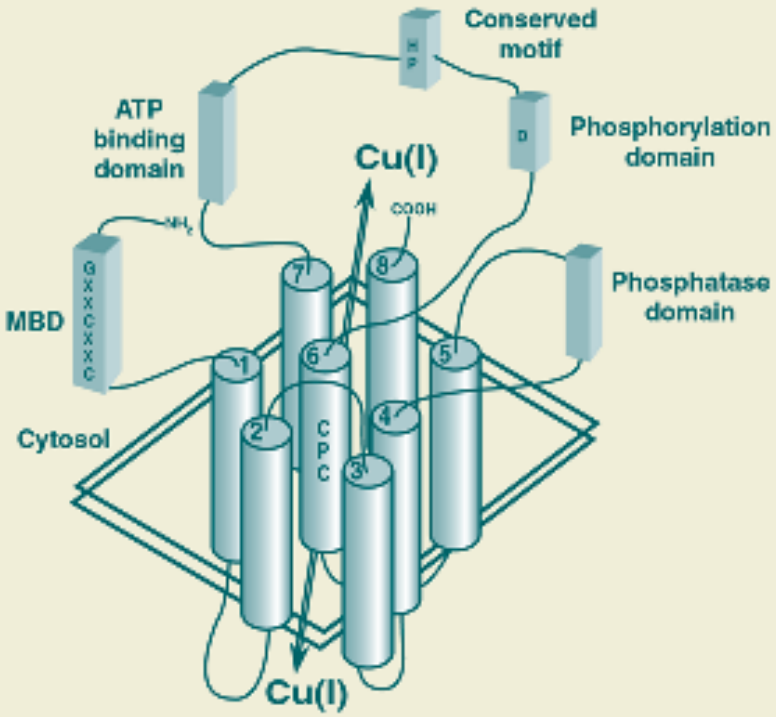
Heavy Metals in the Environment



edited by
Bibudhendra Sarkar

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edited by
Bibudhendra Sarkar
The Hospital for Sick Children and University of Toronto
Toronto, Ontario, Canada

MARCEL DEKKER, INC. NEW YORK • BASEL

ISBN: 0-8247-0630-7

This book is printed on acid-free paper.

Headquarters
Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
tel: 212-696-9000; fax: 212-685-4540

Eastern Hemisphere Distribution
Marcel Dekker AG
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
tel: 41-61-261-8482; fax: 41-61-261-8896

World Wide Web
http://www.dekker.com

The publisher offers discounts on this book when ordered in bulk quantities. For more information, write to Special Sales/Professional Marketing at the headquarters address above.

Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

Preface

Our global environment now consists of numerous natural and artificial metals. Metals have played a critical role in industrial development and technological advances. Most metals are not destroyed; indeed, they are accumulating at an accelerated pace, due to the ever-growing demands of modern society. A fine balance must be maintained between metals in the environment and human health. It is with this view in mind that this book has been written to address diverse issues surrounding heavy metals in the environment. Nineteen chapters have been contributed by 50 experts from around the world, known for their expertise and outstanding research. The book provides a critical review and analysis of the current state of knowledge of heavy metals in the environment.

The volume begins with a chapter on the essentiality and toxicity of metals. The widespread distribution of metals in the environment is of great concern because of their toxic properties; however, some metals are also essential for normal growth and development. This chapter provides a critical assessment of nutritional and toxicological information based on available data on humans. The evaluation has used information available on speciation and bioavailability to identify the critical effects and clinical manifestations of metal deficiency and toxicity. New principles and basic concepts are presented to define the acceptable range of oral intake (AROI) at which no adverse effects occur and the corresponding safe range of population mean intake (SRPMI) of essential trace metals such as selenium, iron, manganese, zinc, and copper. The interdependence of various

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elements is discussed with regard to metabolic and functional interactions involving storage and metabolism.

Analytical measurements of heavy metals in the environment are an integral component of monitoring and assessing their toxic effects. They are required for regulatory purposes and routine monitoring to ensure compliance with allowed levels to determine hazardous conditions. Clean-ups of contaminated locations are commenced on the basis of measurements indicating the site and extent of contamination. Chapter 2, on analytical methods for quantitative determination of heavy metals discusses various analytical tools and speciation analyses of heavy metals as well as their microscopic analyses. Techniques used for speciation analyses are discussed for individual metals such as chromium, arsenic, mercury, lead, and cadmium. This chapter also describes recent developments in the use of microprobe beamline to monitor intracellular distribution of elements in a single cell.

The need to develop and establish new toxicological approaches to assess the potential cytotoxic and genotoxic effects of heavy metals in the environment is addressed in Chapter 3, which focuses on a variety of in vitro toxicological screening methods for the biomonitoring of heavy metals. These methods take advantage of intracellular effects of metals to induce the expression of detoxifying proteins, other protective proteins, and proteins involved in cell cycles and proliferation and apoptosis. Suggestions are made as to the future of heavy metal biomarker research and how it can be more carefully monitored in the human environment.

A large spectrum of radionuclides was produced after the creation of the cosmos. Their radioactive half-lives are very long, and they remain ubiquitous components of the environment. Additionally, as a result of the development of nuclear weapons and nuclear technology, a number of artificial radionuclides have become a part of the human environment. Chapter 4 discusses the distribution and concentration of both natural and manmade radionuclides and the mechanism of their transfer to plants, animals, and humans. Possible long-term effects of their distribution in human tissue in terms of health implications are discussed. Metallic agents, as a class, make up a substantial portion of known human carcinogens. Chapter 5 reviews the topic of metal carcinogenesis, following the International Agency for Research on Cancer (IARC) classification system, with particular emphasis on known human carcinogens.

In recent years, both carcinogenic and noncarcinogenic potential of arsenic have been intensely studied. Chapters 6 and 7 review the global perspective on arsenic in the environment and aspects of arsenic toxicity. Chapter 6 explores the environmental behavior of arsenic with special reference to the abundance and distribution of arsenic in the lithosphere, sediments, soil environment, and groundwater. It also discusses various pathways of arsenic emission into the environment, methods for arsenic determination in drinking water, and techniques

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for remediation of arsenic-contaminated soil and groundwater systems. Chapter 7 discusses the sources of human exposure and aspects of human toxicology with special emphasis on chronic arsenic poisoning and its general effects related to dermatological manifestations, cardiovascular diseases, neurological impairments, and cancer effects.

Individual chapters are devoted to selected metals in the environment, including cadmium, chromium, aluminum, nickel, lead, mercury, and molybdenum. Chapter 8 reviews the pertinent literature of cadmium toxicology, with discussions of the health effects in humans of cadmium exposure and the molecular mechanisms underlying these effects. The connection between inhalation of chromium (VI) compounds and the causation of cancers of the airways and lungs is well established. Chapter 9 describes epidemiological studies along with the toxicokinetics and molecular mechanisms underlying the carcinogenicity of chromium (VI). It is followed by an in-depth consideration of approaches to the biological monitoring of chromium (VI)–exposed subjects. Chapter 10 presents an assessment of the hazards of aluminum exposure to humans, animals, and plants. Chapter 11, on nickel, reviews its distributions in the environment, human exposure, metabolism, systemic and molecular toxicology, and carcinogenesis. This chapter also includes a discussion on the interaction of nickel with other essential metals such as magnesium, calcium, iron, zinc, and manganese. Chapter 12 discusses the release of lead in the environment, human body burdens, and the population at risk. Special emphasis is given to analytical methods for the assessment of lead exposure and its metabolism, treatment of lead poisoning, in vitro and animal studies, molecular mechanisms, reproductive outcome, risk assessment and human epidemiological studies.

