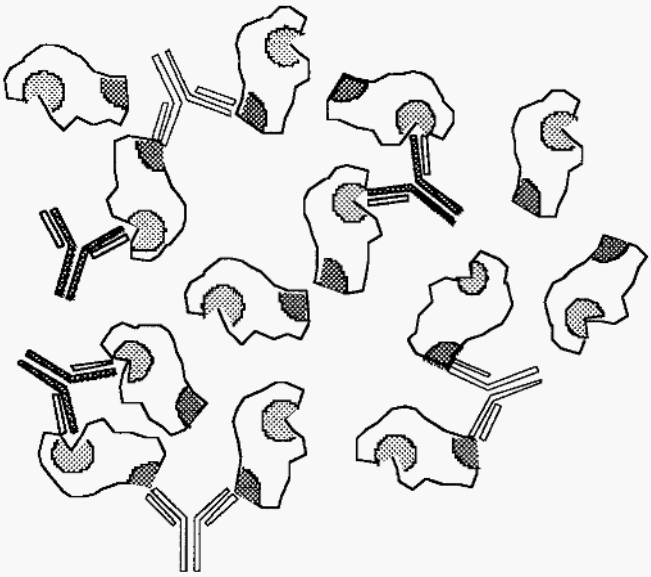


Figure 99. Formation of soluble complexes in the presence of an excess of antigen.

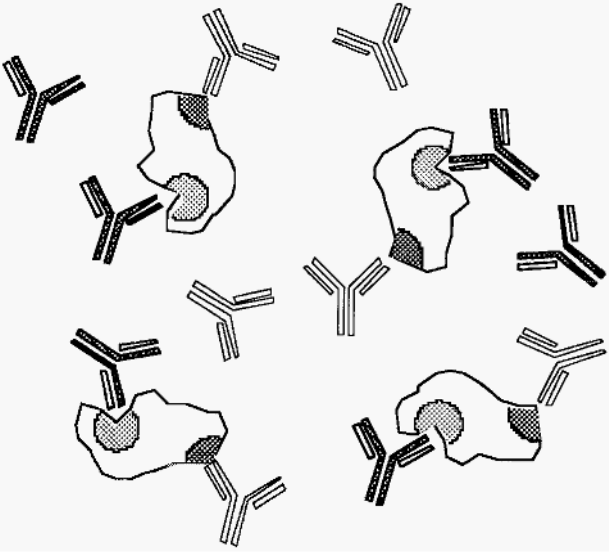


Figure 100. Formation of soluble complexes in the presence of an excess of antibody.

The dependence of immunoprecipitation on the proportions of Ab and Ag can be expressed graphically, as in Fig. 101.

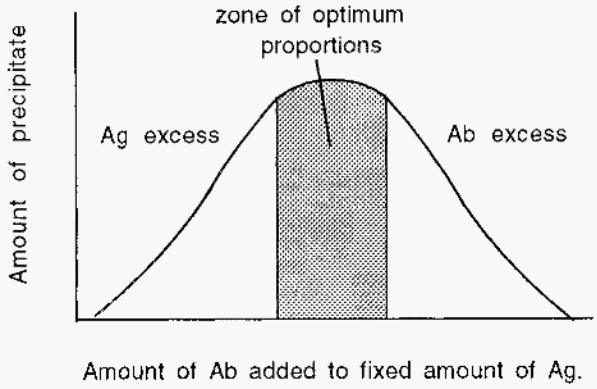


Figure 101. Immunoprecipitation at different proportions of Ab and Ag.

Often, with a novel combination of Ag and Ab, the optimal concentrations are not known. This has led to the use of diffusion techniques, where the diffusion of either the Ab or the Ag generates a concentration gradient of that molecule. The optimal concentration will occur somewhere along the concentration gradient and will lead to formation of an immunoprecipitate at that point.

Immunoprecipitation is also affected by pH and in general precipitation will not occur substantially outside of the range pH $5 \rightarrow 9$.

6.3.1 Immuno single diffusion

Immunodiffusion is usually conducted in macroreticular agarose gels, which serve to stabilise the system against flow-induced disturbances, such as convection, but do not impede diffusion. In immuno single diffusion, the one component is present throughout the gel at a constant concentration, while the other component diffuses into the gel from solution (Fig 6.10).

In immuno single diffusion, the precipitate band moves further into the gel with time, due to the continual diffusion into the gel of the component originally present in solution. The precipitate band also tends to be indistinct as it is spread over an area, with the precipitate band being formed on its leading edge and redissolving on the trailing edge. This “fuzziness” of the bands makes it difficult to determine how many precipitate bands there may be.

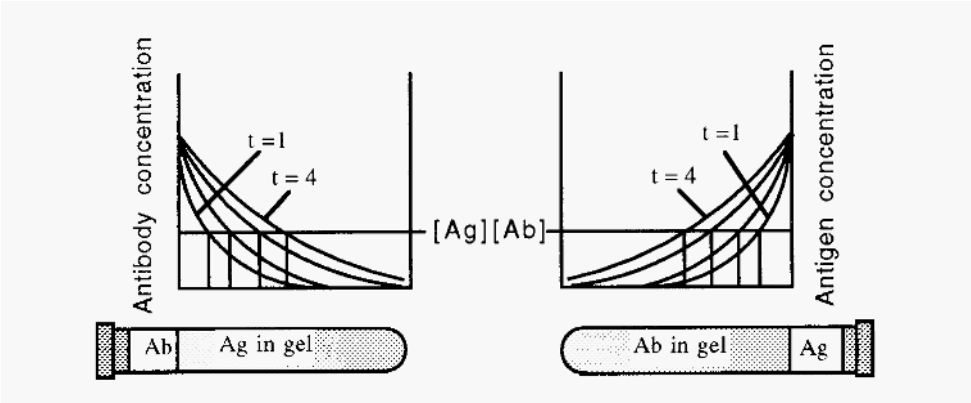


Figure 102. Immuno single diffusion.

The Ag or the Ab is present throughout the gel at a constant concentration and the other component is allowed to diffuse into the gel from solution, where it is present at a higher concentration. Immunoprecipitation will occur at the positions where the Ab and Ag are present in equivalent concentrations (indicated by the vertical lines in Fig. 102). As the one component will continue to diffuse into the gel over time, the position of the immunoprecipitate will move further into the gel with time and the precipitate will not form a sharp line, as it will be re-dissolving on one edge and precipitating on the other.

6.3.1.1 Mancini radial diffusion

A practical, quantitative, single diffusion system is Mancini radial diffusion[6]. In this method the Ab is added to the gel and the Ag to a well cut into the gel. The Ag diffuses into the gel and forms a precipitate where it meets the Ab in optimal proportions, forming a circular precipitin line surrounding the central well. With time, the circular precipitate will grow in diameter until the supply of Ag is exhausted, at which point the growth in diameter ceases. The method gives a quantitative measure of the amount of Ag, because the more there is, the larger will be the diameter of the circle of the precipitin band surrounding the central well. A standard curve can be constructed from the diameters obtained with known concentrations of a standard Ag, and this can be used to determine the concentration of an unknown, from the diameter of its precipitin circle.