It is believed that the global cycling of mercury of natural and anthropogenic sources is responsible for the transport and deposition of mercury in areas remote from the original source. Chapter 13 takes a detailed look at mercury in the environment and its toxic actions, including a discussion on epidemiological studies of prenatal exposure. Molybdenum is essential to a variety of organisms, and is distributed widely in the environment owing to its diverse chemistry and its technological and agricultural applications. Chapter 14 provides a balanced picture of the complex environmental chemistry of molybdenum, including its interactions with copper, which can be either antagonistic or beneficial from the interplay of individual components in the biogeosphere.

The intracellular concentration of heavy metals is kept in balance by a variety of metal-transporters. Many of the metals are toxic in excess. Bacterial metal resistance probably arose early in evolution owing to widespread geochemical sources of metals. Chapter 15, devoted to the microbial resistance mechanism of heavy metals, discusses the mechanisms of resistance to zinc, cadmium, lead, copper, arsenic, and antimony in bacteria. The exposure to metal that is harmless to some bacteria may be destructive to others with specific genetic changes. Chap-

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ter 16 examines genetic susceptibility to heavy metals in the environment, noting how each metal is expected to have its own series of transporters. Transport of several metals is highly dependent upon the concentration of the other metals. This balance can be disrupted when any gene within the balanced system is nonfunctional. The interaction between genes and environment—considered critical for avoiding metal toxicity not only for humans but also for a wide variety of animal species—is described in detail. Selenium has multiple biological actions as an essential trace element, a modifier of other toxic elements, an anticarcinogenic agent, and a toxicant. These are all discussed in Chapter 17, which provides an overview of the entire profile of biological actions of selenium in nutrition and toxicology.

Over the past three decades, elements such as arsenic, antimony, gallium, and indium have been used in the manufacture of semiconductors for computer chips, cellular telephones, and light-emitting diodes. Many tons of these elements have been incorporated into these devices, either as dopants for silicon-based computer chips or in higher-speed semiconductors, such as gallium arsenide and indium arsenide. With the increased demand for higher-speed devices, older devices have been discarded, generating a large stockpile of electronic equipment containing these elements known collectively as ''e-waste.'' This is a new phenomenon, and the magnitude of this growing problem has been recognized only recently, since there are no well-established recycling programs for such item. Chapter 18, on semiconductors, provides an assessment of the present state of knowledge of the role and biological effects of metal/metalloids utilized in the semiconductor industry. The potential human health and environmental effects of these elements, either alone or as mixtures, are discussed in relation to areas of future studies.

There is a growing need for methods of assessing the amount of heavy metals pollution in our natural and industrial environments. While it is relatively straightforward to use the techniques of analytical chemistry to detect heavy metal concentrations in a particular location, they do not indicate how much of this metal is a ''biological hazard.'' Chapter 19 describes biosensors for monitoring heavy metals, and how researchers are exploiting various biological mechanisms to determine the amount of ''bioavailable'' heavy metal in the natural and industrial environments. These methods are still in their infancy compared with the techniques of analytical chemistry, but they clearly offer advantages in terms of ease of use, and biological relevance. The recent progress made in the development of whole-cell and protein-based biosensors is encouraging and holds much promise for the future.

The book was written by contributors in close collaboration with me. I visited some of their laboratories, intensively discussed their work with them, and made a few field trips to environmentally affected areas to obtain first-hand knowledge. Despite conscientious efforts by all concerned, the chapter authors,

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the editor, and the publisher cannot assume any liability for errors that this book may contain. Every effort has been made to keep the error rate as low as possible.

Heavy Metals in the Environment will be an invaluable resource for toxicologists; biochemists; bioinorganic, inorganic, environmental, and medicinal chemists; immunologists; oncologists; physiologists; pharmacologists; geneticists; bacteriologists; molecular biologists; environmental scientists; and upper-level undergraduate and graduate students in these disciplines.

I thank many of my international colleagues who provided valuable suggestions in the selection of topics and other advice. Special thanks are due to Loretta LeBlanc for preparing the manuscript and to Suree Narindrasorasak, Ping Yao, and Negah Fatemi for their assistance in the preparation of the index.

Bibudhendra Sarkar

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Contributors

Charles O. Abernathy Office of Water Quality, U.S. Environmental Protection Agency, Washington D.C.

Khosrow Adeli Division of Clinical Biochemistry, Department of Laboratory Medicine and Pathology, and Program in Structural Biology and Biochemistry, The Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada

George Becking World Health Organization, Research Triangle Park, North Carolina

Prosun Bhattacharya Department of Land and Water Resources Engineering, Royal Institute of Technology, Stockholm, Sweden

Ibolya Bontidean Department of Biotechnology, Lund University, Lund, Sweden

Nigel L. Brown School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

Montserrat Casadevall Centre for Toxicology, The School of Pharmacy, London, England

Thomas W. Clarkson Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, New York

Philippe Corbisier Joint Research Centre of the European Commission, Institute for Reference Materials and Measurements, Geel, Belgium

Max Costa Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York

Diane W. Cox Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada

Elisabeth Csöregi Department of Biotechnology, Lund University, Lund, Sweden

Lara M. Cullen Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada

John R. Forbes Department of Biochemistry, McGill University, Montreal, Quebec, Canada

Bruce A. Fowler Department of Epidemiology, Laboratory of Cellular and Molecular Toxicology, University of Maryland, Baltimore, Maryland

Seth H. Frisbie Better Life Laboratories, Inc., Plainfield, Vermont

Mallika Ghosh Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan

Othman Ghribi Department of Pathology, University of Virginia, Charlottesville, Virginia

Robert A. Goyer University of Western Ontario, London, Ontario, Canada

Mary M. Herman National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland

Seiichiro Himeno School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

J. Thomas Hindmarsh Department of Pathology and Laboratory Medicine, The University of Ottawa and The Ottawa Hospital, Ottawa, Ontario, Canada

Nobumasa Imura School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan

Gunnar Jacks Department of Land and Water Resources Engineering, Royal Institute of Technology, Stockholm, Sweden

Andreas Kortenkamp Centre for Toxicology, The School of Pharmacy, London, England

Jonathan R. Lloyd School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

Emily F. Madden Center for Devices and Radiological Health, U.S. Food & Drug Administration, Rockville, Maryland

R. Bruce Martin Department of Chemistry, University of Virginia, Charlottesville, Virginia

Ross F. McCurdy InNOVAcorp., Dartmouth, Nova Scotia, Canada

Henry H. Murray Corporate Strategic Research, ExxonMobil Research and Engineering Co., Annandale, New Jersey

Ravendra Naidu CSIRO Land and Water, Commonwealth Scientific & Industrial Research Organization, Glen Osmond, South Australia, Australia

Gunnar F. Nordberg Department of Environmental Medicine, Umeå University, Umeå, Sweden

Monica Nordberg Institute of Environmental Medicine, Karolinska Institute, Stockholm, Sweden

Richard Ortega Chimie Nucléaire Analytique Bioenvironnementale, University of Bordeaux, Gradignan, France

Wendy E. Parris Division of Clinical Biochemistry, Department of Laboratory Medicine and Pathology, and Program in Structural Biology and Biochemistry, The Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Gregory R. Peters Philip Analytical Services Inc., Bedford, Nova Scotia, Canada

Barry P. Rosen Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan

Brittmarie Sandström Research Department of Human Nutrition, The Royal Veterinary and Agricultural University, Copenhagen, Denmark

Bibudhendra Sarkar Program in Structural Biology and Biochemistry, The Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada

John Savory Department of Pathology, Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia

Mary J. Sexton Department of Epidemiology and Preventive Medicine, School of Medicine, University of Maryland, Baltimore, Maryland

Donald R. Smith Department of Environmental Toxicology, University of California, Santa Cruz, California

Euan Smith CSIRO Land and Water, Commonwealth Scientific & Industrial Research Organization, Glen Osmond, South Australia, Australia

Edward I. Stiefel Department of Chemistry, Princeton University, Princeton, New Jersey

Jessica E. Sutherland Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York

David M. Taylor Department of Chemistry, Cardiff University, Cardiff, Wales

Michael P. Waalkes Laboratory of Comparative Carcinogenesis, National Cancer Institute and National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina

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1

Essentiality and Toxicity of Metals*

Gunnar F. Nordberg
Umeå University, Umeå, Sweden

Brittmarie Sandström
The Royal Veterinary and Agricultural University, Copenhagen, Denmark

George Becking
World Health Organization, Research Triangle Park, North Carolina

Robert A. Goyer
University of Western Ontario, London, Ontario, Canada

1. INTRODUCTION

Many metals are of concern because of their toxic properties and some metals are also essential for survival and health of animals and humans.

In risk assessments concerning toxicity of essential metals, their essentiality should also be taken into account to avoid too low intakes. This has not always been done in a proper way and authorities responsible for protecting the public from adverse health effects from toxicity have issued recommendations that have

* Part of this chapter was a contribution to the draft WHO/IPCS working paper ''Methodology for Risk Assessment of Toxicity for Essential Trace Elements.''

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been partly in conflict with those issued with an aim of protecting from deficiency. There is an obvious need for an approach including a balanced consideration of nutritional as well as toxicological data for these metals.

These new principles of evaluation take into account some basic concepts of interindividual variability in sensitivity to deficiency and toxicity. Such variation translated into one interval of (low) daily intake, at which there is a risk of developing deficiency, and another interval of (high) dietary intake, at which toxicity may occur. In between there is a set of intakes that represent the acceptable range of oral intakes (AROI), at which no adverse effects occur. While it is possible to define such a range that will protect most people, a range will not usually be found that protects all persons from adverse effects. Those with genetically determined sensitivity may require higher intakes to avoid deficiency or lower intakes to avoid toxicity than those defined by the acceptable range. AROI is defined as protecting 95–98% of healthy individuals in specified gender and life stage population groups from even minimal adverse effects of deficiency or toxicity. While AROI is defined for intakes by individuals in a population group, the corresponding range for mean intakes is the safe range of population mean intakes (SRPMI).

This chapter reviews principles and methodologies that may be applied in defining limits of safety for nutritionally essential metals. Excessive intake of these metals can give rise to toxicity. There is increasing use of various standards worldwide that express the maximum acceptable limits for human exposures for various substances present in the environment including nutritionally essential trace elements. In some instances, the methodology applied to standard setting for these nutritionally essential substances has been the same as applied to toxic metals. In the case of zinc, RDA (recommended daily allowances set by the U.S. National Research Council) and RfD (reference dose, set by the U.S. Environmental Protection Agency) were found to be almost identical and, for certain age groups, the RDA was higher than the Rfd (1,2). It is becoming apparent that standard setting for nutritionally essential trace elements requires consideration beyond approaches traditionally applied to metals that have no biological requirement for good health.

Recognition of this problem has prompted a number of conferences on this topic that have resulted in publications and discussions of potential approaches as well as potential problems. These activities include a workshop sponsored by the U.S. Environmental Protection Agency, the Agency for Toxic Substances and Disease Registry, and the International Life Sciences Institute's Risk Sciences Institute held in March 1992 in Herndon, Virginia, which reviewed the problem and identified a number of topics that had been inadequately considered to date (1). Similarly, a Nordic Working Group on Food and Nutrition and the Nordic Group on Food Toxicology have prepared a report (3), and a conference on ''Trace Elements in Human Health'' held in Stockholm in May 1992 has also

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been reported (2). The conceptual framework for the preparation of a WHO/IPCS methodology document was based on discussions from a WHO planning meeting held in Washington in April 1996 (4) and on IPCS Workshop held in Santiago de Chile in 1998. The methodology [reviewed in part also by Nordberg et al. (5)] proposed in the present chapter has evolved from the activities.

Metals have been classified as essential, beneficial, or detrimental. Trace elements recognized as essential for human health include iron, zinc, copper, chromium, iodine, cobalt, molybdenum, and selenium (6). For the purpose of this chapter all metallic elements in this listing will be considered in discussions of methodology. Later in the chapter, the methodology described will be applied to a few of these as examples. There is also a second group of elements thought to be beneficial to life (e.g., silicon, manganese, nickel, boron, and vanadium). Some of these elements may be essential to vegetative life and perhaps beneficial to human health but, generally, they are not yet accepted as essential for human health. If any of these or other elements become accepted as essential for humans and quantitative nutritional requirements are established, the approaches outlined in this chapter should be applicable for setting an acceptable range of oral intakes (AROI).

The methodology described for determining the AROI is not intended to be applied to detrimental metals or metals that are regarded as purely toxic metals such as lead, cadmium, and mercury, which are not known to provide any essential or potentially beneficial health effect at any level of exposure. Also this methodology, in its present form, is not intended to assess risk for carcinogenicity, although at present this is only of concern for one essential element, chromium, and is probably limited to inhalation and not oral exposure. While the essential elements are not by themselves known to be carcinogenic by the oral route, several play important roles as modulators of carcinogens by promoting or protecting from oxidative damage. For example, selenium deficiency and excess iron intake may act synergistically to enhance oxidative damage of macromolecules, nucleic acids, and lipid membranes.

Presently recommended dietary intakes of essential trace metals are based on estimates of amounts needed to prevent clinical or biochemical deficiency. It is increasingly recognized that higher intakes of some of the trace elements may have beneficial health effects in relation to risk reduction of degenerative diseases such as cardiovascular disease and cancer. These suggested higher levels are usually not met by ordinary foods but require supplements, often administered in a more available form than dietary minerals. Long-term intake of high but not immediately toxic doses of trace elements may lead to interaction with other trace elements and/or other changes not identified with a classical toxicological approach. Thus the safety of essential trace elements is a subtle issue and as new criteria for estimates of requirements are emerging there may be a need to redefine also the criteria used to estimate adverse-effect levels.

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For essential metals health risk assessment requires consideration of both toxicity from excess exposures and health effects as a consequence of deficiencies from severe restriction of intake. Such an approach involves principles from nutrition as well as toxicology. The objective is to make recommendations that result in a range of recommended intakes that recognizes consequences of both nutritional deficiency and toxicity.

The approach described in this chapter outlines the principles that support the concept of AROI, or a ''homeostatic model'' for determining the distribution of intakes for essential trace metals (ETM) that meet nutritional requirements of a healthy population as well as preventing toxicity. The methodology presented in this document recognizes the importance of variability in exposure and biokinetics arising from age, gender, physiological conditions, and nutritional status. In addition it should be noted that dietary/food intake is only part of oral intake. Oral intake also includes intake from water, dietary supplements, and a fraction of inhalation exposures that are subsequently ingested after coughing and swallowing.

1.1 Nutritionally Essential Trace Metals (ETM)

The traditional criteria for human health are that absence or deficiency of the element from the diet produces either functional or structural abnormalities and that the abnormalities are related to, or a consequence of, specific biochemical changes that can be reversed by the presence of the essential metal (6).

The criteria for identifying ETM have evolved over the past 50 years and may be expected to expand as the result of future research. New end points reflecting effects of deficiency have been considered in recent investigations of essentiality of ETMs in experimental animals (7). These have included mild reductions in growth rate, impairment of reproductive performance, decreased life span, sudden unexpected death, and some anatomical lesions.