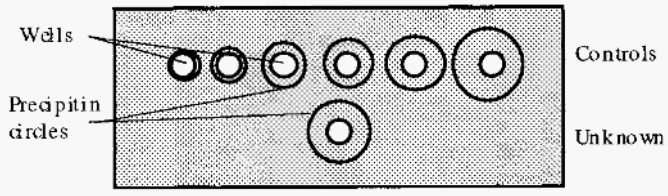


Figure 103. Mancini radial diffusion.

6.3.2 Immuno double diffusion

The limitations of immuno single diffusion can be overcome by using immuno double diffusion. In this case the Ab and the Ag diffuse into opposite ends of the gel and form a precipitate where they meet in equivalent concentrations. With time, the position of the immunoprecipitate will not change, but simply more precipitate will form at the same position as the Ab and Ag continue to diffuse into the gel (Fig. 104). This is assuming that the Ab and Ag continue to diffuse at the same relative rate, which they will do if the temperature is kept constant.

If there is more than one antigen/antibody couple present, then each of these systems will form their own precipitate band. If the antigens are of different sizes or shapes, they will diffuse at different rates and will form precipitate bands, at different places. (IgG antibodies are all of the same size and gross shape and so will all diffuse at the same rate). Because the precipitate band(s) formed by double diffusion are very sharp, different bands are easily distinguished from one another and so it is relatively easy to determine how many bands have been formed.

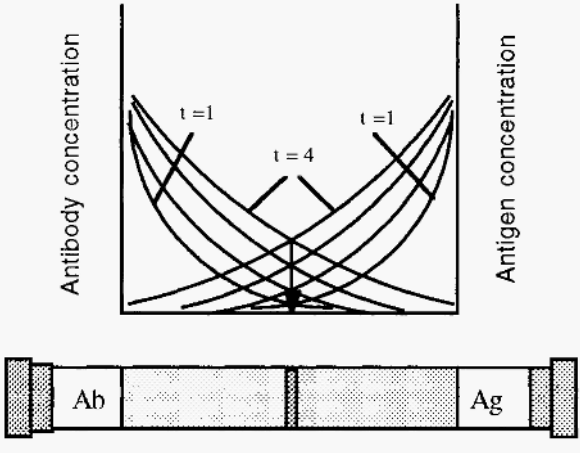


Figure 104. Immuno double diffusion.

6.3.2.1 Ouchterlony double diffusion analysis

Although the concentration gradients formed during immuno double diffusion enable the Ab and Ag to "find" one another at optimal proportions, sometimes the starting concentration of either the Ab or the Ag is out of range, i.e. is too high or too low, and so it is necessary, with an unknown system, to repeat the experiment using different Ab and Ag concentrations. An efficient way of doing this was devised by Ouchterlony[7]. In this method the gel is cast as a layer, 1→1.5 mm thick, on a support (a Petrie dish is convenient) and a pattern of wells (Fig. 105) is cut into it using a die and template. Ab and Ag are added to appropriate wells; typically, Ag may be added to the central well and a serial dilution of Ab added to the surrounding wells.

Fig. 105 shows a single central well surrounded by six circumferential wells, but the Ouchterlony technique is not limited to this arrangement. The pattern can be extended *ad infinitum,* to accommodate different Ab/Ag concentrations.

The serial dilution series is conveniently constructed in the wells of a microtitre plate. A constant volume, say 100 µl, is added to 5 wells of the plate, 100 µl of antiserum is added to the first well and mixed in, 100 µl of this mixture is transferred to the next well, mixed in, and 100 µl transferred to the next well etc., until the last well contains 200 µl of mixture containing 1/32 of the original concentration of Ab.

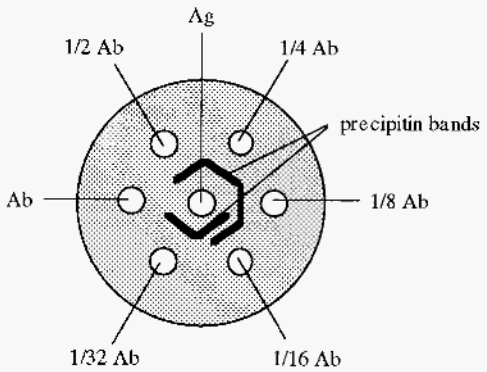


Figure 105. Ouchterlony double diffusion.

Three practical points must be borne in mind:-

- Development of the precipitin bands must be done at a constant temperature, usually 37 C, to prevent changes in diffusion rates, which can give rise to indistinct bands.

- To prevent the gel from drying out, it must be incubated in a sealed container with an atmosphere saturated with water vapour. A Tupperwareô-type container, containing several sheets of filter paper, saturated with water, is suitable.
- The gel and the Ag and Ab solutions must contain a preservative, such as merthiolate, to prevent microbial growth. A moist environment, at 37 C, in the presence of protein (the Ab and Ag) and carbohydrate (the agarose gel) is ideal for microbial growth.

Ouchterlony double-diffusion can be used in a test of identity of two antigens. If these are identical, their immunoprecipitin lines will fuse into a single line (Fig. 106 a) whereas if they are non identical their precipitin lines will cross (Fig. 106 b). Partial identity is indicated by a fused arc, with a spur (Fig. 106 c).

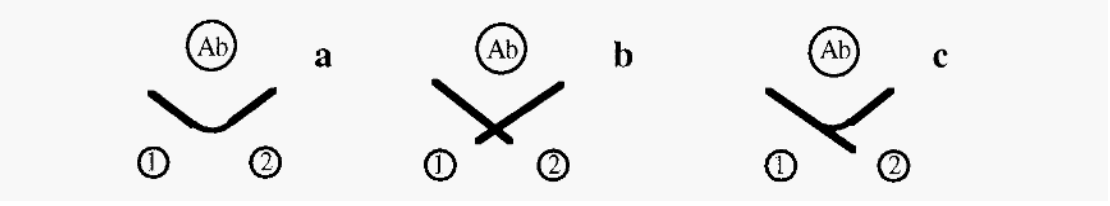


Figure 106. Test of identity using immuno double diffusion.

6.3.2.2 Determination of diffusion coefficients

As mentioned above, the development of different precipitin bands in double diffusion is due to differences in the rate of diffusion of different antigens, which is reflected in their different diffusion coefficients. Diffusion coefficients give information about the size and shape of molecules. Because the position of the precipitin band is a function of the diffusion coefficient of the antigen, immunodiffusion can be used to measure diffusion coefficients.

One such method, devised by Polson[8], uses a device which Polson refers to as his "mouth organ" apparatus (Fig. 107). This consists of four perspexô blocks, marked A, B, C and D, with holes drilled through three of them and part way into D. The blocks are able to slide relative to one another on joints greased with petroleum jelly.