1.2 Homeostatic Mechanisms

A defining characteristic of ETMs is that there are homeostatic mechanisms that maintain optimum tissue levels over a range of exposures for the performance of essential functions (8). Homeostatic mechanisms involve regulation of absorption, tissue retention, and excretion of ETMs. There are specific homeostatic mechanisms for each ETM (6). For some ETMs, namely those that are handled as cations (zinc, iron, copper, manganese, chromium), the homeostatic mechanisms operate predominantly via the gastrointestinal tract and the liver. They may regulate uptake and transfer by the gut (Fe, Zn) or by biliary excretion (Cu). For each of these ETMs there may be a specific chain of protein carriers and receptors to effect uptake into cells. Anionic ETMs, like molybdenum and selenium, are more

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soluble and systemic absorption is less regulated than for cationic ETMs. They are absorbed very efficiently and subsequent control, tissue deposition, and excretion are managed by oxidation state. Total body burden is regulated by renal excretion. A third category of homeostatic mechanism is illustrated by cobalt, which is a highly reactive element with several oxidation states. The physiological role for this metal is as one of highly regulated form in cobalamin and there is no evidence that humans require inorganic cobalt. Whether or not an analogous situation applies to chromium and the ''glucose tolerance factor'' is unclear particularly since inorganic chromium has been effective in alleviating chromium deficiency in patients on total parenteral nutrition. However, there is a wide diversity within populations and individuals as to the efficiency of these mechanisms.

2. CONCEPTS OF EVALUATION—CONSIDERATION OF TOXICITY AND ESSENTIALITY

Interindividual variation that occurs in human populations is considerable. This applies to the expression of toxicity from higher doses of an ETM as well as for the expression of deficiency symptoms as a result of too low intakes of the same essential element.

2.1 Acceptable Range of Oral Intake (AROI)

In Figure 1 the interindividual distribution of sensitivity is shown for nutritional requirements and for expression of toxicity. For a specific adverse effect of deficiency, individuals exist in a population who display average sensitivity to developing symptoms (mean nutrient requirement, Fig. 1) as well as more sensitive individuals, i.e., those developing deficiency symptoms at somewhat higher intakes (+1.5–2.5 D nutrient requirement), and individuals with less sensitivity, i.e., those that develop deficiency symptoms first when intakes are lower (−1.5–2.5 D nutrient requirement). A similar situation applies for a specific toxic effect; i.e., some individuals display symptoms at doses lower than those giving rise to symptoms in the individuals of average sensitivity and there are also individuals who are less than average sensitive and they require higher doses to develop symptoms. The situation can be depicted as two bell-shaped curves describing the distribution of sensitivity to deficiency and toxicity. In most cases an interval between these curves describes the AROI in which no adverse effects occur in the large majority (95–98%) of subjects (cf. Fig. 1). If these conditions are instead depicted with curves in cumulative forms, a U-shaped curve is formed and the AROI appears at the bottom of the U (Fig. 2). Further aspects and examples of how AROI for specific essential metals can be derived will be given in later sections.

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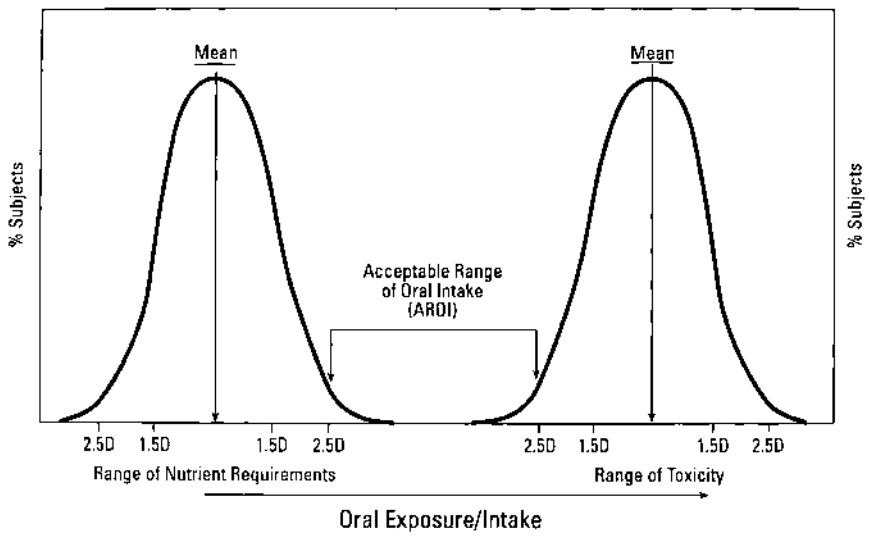

FIGURE 1 Theoretical model describing distribution of intakes to meet nutritional requirements (left) and distribution of intakes giving rise to toxicity (right), with the acceptable range of oral intakes (AROI) in between. Lower limit to the AROI should cover the requirements of most (97.5%) of the population; the higher limit if AROI should protect a similar proportion of the population from toxic effects.

2.2 Groups with Special Sensitivity/Resistance

2.2.1 Genetically Determined

In Figure 3, the same model for distribution of intakes to meet nutritional requirements and to prevent toxicity is displayed as in Figure 1. The low limit of the AROI covers the requirement of most of the population and the high limit of the AROI should protect most of the population from toxic effects. Special population subgroups, such as persons with Wilson's disease, may exhibit toxicity at relatively low intakes of copper, lower than the acceptable range for normal persons. On the other hand, some population subgroups such as B may have requirements higher than the upper limit of acceptable range (for example, zinc intake in subjects with acrodermatitis enteropatica). Further aspects on genetically determined variation in sensitivity have been given by WHO 1996 (6) and will be given in a future document on ''Principles of Risk Assessment for Essential Trace Elements'' by IPCS/WHO.

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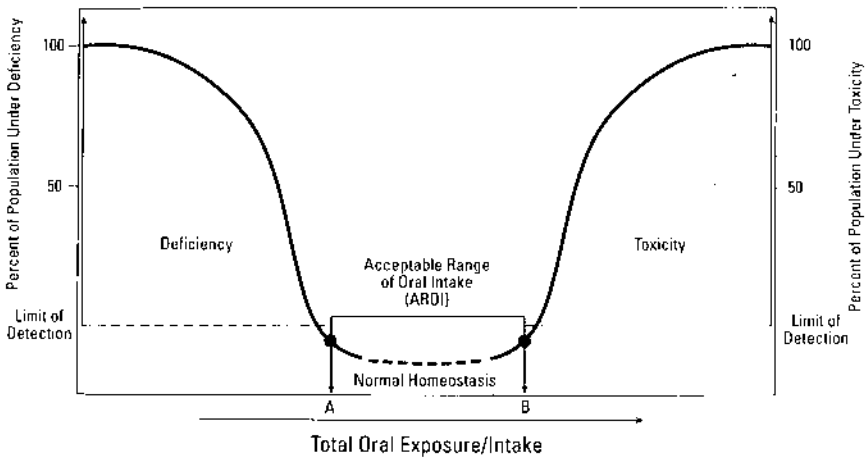

FIGURE 2 Percent of population subjected to deficiency or toxicity to oral exposure/intake. This is the same model as in Figure 1, but in a cumulative form. As intake drops below A (lower limit of AROI), risk for deficiency increases; at extremely low intakes all subjects will manifest deficiency. As intakes increase beyond B, a progressively larger proportion of subjects will exhibit effects of toxicity.

2.2.2 Nongenetically Determined

Many diseases are genetically determined but this is not considered here if the disease is not known to involve a specific metabolic defect related to essential elements. In celiac disease, there is a deficient uptake of several nutrients including essential elements such as iron (9) and zinc (10). In addition, gastrointestinal losses of trace elements can be increased due to diarrhea. If the disease is not well controlled by exclusion of gluten from the diet, and/or the decreased uptake is not compensated by an increased intake of these elements, iron and/or zinc deficiency may develop. Increased urinary losses of zinc are observed in patients with alcoholic cirrhosis (11) and diabetes (12). It has been shown that iron deficiency gives rise to an increased uptake of manganese from the diet (13). It can therefore be assumed that there would be an increased risk of manganese toxicity if persons with iron deficiency were exposed to high oral intakes of manganese.

Sensitivity to manifestations of zinc deficiency is known to be dependent on certain metabolic situations. When discussing effects of deficiency or toxicity of an essential element it is therefore of fundamental importance to specify the

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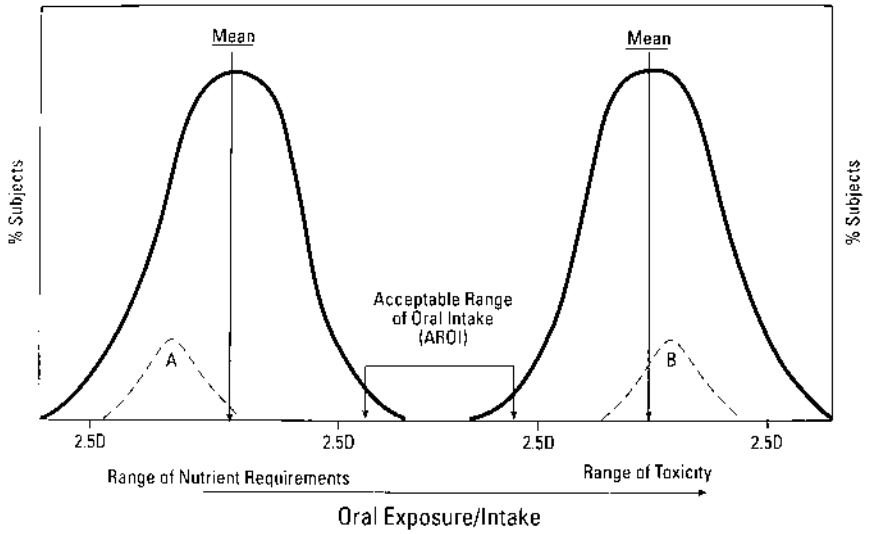

FIGURE 3 Theoretical model for distribution of intakes to meet nutritional requirements of healthy population and prevent toxicity. The lower limit to AROI should cover the requirements of most (97.5%) of the population; the higher limit should protect most of the population from toxic effects. Special population subgroups, such as A, may exhibit toxicity at intakes lower than the acceptable range (e.g., Wilson's disease and copper intake), or in contrast, some population subgroups, such as B, may have requirements higher than the upper limit of acceptable range (e.g., zinc intakes in subjects with acrodermatitis enteropathica).

background conditions of the group of persons under consideration. Such background conditions can be all-determining for the dose-response relationships. For example, at a certain low zinc intake (e.g., under conditions of total parenteral nutrition) clinical symptoms of zinc deficiency may not develop in a group of individuals who are in metabolic balance, but may be clinically manifest in persons who undergo growth or who are in an anabolic phase (14). Dose-response relationships for zinc deficiency thus can be quite different depending on metabolic state.

2.3 Nutritional Requirement and Safe Range of Population Mean Intake (SRPMI)

Public health aspects concerning adverse effects as a result of deficiency from an essential element have been discussed in many documents. Definitions relating

to the needs of individuals were defined by the Joint FAO/IAEA/WHO Expert Consultation on Trace Elements in Human Nutrition (6).

> Requirement. This is the lowest continuing level of nutrient intake that, at a specified efficiency of utilization, will maintain the defined level of nutriture in the individual.
>
> Basal requirement. This refers to the intake needed to prevent pathologically relevant and clinically detectable signs of impaired function attributable to inadequacy of the nutrient.
>
> Normative requirement. This refers to the level of intake that serves to maintain a level of tissue storage or other reserve that is judged [by the Expert Consultation] to be desirable.

Ideally the establishment of estimates of requirement of essential element should be based on functional criteria for adequacy. For many trace elements sensitive and specific criteria are lacking and other approaches have to be adopted. The *factorial technique*, i.e., the sum of the obligatory endogenous losses of the element via skin, kidney, and intestine with the addition of requirements for synthesis of new tissue in periods of growth, has been the basis for the estimates of zinc requirement in the WHO 1996 report (6). This approach was originally used for estimates of protein requirements. The obligatory losses are usually determined in balance studies at different intakes. The principal problem in relation to zinc has been to account for the ability to adapt to different intakes by changes in endogenous losses.

Most early reports on recommended intakes of essential elements have provided estimates of the requirements of individuals and the ''recommended'' or ''safe'' level of intake has been defined as the average requirement + 2 SD in requirement. Thus for an individual consuming this amount of element there would be a very low probability that the individual's requirement was not met. The WHO 1996 report is concerned with population (group) mean intakes rather than intakes of individuals. The lower limit of population mean intake is set so that very few individuals in the population (group) would be expected to have intakes below their requirement; i.e., the population mean intake corresponds to the estimates of average individual requirement + 2 SD in intakes. The term ''population'' in the WHO 1996 report refers to a group that is homogeneous in terms of age, sex, and other characteristics believed to affect requirement and not, for example, to demographically or culturally defined groups. The variability in usual intakes within a population group is usually larger than estimates of variability of requirements and it has empirically been demonstrated that the evaluation of the prevalence of inadequate intakes is relatively insensitive to the variability in requirements.

WHO/FAO/IAEA Expert Consultation (6) identified the need to work with recommendations of mean population intakes, since it is difficult in practice to

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give a precise description of upper and lower ends of intake distributions. They defined the mean normative requirement so as to protect 98–99% of the population from minimal adverse effects (including undesirably low tissue stores). The upper limit of population mean intake to avoid (minimal) toxicity in 98–99% of the population was also discussed (6). If 98–99% of the population is protected from deficiency and the same percentage is protected from toxicity, this means that 96–98% of the population is protected from both deficiency and toxicity. Based on these considerations, SRPMI was derived (6) thus protecting 96–98% of healthy persons in specific gender and life stages from even minimal effects of deficiency or toxicity.

3. FACTORS MODIFYING DOSE-RESPONSE RELATIONSHIPS

As mentioned earlier, homeostatic mechanisms regulating ETMs are dependent on basic properties of the essential metals, i.e., their belonging to one of three groups, namely those that are handled as cations (zinc, iron, and copper), anions (molybdenum and selenium), or bioinorganic complexes (e.g., cobalt as cobalamin). These properties in terms of speciation of the elements are of fundamental importance to their behavior in the human body including bioavailability and also influence interactions. There is a considerable body of literature available on factors influencing dose-response relationships of metals. Early literature on this subject was reviewed by Nordberg (15) and a recent review is included in the 1996 WHO document (6). The following are a few aspects.

3.1 Bioavailability, Uptake, and Utilization

Bioavailability of trace metals is influenced by chemical form or species but also by such factors as food source, or dietary media, nutritional state, age, pathological conditions, and interactions with other nutrients or toxic substances.

These factors are important and should be considered both when estimating the nutritional requirement and when assessing toxicity. A number of physiological and dietary variables influence trace element utilization (6). Intrinsic (or physiological) variables that influence the absorptive process, such as the poorly regulated situation during infancy for the absorption of a number of elements such as chromium, iron, and zinc and the probable decline in absorptive efficiency during senility (copper and zinc). There may be an adaptation to low trace element status or high demand by modifying activity/concentration of receptors involved in uptake from gastrointestinal tract (chromium, copper, manganese, zinc).