With the blocks aligned, a serial dilution of Ab can be introduced into the wells in block D after which block D is moved to one side to seal the wells. Molten 1% agarose is introduced into the wells in block C and sealed offby moving blocks A and B to one side, before the agarose sets. Finally, Ag is added to the wells in block B and sealed off by moving block A to one side.

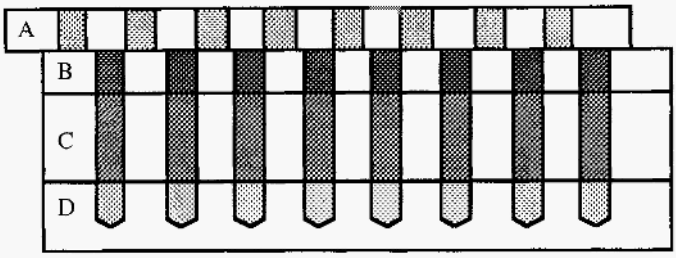


Figure 107. Polsonís ìmouth organî apparatus for the determination of diffusion coefficients by immunodiffusion.

The apparatus is incubated at 37 C and Ab and Ag diffuse through the column of agarose, of known precise dimensions, to form a sharp precipitin band where they meet in optimal proportions. Where the proportions are not optimal the bands are relatively fuzzy (Fig. 108).

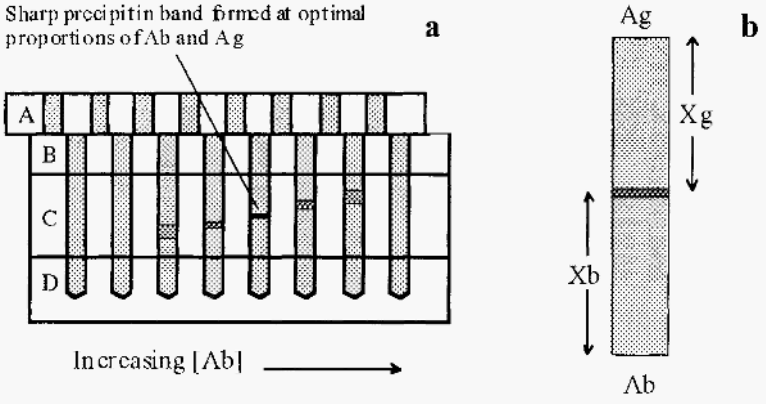


Figure 108. Quantitative immunodiffusion in Polsonís apparatus.

From the column in which the precipitin band is most sharp, the measurements Xg and Xb are taken (Fig. 108b). From these the diffusion coefficient of the Ag can be calculated by substitution in the equation[8]:-

$$\frac{Xg^2}{Xb^2} = \frac{Dg}{Db}$$

Where, Dg = diffusion coefficient of the antigen,
Db = diffusion coefficient of the antibody

Since all IgG molecules have essentially the same gross structure, they will all have the same diffusion coefficient. Db is thus a constant (4.6 x 10-7 $cm^2\ sec^{-1}$

Diffusion coefficients can also be measured by molecular exclusion chromatography, since the separating mechanism in this technique is also diffusiondependent. A standard curve of 1/D vs K_{av}, constructed using proteins of known diffusion coefficient (D), can be used to determine the diffusion coefficients of unknowns[9]. It is useful to have two independent measures of the diffusion coefficient - in this case by immunodiffusion and MEC - as the results from the different methods serve as a check on one another.

6.4 Immunoelectrophoresis

Immunoelectrophoretic methods combine electrophoresis with subsequent immuno-detection, and the conditions used are a compromise of the optimum for each of the two steps. A number of immunoelectrophoresis methods will be discussed below, as it is useful to have an appreciation of the principles of each method, but it must be emphasised that methods involving immunoprecipitation have today largely been replaced by methods involving amplification, most commonly with enzymes.

6.4.1 Cross-over electrophotesis

One of the limitations of the Ouchterlony double diffusion system is that a large proportion of the Ab and Ag diffuse in non-productive directions and are thus wasted. Only that proportion of the Ab that diffuses towards the Ag, and *vice versa,* will productively form immunoprecipitate.

It will be recalled from the discussion of paper electrophoresis that due to electroendosmosis the γ-globulins which are the antibodies migrate towards the cathode, whereas all of the other serum proteins migrate towards the anode (See section 5.3.1). For the analysis of blood proteins, this provides a way of ensuring that all of the Ag meets all of the Ab, by a process known as cross-over electrophoresis[10]. In this process, the Ab and Ag are placed in two wells in an agarose gel and subjected to electrophoresis as illustrated in Fig. 109. In this way all of the Ag encounters all of the Ab and wasteful diffusion is obviated. This may be useful when the amount of Ag available is limited, for example in forensics.

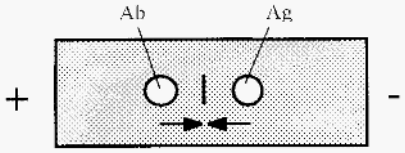


Figure 109. Cross-over electrophoresis.

6.4.2 Rocket electrophoresis

A limitation of Mancini radial diffusion (Section 6.3.1.1) is that differences in the diameters of the precipitin rings may be small. The reason for this is that diffusion is in all directions, and a consequence is reduced sensitivity In the "rocket" electrophoresis method of Laurell[11], diffusion of the Ag is replaced by electrophoresis of the Ag into the gel containing the Ab. Electrophoretic migration of the antigen occurs in one direction only so that immunoprecipitation, instead of occurring in a circle. occurs in a rocket shape (Fig. 110). hence the name.

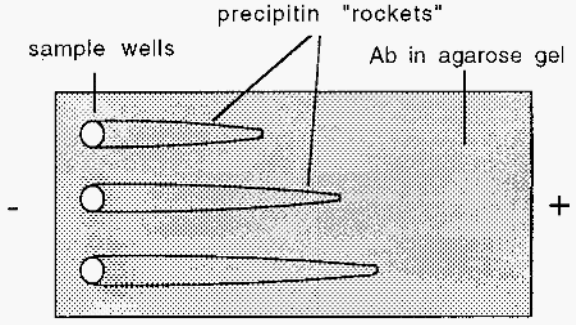


Figure 110. Rocket electrophoresis.

An advantage of rocket electrophoresis is that it is more sensitive than Mancini radial diffusion. because, being "pulled out" in one direction only, differences in the length of the rockets are greater and are more easily measured. A standard curve of rocket length vs antigen concentration can be constructed and used to determine the concentration of unknowns analysed under the same conditions.

6.4.3 Grabar-Williams immunoelectrophoresis

Grabar-Williams immunoelectrophoresis[12] is a binary method in which an Ag mixture is first separated by electrophoresis and the separated

Binary having two parts

components are subsequently detected by immuno-diffusion. It may be considered as a development of the Ouchterlony technique, with better resolving power because of the separation of the Ag mixture in the electrophoresis step.