With regard to metabolic/functional interactions there is an interdependence of elements in processes involved in storage and metabolism, e.g., in relation to catecholamine metabolism (copper and iron) and in relation to iodine

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utilization (selenium) and protein synthesis (zinc). With regard to extrinsic (dietary) variables, mucosal uptake may be influenced by the solubility and molecular dimensions of trace-element-bearing species within food, digesta, and gut lumen (iron oxalates, zinc, and iron phytates associated with calcium). Dietary components may also increase the mobility of an ETM; e.g., citrate enhances zinc absorption.

Antagonism involving, for example, competition with receptors regulating absorption transport and storage have been shown to limit ETM uptake and storage (e.g., in cadmium/zinc/copper antagonism).

3.2 Age, Gender, Pregnancy, and Lactation

Growth and development of the fetus is dependent on availability of essential trace metals, for example zinc. Developmental abnormalities of the nervous system have also been shown in experimental animals as reviewed by Sandstead (16). In infancy, gastrointestinal absorption may be higher, that is, less well regulated, than in later stages of life. In the elderly, intestinal uptake of trace metals may decline even in those with normal health (17). There are also differences in nutritional requirements depending on differences in metabolic handling of trace metals between the two sexes. For example, men are larger of stature than females. Skeletal size is related to height as is body calcium. Protein and energy requirements are considerably lower for females than for males. For these reasons it is important to define specific nutritional requirements and toxic levels separately for men and women and sometimes in relation to age. Increased demand for ETM, particularly iron, zinc, and copper, occurs during pregnancy and lactation (6,18). Specific recommendations for pregnant and lactating women are therefore warranted.

3.3 Interactions

Interactions between ETMs are important in determining bioavailability and, in turn, the AORI for metals involved. For example, copper can interact with a number of other nutrients (19) leading to reduced availability.

Copper uptake in the gut is inhibited by high zinc intakes. Both a direct interaction and an interaction mediated by metallothionein induction may explain this phenomenon. It is well known that zinc can induce metallothionein synthesis in intestinal cells. Such metallothionein may sequester copper, since copper has higher affinity to SH groups on metallothionein than zinc. By this mechanism copper may be trapped in the intestinal mucosa, not reaching the systemic circulation to the same extent as in persons with normal zinc intake and therefore increasing susceptibility to copper deficiency. The critical effects of zinc toxicity are considered to be related to induced copper deficiency (20).

There are some studies in animals indicating a decrease of copper absorption when intake of iron is high. In human preterm neonates the usual increase

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in erythrocyte superoxide dismutase (E-SOD) was impaired when mothers took iron supplements. There may be a possible relationship between E-SOD concentration in neonates and changes in copper availability in association with high Fe intakes (21).

High levels of molybdenum in the diet may induce or aggravate copper deficiency by making copper unavailable for absorption (19). This interaction has been applied in the use of molybdates in the treatment of Wilson's disease (22). Selenium may also compete for the gastrointestinal absorption of copper (23).

4. EFFECTS OF DEFICIENCY AND TOXICITY

4.1 Basic Principles for Classifying Effect

Evaluation principles concerning adverse effects from metal toxicity have been previously developed by the ICOH Scientific Committee on the Toxicology of Metals, i.e., by the Task Group on Metal Toxicity 1976 [cf. Nordberg (24,25)]. Adverse effects can occur from too low or too high an intake of an essential element. As mentioned, it may not be possible to arrive at recommendations that protect *all* individuals in a population from such adverse effects, since some individuals with genetically determined metabolic disorders may require intakes that are higher or lower than those that represent AROI for normal persons. AROI can therefore only be applicable for persons without such disorders. It is further considered that the use of sensitive indicators of adverse effects with clinical or functional significance as markers of critical effect will provide further safety from more severe clinical disease caused by deficiency or toxicity. Based on present knowledge, it seems possible in most cases to define an AROI for the essential elements that will protect 95–98% of the population from adverse effects (i.e., the protective level at each end of the U-shaped curve would be 97.5–99%).

These considerations are based on the assumption that adverse effects from deficiency or toxicity follow an S-shaped (or Z-shaped for deficiency) dose-response curve. As mentioned, such curves represent the cumulative conversion of a gaussian distribution (or other defined distribution) of individual thresholds of effect in a population. Such effects, which occur when a threshold concentration is exceeded in a specific tissue of an individual, are termed *deterministic effects*. Many adverse effects, which result from, e.g., enzyme inhibition and similar biochemical mechanisms, are considered to be of this nature.

In Figure 4 (right side), a theoretical example of dose-response curves for a metallic compound is illustrated. Effects of increasing severity occur when the daily oral intake increases above a certain level (curves 5–8). Slight changes in biochemical markers without functional or clinical significance (curve 5) are of limited importance in relation to public health. Subclinical markers of effects

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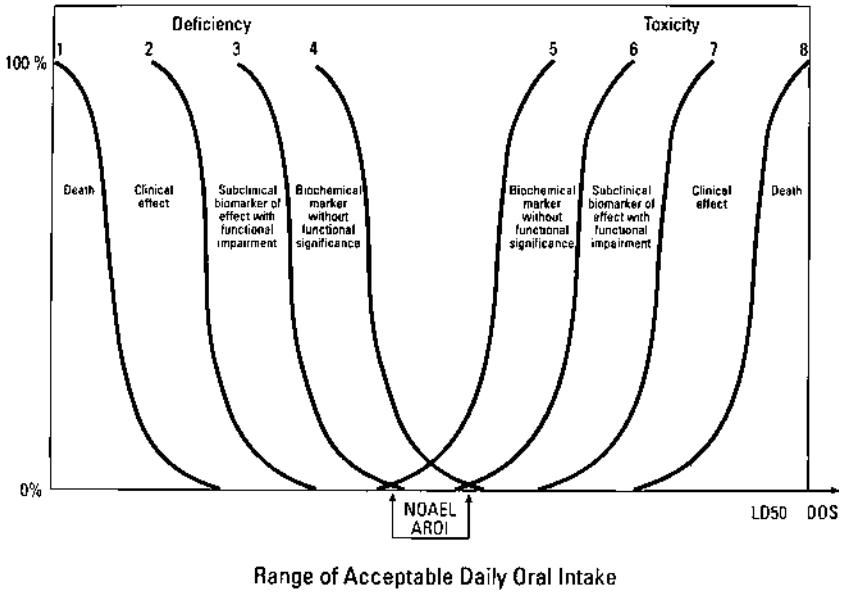

FIGURE 4 Theoretical dose-response curves for various effects occurring in a population at various levels of intake (doses) of an essential trace element. Lethal effects and clinical disease must always be prevented, but subclinical effects indicating impairment of organ function are often identified as critical effects. The lower end of the dose-response curve for such critical effects related to deficiency (curve 3) and toxicity (curve 6) defines the range of acceptable daily oral intakes (AROI). Biochemical effects without functional significance (4 and 5) are considered without health impact and should not be taken as critical effects.

that are related to functional deficits or that signal the development of clinical disease are more relevant to public health and such markers are often crucial for preventive action. These effects and their dose-response curves can be regarded as a critical piece of information when discussing preventive measures such as exposure limits and recommendations of safe intakes and such effects are termed *critical effects*. Sometimes subclinical biomarkers of effects have not been identified, and the clinical effect that occurs at the lowest dose must be used as critical effect.

These evaluation principles focusing on the critical effect, i.e., the adverse effect that occurs at relatively low exposures and implies some kind of clinical or functional impairment, have been adopted by several international bodies and have been used by WHO/IPCS (26).

In toxicological cancer research and in radiation biology, considerable research efforts have been devoted to characterizing another type of effects, so-called *stochastic effects*, which are considered to occur as a result of a random process of interaction between the agent and DNA in the cell nucleus. Cancer induction is considered to be such a stochastic effect. For such effects it has been assumed that the dose-response curve has a linear component in the low dose range (e.g., 27) and for safety reasons it has been the policy in some situations to set very low acceptable exposures, since only extremely low risks have been considered as acceptable for cancer from environmental pollution with chemical substances. Risks of one per one million or one per hundred thousand have sometimes been used. Under such acceptance conditions the carcinogenic effect will be the critical effect.

The application of linear low-dose extrapolation in cancer risk estimation for chemical substances is controversial. The existence of a linear component in the low dose range has been refuted by other groups of scientists (28). Even one of the authors (29) of the original Crump et al. paper considers that it creates a false sense of scientific security in low-dose extrapolation.

The International Agency for Research on Cancer considers that there is no scientifically valid basis for a generalized model for low-dose extrapolation in chemical carcinogenesis. In the preamble to the Monographs, it is stated:

> The form of the dose-response relationship can vary widely, depending on the particular agent under study and the target organ. Both DNA damage and increased cell division are important aspects of carcinogenesis, and cell proliferation is a strong determinant of dose-response relationships for some carcinogens (30). Saturation of steps such as absorption, activation, inactivation and elimination may produce nonlinearly in the dose-response relationship, as could saturation of processes such as DNA-repair (30–32).

In radiation protection, on the other hand, there has been general agreement on the usefulness of linear extrapolation and use of the ''collective dose'' approach when establishing exposure limits (33–35). However, the possibility cannot be excluded that there may be a threshold for cancer induction in the millisievert (mSv) range (35). Radiation levels considered acceptable by the International Commission on Radiological Protection (1 mSv per year) are likely to correspond to a considerably higher risk than one in a million for lifetime exposure, suggested as an acceptable risk level for carcinogenic chemical agents.

While the approach with linear low-dose extrapolation may serve a good purpose for the protection of public health, when regulating ionizing radiation or artificial nonessential chemical pollutants, similar safety considerations cannot

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be applied to essential trace elements, even if they may be carcinogenic at excessive intakes.

For essential elements it is obvious that the balance between risks of deficiency and risks of toxicity must be considered. To use policy considerations, in the absence of sufficient scientific evidence, as a basis for a linear low-dose extrapolation down to zero dose is not in the interest of public health, since such extrapolation may lead to recommendations of too low intakes that may cause severe clinical deficiency and death. Also, for considerations relating to carcinogenicity, there is obviously a need for a balanced consideration and there is an urgent need to make a detailed examination of available evidence concerning carcinogenicity of essential elements and to develop a policy of extrapolation that is compatible with maximal public health benefit.

4.2 Examples of Effects of Varying Severity

4.2.1 Deficiency

Lethal Deficiency. Severe deficiency sometimes with lethal outcome may result from situations when individuals are completely devoid of the nutritional element in question. Such situations may lead to clinical disease and in extreme cases death such as in severe cases of myxedema that may occur in pronounced iodine deficiency (36). Severe iron deficiency may also lead to severe anemia with lethal outcome in rare cases (9). In China, Keshan disease, with myocardial abnormalities and sometimes death, may occur as a result of selenium deficiency, possibly in combination with viral disease (37,38). Lethal outcomes in deficiency cases are often related to some interacting agent or intercurrent disease and can then be considered as deficiency in a sensitive subgroup of the population.

Deficiency—Clinical Disease. Cases of myxedema and goiter with decreased thyroid functions were common in the past before dietary fortification with iodine was undertaken (36). Goiter results from TSH stimulation of growth of thyroid cells. In rats it has been shown that iodine deficiency with resulting high TSH levels will promote the development of thyroid cancer when a carcinogen is also administered (36).

Iron deficiency with clinically observable anemia with pallor and fatigue is still not uncommon among women of childbearing age (36). Psychomotor development has been shown to be impaired in children whose mothers were iron-deficient during pregnancy (39).

Clinical deficiency symptoms as a result of copper deficiency are more rare, but anemia unresponsive to iron therapy sometimes can be ascribed to copper deficiency (40). Growth failure, poor immunity, impaired wound healing, and impairment of special senses and cognition are clinical signs of zinc deficiency (20,41). Selenium deficiency has been shown to be related to an endemic disease

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in China with cardiomyopathy and sometimes changes in the thyroid called Keshan disease, and also to Kashin-Beck disease, a joint and muscle disease occurring in the same area (42). Muscular symptoms have been reported in patients on total parenteral nutrition (TPN) (43).

Subclinical Markers of Deficiency With or Without Clinical Significance. A number of markers related to decrease in enzyme levels etc. have been shown to be changed in persons with low intakes of some essential elements. In some cases such influences on enzyme levels are believed to be of functional significance and may even be precursors of clinical disease. In other cases a slight decrease in an enzyme level may be tolerated without any functional significance, namely if there is a great surplus of this enzyme in the medium in which it is measured. It is sometimes necessary to devote considerable research efforts to the demonstration of relationships between such indicators and the occurrence of disease or impaired physiological performance. The limited information available makes it difficult in some instances to determine whether or not a specific indicator should be considered of clinical or functional significance.

An example of a subclinical marker of deficiency is the saturation of glutathione peroxidase (GSHPx) in plasma, erythrocytes, and platelets, which is impaired when selenium intakes are low. Incomplete GSHPx saturation in plasma does not occur until Se intakes fall below approximately 40 μg/day (44,45). For saturation of GSHPx in erythrocytes higher intakes are required and for saturation in platelets more than 100 μg/day is required (45,46). The clinical or health importance of such incomplete GSHPx saturation is debated, particularly in the most sensitive compartment, i.e., platelets.

For some essential elements, e.g., zinc, specific and sensitive biochemical indicators of deficiency are lacking. In the absence of such indicators, the estimates of requirements have to be evaluated by a factorial technique, i.e., by adding together the requirements for tissue growth, maintenance, metabolism, and endogenous losses. It is notable that the activity of zinc-dependent processes as well as plasma and tissue zinc concentrations can be maintained over a long time at low intakes by substantial reductions in endogenous losses of zinc. This adaptive ability was taken into account by WHO in 1996 for estimates of the physiological requirement of absorbed zinc using data from long-term balance studies at very low intakes (47,48). At the basal requirement level the ability to increase the efficiency of zinc retention has been fully exploited. Observations made during the early phase of the same studies of zinc depletion were used to estimate the normative physiological requirement. For adult males an uptake of 1.4 mg/day was judged to maintain zinc equilibrium without the need of adaptive changes in endogenous losses. With 30% fractional uptake from the diet this corresponds to a daily normative dietary requirement of 4.7 mg/day. Lacking corresponding long-term studies in other age groups endogenous losses in relation to basal metabolic rates were used as the basis for extrapolation.

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Another example is a decrease in ceruloplasmin levels in low copper intakes and decreased superoxide dismutase (SOD) when copper intakes are low (19). While these changes are considered useful indicators of decreased biological copper activity, their clinical significance has not been well defined.

A reduction of maximal oxygen consumption has been demonstrated in physical-performance tests in young women with low serum-ferritin concentrations without anemia suggesting effects on tissue level unrelated to oxygen-transport capacity (49).