Conditions used have to be a compromise between the requirements of the two stages. For example;-

- Electrophoresis is best performed in a sieving gel, such as polyacrylamide (Section 5.7), partly because this restricts diffusion. However, immunodiffusion is dependent upon diffusion and so agarose, a non-sieving gel, is used as this does not impede diffusion.
- The requirement for immunoprecipitation restricts the buffer pH, which must not be too far from physiological pH.
- Both electrophoresis and immunoprecipitation require a buffer of low ionic strength.

The sample is introduced into a well cut into an agarose gel, supported on a glass slide, and is separated by electrophoresis. To prevent protein from migrating off the end of the gel, a sample of bromophenol blue may be run in parallel with the protein and the run stopped when the bromophenol blue reaches the end of the gel. A trough is then cut, parallel to the direction of electrophoresis and a few millimeters from the well (Fig. 111). Ab is introduced into the trough and the gel is incubated as described for the Ouchterlony technique for several days to allow formation of the immunoprecipitin bands.

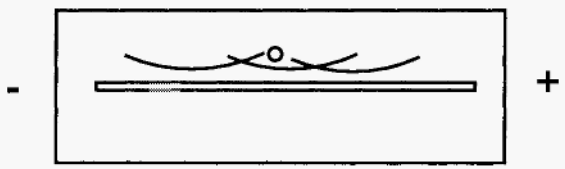


Figure 111. Grabar-Williams lmmunoelectophoresis.

Optimal proportions of Ab and Ag may be established by a prior Ouchterlony analysis, or replicate runs, covering a range of Ab and Ag concentrations, may be carried out.

6.4.4 Clarke-Freeman 2-D immunoelectrophoresis

Rocket electrophoresis (Section 6.4.2) is a method for the quantitation of Ags by electrophoresis into an Ab-containing gel. In the

rocket method different Ags are placed in different wells and either the Ag must be pure or, more usually, a mono-specific antiserum is used. In Grabar-Williams immunoelectrophoresis (Section 6.4.3) impure Ag mixtures are first separated by electrophoresis, before being analysed by immuno-precipitation. The Clarke-Freeman technique13 combines aspects of Grabar-Williams immunoelectrophoresis and of rocket electrophoresis. The sample is first separated by electrophoresis in one dimension. as in the Grabar-Williams technique, and then at right-angles to the first electrophoresis is electrophoretically drawn into an Ab-containing gel, as in the rocket technique. Like rocket electrophoresis, the latter stage is a form of cross-over electrophoresis and so an advantage over the Grabar-Williams technique is that all of the Ag meets Ab and there is no wasteful, unproductive, diffusion.

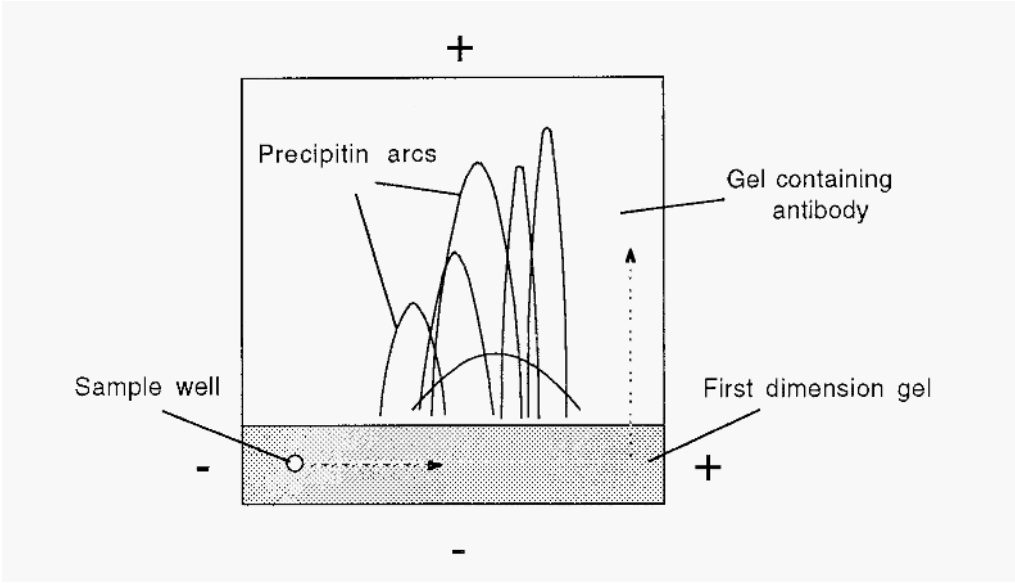


Figure112. Clarke-Freeman immunoelectrophoresis.

Sample in the well is separated by electrophoresis in the first dimension. A gel containing Ab is then cast adjoining the first gel along one of its edges. The separated sample components are then drawn into the Ab-containing gel by electrophoresis in the second dimension.

6.5 Amplification methods

At low levels of Ab and Ag, no visible precipitation may be formed. In modem methods of immunological analysis, therefore, use is made of amplification methods which enable the Ab/Ag reactions to be visualised and quantitated. Amplification methods will be discussed here in more-or-less historical order. the enzyme and immunogold methods being more recent.

6.5.1 Complement fixation

ìComplementî is a name given to a group of serum proteins which bind Ag/Ab complexes and cause lysis of cells displaying surface antigens. Complement thus functions *in vivo* as part of the immune response, aimed at the lysis of foreign cells. This activity is exploited in the complement fixation assay, which is a sensitive means of detecting Ag/Abcomplexes. The complement fixation assay is about 100-fold more sensitive than the precipitin reaction.

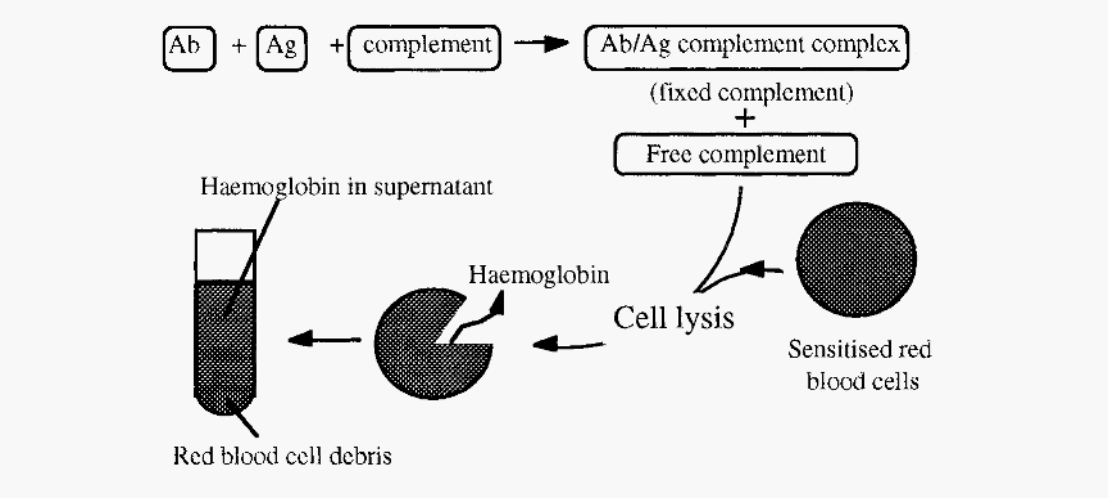


Figure 113. The complement fixation assay.

The complement fixation assay depends on the fact that complement is consumed (fixed) by Ag/Ab complexes, making less complement available. The amount of complement left (i.e. not fixed by the Ag/Ab complexes) can be measured by its ability to lyse red blood cells sensitised by antibodies binding to surface antigens. The amount of lysis can be quantitated by measuring the haemoglobin released into the supernatant, by its absorbance at 541 nm.

If all of the complement was bound by Ag/Ab complexes, none would be available to bind to the red blood cells and no lysis, and consequently no release of haemoglobin, would occur. On the other hand, if no Ag/Ab complexes were formed, no complement would be fixed, so all of it would be available to lyse red blood cells and the absorbance of the released haemoglobin would be at a maximum.

In order to measure the amount of a specific Ag present in a complex mixture, it is necessary to first establish a standard curve by measuring the complement fixed when different amounts of Ag are added to a fixed amount of complement and Ab. The standard curve (Fig. 114) has a shape similar to that of an immunoprecipitation curve (Fig. 101). Because of the shape of the standard curve, two different Ag concentrations can give the same degree of complement fixation (dotted lines in Fig. 114). In measuring an unknown, therefore, it is necessary to test several dilutions of the Ag to establish on which side of the curve the Ag concentration falls. The [Ag] can then be read off from the standard curve.

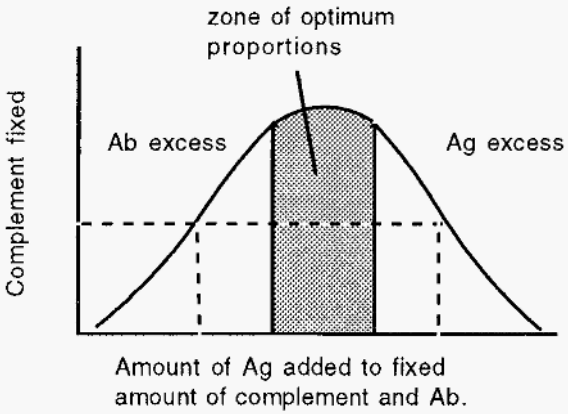


Figure 114. Standard curve for complement fixation.

The method has two principal disadvantages:-

- Certain crude mixtures cause haemolysis by mechanisms unrelated to complement, and,
- Some crude mixtures inactivate complement in the absence of the appropriate Ag/Ab complex.

6.5.2 Radioimmunoassay (RIA)

Radioimmunoassays (RIAs) combine the sensitivity of radioisotope detection with the selectivity of immunoassays. RIAs can be used to detect molecules that do not fix complement when combined with a specific antibody, for example *haptens* (see p152), and RIAs are mostly used for the assay of small molecules. Examples of compounds that can be assayed by RIA are peptide hormones, steroids (such as testosterone), cyclic AMP, morphine, digitalis etc.

The principle of the RIA is the same as that of the chemical assay technique known as an *isotope dilution assay.* In an isotope dilution assay a known amount of a radioisotope, of known specific activity (i.e radioactivity per unit mass), is added to a sample and a pure sample of the element of interest is subsequently extracted and its specific activity determined. The decrease in the specific activity of the isolated element is due to the presence of the non-radioactive isotope which dilutes the radioactive isotope. From the extent of this dilution, the concentration of the endogenous, non-radioactive isotope can be determined.

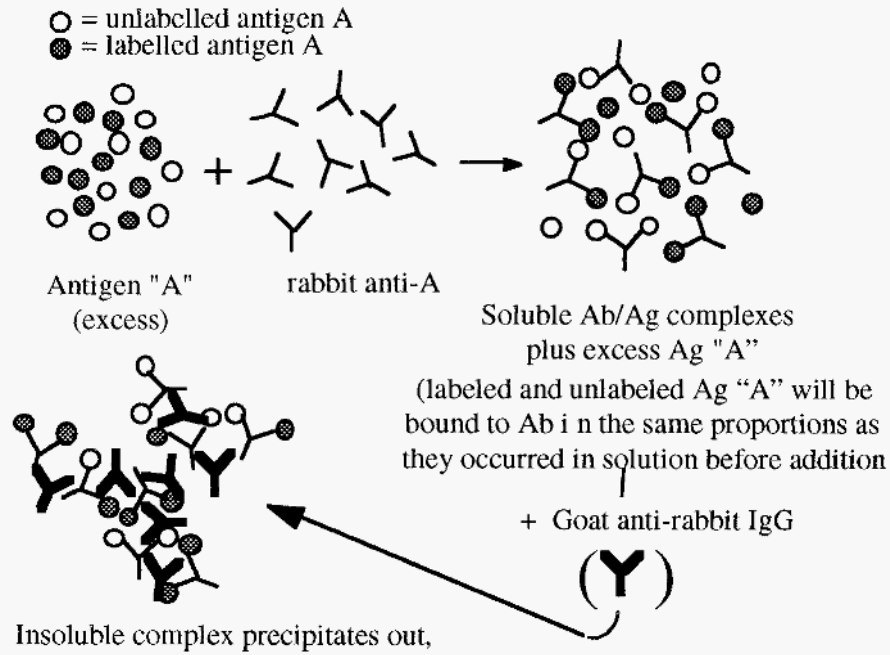


Figure I15. Radioimmunoassay.

A RIA is illustrated in Fig. 115. In a RIA, a radio-labelled antigen is used to dilute an unknown amount of an unlabelled antigen, present in the sample. A non-saturating amount of, say, rabbit antibody to the antigen is added (i.e. the antigen must be in excess). The labelled and unlabelled antigens will bind to the antibodies in the same ratio as they are present in the sample. The Ab/Ag complexes can then be precipitated, for example by addition of a goat anti-rabbit IgG antibody. The radioactivity in the precipitate will be inversely related to the amount of the antigen originally present in the sample. The concentration of the unknown antigen in the sample can thus be measured by reference to a straight line standard curve in which the % inhibition is plotted against log[non-radioactive Ag].

Radioimmunoassay has the following disadvantages:

- A radioactively labelled Ag may not be available, especially for an antigen which has not been extensively studied.
- Associated with the radioimmunoassay are all of the hazards of radioisotopes, which means that specially equipped laboratories and special licences are necessary.

6.5.3 Enzyme amplification

The advantage of enzyme methods over radioisotope methods of amplification is that, because enzymes are safe and biodegradable, no special licences or safety facilities are required, and disposal after use is no problem.

6.5.3.1 Enzyme linked immunosorbent assay (ELISA)

The ELISA method was introduced in its modern form by Engvall and Perlmann[14] and van Weemen and Schuurs[15]. The principle of an ELISA is that an enzyme, linked to an immunoreactive molecule (an antibody or protein A) can be used to detect the presence of an antigen with great sensitivity, due to the amplification achieved by the enzyme catalysed reaction. The method can also be turned about and used to detect Ab. Several different formats of ELISA are possible. Only some concepts pertaining to ELISAs are discussed here. For details on how to conduct an ELISA, a specialist text[16,17] should be consulted.

The competitive ELISA for measurement of Ag is conceptually similar to a RIA (Section 6.5.2), in that a labelled Ag competes with an unlabelled Ag for binding to the antibody, but in this case the label is an enzyme (Fig. 116).

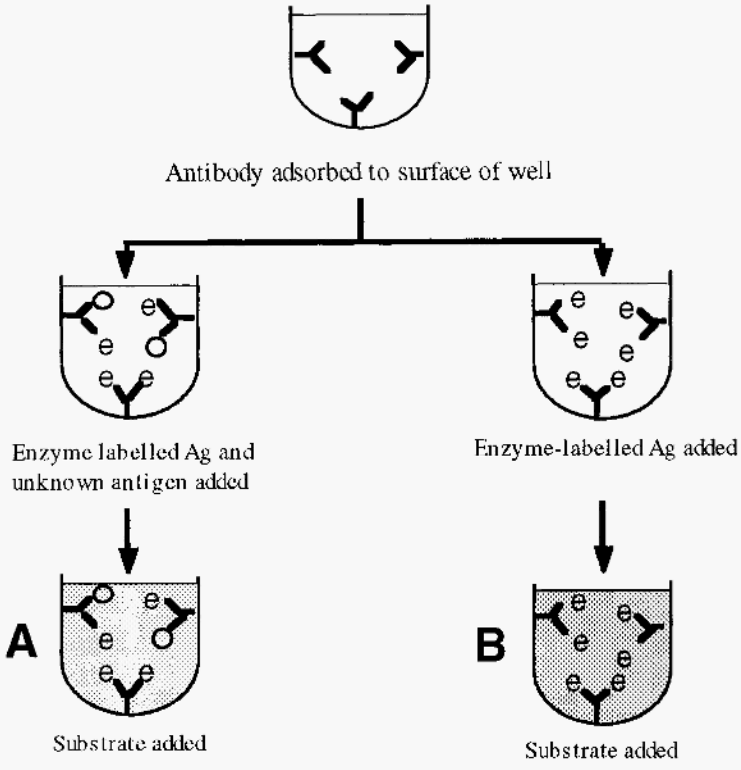


Figure 116. A competitive ELISA for measuring [Ag]. Unknown [Ag] is proportional to the absorbance difference between the wells A and B.

Antibody is inmobilised by adsorption onto the walls of a plastic microtitre plate. Excess antibody is washed off and the enzyme-linked Ag is added to one set of wells while the unknown sample, mixed with enzyme-linked Ag is added to another set. The unknown sample Ag thus serves to dilute the amount of labelled Ag which reacts with the immobilised Ab After incubation, excess antigen is washed off and a solution of the enzyme substrate is added. The enzyme catalysed reaction generates a colour which can be measured. The intensity of this colour is inversely proportional to the amount of Ag originally present. The microtitre plate contains 96 wells in an 8 x 12 array, which permits the exploration of a number of different combinations of coating and Ab/Ag concentrations, permitting optimisation of the reaction[16,17].

In another format, related to immunoblotting (Section 6.5.3.2), the Ag (usually a solution containing a mixture of proteins, including the protein of interest) may be adsorbed onto the walls of a microtitre plate and the Ag of interest subsequently detected with an enzyme-labelled Ab. For increased sensitivity, further amplification may be obtained by using two antibodies, an Ag-specific primary Ab and an enzyme-labeled secondary Ab that is specific for the type of primary Ab. This has the advantage that the secondary enzyme-labeled secondary Ab can be a universal reagent, targeting primary Abs of the same type but with different specificities.

6.5.3.2 Immunoblotting

In one ELISA format the principle of amplified detection of an immobilised Ag using an enzyme labeled Ab is used. The Ag, in this case, may be coated onto the walls of a microtitre plate. A similar principle applies to immunoblotting, whereby Ag immobilised on for e.g. a nitrocellulose filter, can be detected using an enzyme-linked antibody system (Fig. 117). An example is *dot blotting,* in which a small volume of the antigen of interest is dotted onto a nitrocellulose filter[18]. The surrounding protein-binding sites may be blocked with milk proteins, which do not tend to bind proteins non-specifically. The dot can then be probed with a primary antibody, followed by an enzyme-labelled secondary antibody, as in an ELISA. The only difference is that in an ELISA, the enzyme product is soluble, whereas in an immunoblot an insoluble product is necessary. A commonly used combination is horseradish peroxidase as the enzyme label, with 4-chloro-1-naphthol as the substrate. The blue/grey product is insoluble in water[19]. Alternatively, a gold-labelled Ab or protein A may be used[18].

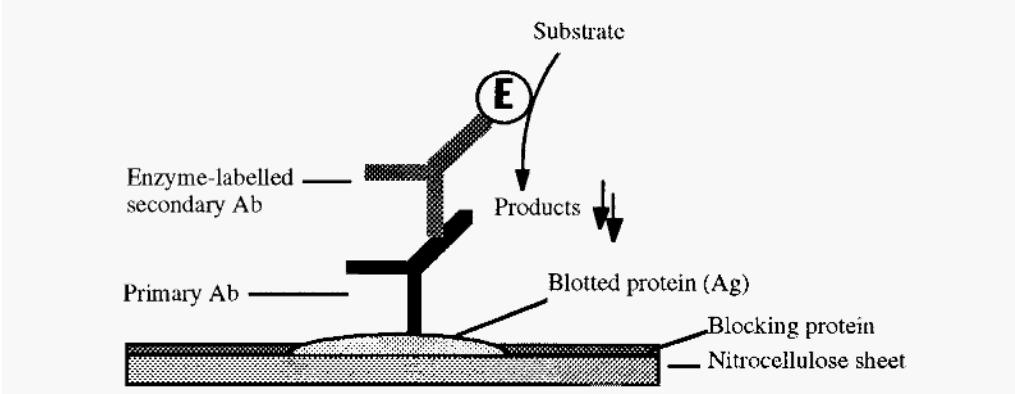


Figure 117. A schematic sketch of immunoblotting.

A related technique is *western blotting*[20]. The curious name of this technique is due to a pun. A technique for the blotting of DNA fragments, and probing these with labelled RNA, was named ìSouthern blottingî after its originator, E. M. Southern. The reverse process: blotting RNA and probing this with DNA fragments, was then named ìnorthern blottingî, to indicate that it was an opposite process. The blotting of proteins was subsequently called "western blotting" to indicate a similar process but with different molecules, i.e. in a different direction.

In western blotting, proteins are first separated by SDS-PAGE (p 133). The separated proteins are then drawn out of the gel, and blotted onto nitrocellulose, by transverse electrophoresis, forming a replica of the gel separation (Fig. 118). In this process the nitrocellulose sheet must be on the anodic side of the gel, as protein/SDS complexes are negatively charged and have an anodic migration.

Within the gel the proteins are not accessible to probing by antibodies, but they become accessible after electroblotting onto the surface of a nitrocellulose sheet. Antibodies bound to the blotted proteins can be detected with an enzyme-labelled secondary antibody, as in a dot blot.

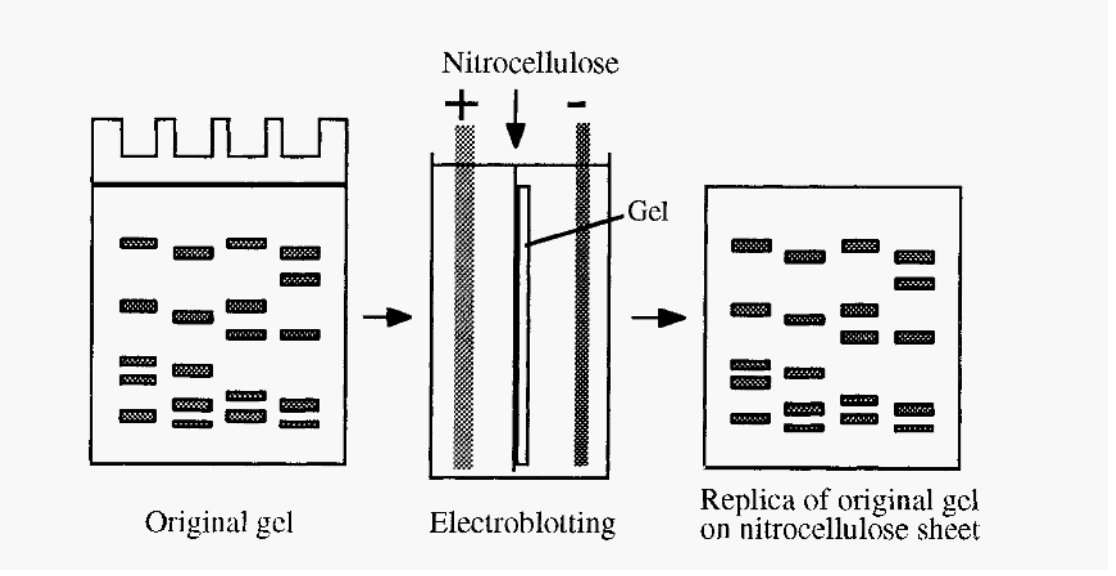


Figure 118. Electroblotting.

Treatment with SDS tends to denature proteins, although the effects can be minimised if the sample is not boiled in SDS (see Section 5.8.1). The denatured protein might not be recognised by the antibodies used to

probe the blot. In this case a renaturing blot system[21], can be used to advantage. In this technique the transfer buffer does not contain SDS and the SDS may be removed from the gel before the transfer step. The nitrocellulose sheet must be placed on the appropriate side of the gel, depending on the direction of migration of the protein at the pH of the transfer buffer. Normally a high pH is used, giving an anodic migration, but this is not appropriate if the protein is not stable at a high pH.

In a gel of constant composition, proteins separate by virtue of their differential rates of migration, smaller proteins migrating faster than large ones. This same differential will apply to the migration of proteins during the transfer step, i.e. smaller proteins will transfer to the nitrocellulose more rapidly. If insufficient time is allowed for the transfer, the blot will be biased in favour of smaller proteins. This effect can be overcome by running the first electrophoresis in a gradient gel (Section 5.9). In this case the proteins will each reach a point in the gradient where they are about equally impeded by the gel and during the lateral transfer step they will all migrate out of the gel at about the same rate.

Western blotting is useful for determining the presence or absence of a specific Ag in a complex mixture. It is also useful for testing the specificity of an Ab, before this is used in immunocytochemistry, for example. Blotting (not immunoblotting) is also used as a step in the sequencing of a protein band purified by gel electrophoresis. For this purpose the protein is electroblotted onto a polyvinylidene difluoride (PVDF) membrane, the blot excised and transferred into the sequenator.

6.5.4 Immunogold labeling with silver amplification

Colloidal gold particles, ranging in diameter from 1 to 30 nm, can be prepared by reducing dissolved gold chlorides with various reducing agents[22]. Proteins bind readily to such particles and stabilise the colloids against a salt challenge. Colloidal gold particles can thus be used as labels, attached to either antibodies or protein A, to form immunogold probes. Such immunogold probes find their greatest use in electron microscopy immunocytochemistry, whereby the subcellular distribution of an Ag of interest may be determined. Colloidal gold particles are very electron dense and show up readily in electron micrographs.

With silver amplification[23], immunogold probes may also be used at the light microscopy level and for staining immunoblots[18]. In the immunogold-with-silver-amplification (IGSS) technique, the colloidal gold label serves as a nucleation centre for the deposition of metallic silver. This yields a black stain which marks the position of the Ag of interest.

6.5.5 Colloid agglutination

As mentioned above (Section 6.5.4), proteins can stabilise colloids. The proteins bind to the colloidal particles and similar charge repulsion between the bound proteins keeps the colloidal particles apart, thus preventing flocculaton. Latex beads are commonly used as colloidal suspensions for analysis. A natural system, which is virtually colloidal, is blood, in which the red cells are prevented from aggregating by virtue of their similar surface charges.

Antibodies are divalent and are thus able to simultaneously bind to two similar antigens on two different colloidal particles. Such cross-linking of the colloidal particles causes them to flocculate out of suspension, and this provides a very sensitive method for the detection of Abs specific for the colloid-bound Ag. The method is particularly useful for medical diagnosis in the field. Since flocculation can easily be detected by eye, no sophisticated instrumentation is required. The method gives a simple yes-or-no answer, but it can be made semi-quantitative by dilution of the Ab, until the "definite yes" becomes a "maybe". The dilution at which this happens is inversely related to the initial antibody concentration.

An elegant diagnostic method uses agglutination of endogenous red blood cells as the reporter system[24,25]. Monoclonal Abs are raised against glycophorin, a glycoprotein present on the surface of all red blood cells. These Abs are species specific, i.e. they only recognise glycophorin from a particular species. From these monoclonal Abs, F(ab'), fragments can be made by proteolysis with pepsin. $F(ab')_2$ fragments consist of the two Fab arms of the Ab, bound together by disulfide bridges. The presence of the disulfide bridges is useful as these can be reduced and subsequently used to conjugate a peptide epitope to the free -SH groups of the two separate Fabí fragments. This generates a specific diagnostic reagent (Fig. 119).

Addition of this reagent to a drop of blood will cause haemagglutination, if Abs targeting the peptide epitope are present. For example, a person infected with the AIDS virus will, in the early stages, have anti-AIDS virus Abs present in their blood. These Abs will target specific epitopes on the AIDS virus proteins. These epitopes can be identified and corresponding peptides can be synthesised. Conjugation of one such peptide to an anti-human glycophorin monoclonal Ab half-$F(ab')_2$ fragment will generate a specific diagnostic reagent. Addition of an appropriate dilution of this reagent to a drop of a patient's blood will give a yes/no indication of the presence of anti-AIDS virus Abs in the patientís blood. A positive answer is given by the agglutination of the red blood cells. Such diagnostic analyses are useful for screening in the

field but they should be followed by confirmatory laboratory-based tests, such as ELISA

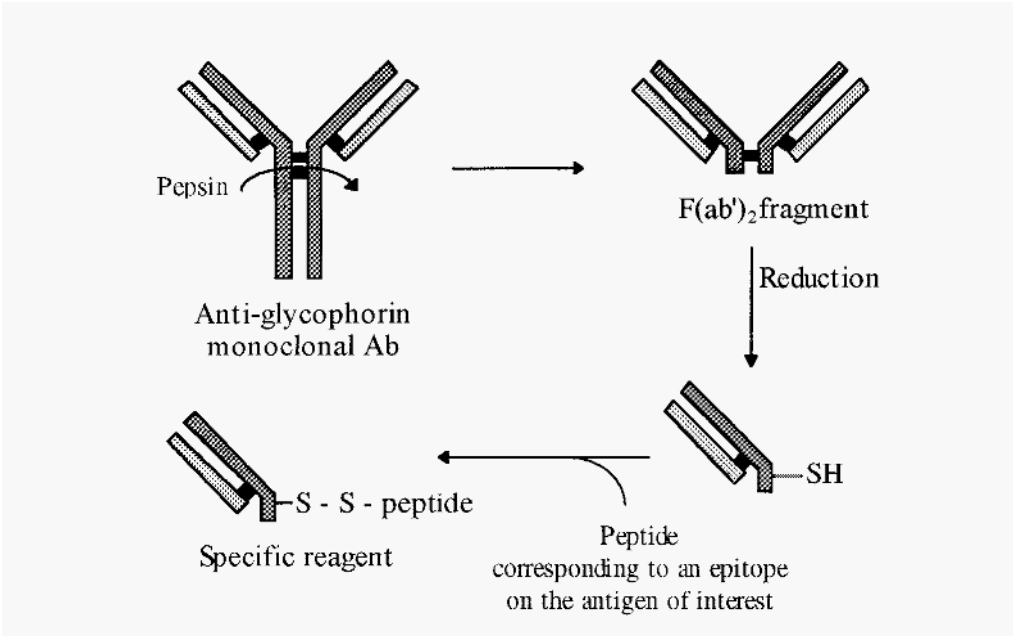


Figure 119. Generation of a reagent for detecting the presence of specific antibodies, using endogenous red blood cells as the reporter system.

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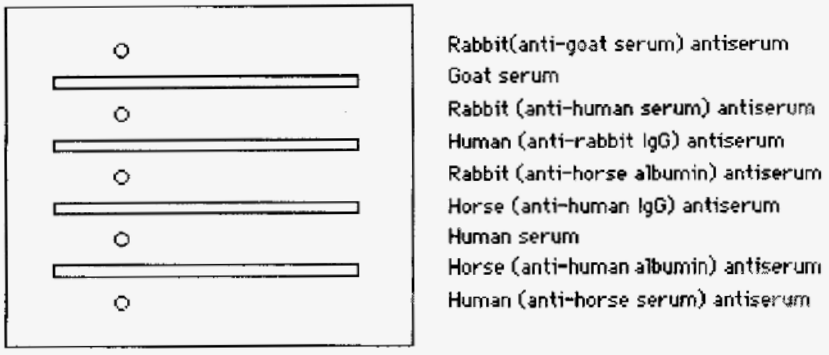
6.6 Chapter 6 study questions

1. Explain the difference between an immunogen and an antigen.
2. Define an ìepitopeî and a "hapten" and describe how anti-hapten antibodies may be raised.
3. An antiserum is described as being a "rabbit (anti-sheep IgG) antiserum". Describe in some detail how it would have been prepared, giving attention to the timing of doses to the animalís response, the role of adjuvants, etc.
4. In immunoelectrophoresis, the pH must be_____?
5. The ionic strength should be _____?
6. The temp. must be _____?
   Would it be possible to arrange an immuno-IEF system? Explain briefly.
8. Describe the operation of Polson's "mouth organ" apparatus, and calculate the diffusion coefficient of an unknown protein which formed an optimal precipitin band 1.25 cm from the Ab origin in an apparatus having gel chambers 3 cm long. ($D_b = 4{,}6 \times 10^{-7}$ cm^2 sec^{-1})
9. Two protein molecules, having the same molecular weight, have different diffusion coefficients. What does this tell one about the proteins?
10. Briefly describe how one would conduct an immuno-electrophoresis experiment, using the method of Clarke and Freeman, and sketch the result which would be expected from the analysis of human serum by this method. Label all relevant points.
11. Outline an immunoassay suitable for the measurement of haptens.
12. Describe, in principle, how one would conduct a "western blot" experiment. Include in your answer discussions of optimisation, reagent versatility, and possible reasons why western blotting is one of the most commonly-used immunological techniques in protein chemistry today.
13. In Grabar-Williams immunoelectrophoresis, the pH must be which of the following: a) Equal to the pI of the protein of interest, b) pH 7.0, c) Between pH 4 and pH 9, preferably as close to pH 7.0 as possible, d) Less than pH 7.0. or, e) More than pH 7.0?
14. In immunoelectrophoresis, the ionic strength should be:- a) low, b) high, or, c) physiological?
15. In immunoelectrophoresis the temperature should be, a) physiological, b) high, c) low, or, d) constant?

16. In western blotting, following SDS-PAGE, on which side of the gel should the nitrocellulose membrane be placed, the cathodic side or the anodic side?
17. The following sketch represents the protocol for a Grabar-Williams



immuno-electrophoresis experiment. Show the polarity of the applied electric field and sketch the precipitin bands which are likely to result from the experiment.

18. Which of the following statements is true? A hapten is a molecule, a) which, by itself, is able to elicit the formation of antibodies, b) which, by itself, is unable to elicit the formation of antibodies, c) which reacts with antibodies, d) of large molecular weight, e) of small molecular weight.
19. An immunogen is a molecule, a) which elicits the formation of antibodies, b) which reacts with specific antibodies?
20. An antigen is a molecule, a) which generates antibodies, b) which reacts with specific antibodies?

Index

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