When iron intakes are low, serum ferritin levels decrease and there is a decreased transferrin saturation and increased erythrocyte protoporphyrin. While these indicators are excellent indicators of low body iron stores, slight decreases in iron stores are of debatable clinical significance.

4.2.2 Toxicity in Humans from Excessive Exposure

Lethal Effects. Excessive doses of soluble salts of iron or copper by the oral route, which may be ingested by accident or with suicidal intent, give rise to extensive and severe gastrointestinal manifestations, systemic toxicity, shock, and death (19,50).

Toxic Effects with Clinical Significance. Clinical disease (without fatal outcome) may occur as a result of ingestion of high doses of soluble selenium salts. Such poisoning cases display nausea, vomiting, and subsequently hair and nail changes and skin lesions (51). Persons ingesting large doses of copper in addition to vomiting, nausea, and diarrhea may develop hematuria and jaundice (19). Also ingestion of soluble iron salts give rise to gastrointestinal manifestations with vomiting and diarrhea often with bloody stools and later cirrhosis may occur (50).

Subclinical Toxic Effects With or Without Functional Significance—Biomarkers of Critical Effect. Like the situation with biochemical markers of deficiency, subclinical markers of toxicity have been identified in the form of changes in enzyme levels, etc. To use such a biomarker as a critical effect, it is of great importance to be able to determine the extent to which increased levels of such biomarkers in media easily accessible for analysis (blood, urine, hair, etc.) signal a subclinical stage of a disease or abnormality in organ function. Unfortunately, for several potentially useful markers, there is a lack of such information. Further definition of the clinical and functional significance of biomarkers is needed. For example, decreased E-SOD levels may occur as a result of excessive zinc intakes. Most probably, this is a result of interference by zinc with uptake of copper and the balance between zinc and copper is of fundamental importance for the expression of this effect (19). For excessive iron intakes, excessive transferrin saturation and excessive levels of serum ferritin can be used as markers of iron overload (50). The clinical significance of marginally increased iron overload is still under discussion. Large excesses like those occurring in persons with hemochromatosis

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are clearly of clinical importance. Homozygotes for hemochromatosis are at several hundred-folds increased risk of liver cancer and an increased risk of coronary heart disease (52,53). Moderate levels of iron overload may also be related to increased risks for carcinogenicity in several organs including the liver, but such effects are not well documented. A relatively large proportion of the population are heterozygotes for the hemochromatosis gene and are potentially at risk for iron overload.

High selenium intakes may give rise to prolonged plasma prothrombin time and increased ALAT.

5. ESTIMATION OF AROI AND SRPMI

5.1 Selection of Critical Effects

Theoretical dose-response curves for various effects that might occur in a population at various levels of intake (doses) of an essential element are given in Figure 4. When defining AROI for individuals in a population, it is obvious that both lethal effects and clinical disease arising from deficiency or toxicity cannot be accepted as indicators of the limits for the AROI. For such severe adverse effects there is usually no conflict between the dose-response curves for toxicity and deficiency. Subclinical effects indicating impairment of organ function are often identified as critical effects. The lower end of the dose-response curve for such effects related to deficiency (curve 3) and toxicity (curve 6) defines the range of acceptable daily oral intakes. As discussed earlier, substantial research efforts are required to establish relationships between physiological (functional) and biochemical effects and usually such research data are needed to determine whether a biochemical effect is with or without functional significance. Biochemical effects without functional significance, i.e., curves 4 and 5 (Fig. 4), are considered without health impact and should not be taken as critical effects. However, given the present database on essential metals, it may be necessary to accept a low prevalence of biomarkers without functional or clinical significance, either on the toxicity or the deficiency side, to establish a range of acceptable intakes, since it is essential that more obvious and clinically important risks of disease are prevented on both the deficiency and toxicity side of the AROI.

5.2 Level of Protection and Methodology for Estimation of Boundaries

The level of protection from adverse effects of toxicity and deficiency may depend on practical possibilities of establishing an AROI. Usually, a range protecting 97.5 or 98% of the population from minimal deficiency or minimal toxicity at each end of the range should be aimed for. If 97.5% of the individuals in a population group are protected from deficiency and the same percentage are protected from toxicity this means that 95% of the population group are protected

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from both deficiency and toxicity. Since requirements as well as sensitivity to development of toxicity vary according to age and sex, AROI is often defined for individuals in specific gender and age groups.

As discussed earlier, a similar level of protection as the one for AROI has been considered by the WHO/FAO/IAEA Expert Consultation (6). This Expert Consultation identified the need to work with recommendations of mean population intakes, since it is difficult in practice to give a precise description of upper and lower ends of intake distributions. They defined the mean normative requirement so as to protect 97–98% of the population from minimal adverse effects (including undesirably low tissue stores). The upper limit of population mean intake to avoid (minimal) toxicity in 98–99% of the population was also discussed (6). Based on these considerations SRPMI was derived (6). As discussed earlier, such a level of protection means that 96–98% of the population group will be protected from both (minimal) toxicity and (minimal) deficiency.

The methodology for the estimation of the limits of the AROI described here as well as that described by the World Health Organization for the determination of the SRPMIs (6) and the methodology used to estimate Dietary Reference Intakes by the U.S. Food and Nutrition Board, Institute of Medicine (54) estimate a range of intakes for nutrients (e.g., essential metals) that protects most of the healthy individuals in specified gender and life-stage population groups from the risk of adverse effects from low intakes (deficiency) or excess intakes (toxicity). In deriving the limits of the AROI, uncertainties in the data as well as the variability in the intakes of individuals within the group must be considered.

The lower limit is, in general, derived from nutritional data from studies in humans, and the uncertainties are usually taken into account through the development of an intake adequate for 50% of the individuals within a population group plus 2 SD. This limit is considered protective for a risk from deficiency in 97–98% of the group. The SRPMIs (6) are derived either from the basal requirement or from the normative requirement of an element, assuming a normal distribution of intakes and the coefficient of variation of the intake distribution. As for the AROI, it is assumed that where the intakes of a population are maintained within the SRPMI, the risks to individuals within the group from deficiency or toxicity will be extremely low. More details on these methodologies can be found elsewhere (6,54–56).

With the exception of the SRPMI methodology, the upper limit of intake for an ETM by an individual within a population group has been determined using the four-step risk assessment framework described by the U.S. National Research Council (57), that is, hazard identification, dose-response assessment, intake (exposure) assessment, and risk characterization. This method has been further developed into a seven-step procedure to be described later.

Interindividual variability in sensitivity to toxicity or deficiency can be taken into consideration in two different ways, either by estimating thresholds

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of adverse effects combined with uncertainty (or safety) factors (UF) or by using all data of importance for the dose-response curve for the critical effect.

When using the threshold + UF approach, the level of intake that is so low that the critical effect can no longer be observed, i.e., the no observed adverse effect level (NOAEL), and/or the lowest intake level at which the critical effect is observable, i.e., the lowest observed adverse effect level (LOAEL), is used in combination with an UF to derive a safe level of intake. The UF takes into account interindividual variability in sensitivity, interspecies differences as well as other uncertainties of data quality. Since ETMs are not considered carcinogenic in humans, a threshold is assumed and the NOAEL, or LOAEL, obtained from the dose-response assessment is divided by the product of the UFs chosen to obtain the upper limit of the AROI. The special problems associated with the application of the risk assessment model to nutrients have been discussed by the IOM (54). When applied to nonessential environmental chemicals zero exposure is assumed to be without risk; however, the fact that nutrients, within certain ranges of intakes, are essential for human life limits the need for some of the large uncertainty factors often used in the assessment of nonessential chemicals. Also, the database used to evaluate nutrient toxicity often comes from studies of human populations and is thus less uncertain with respect to its use in human health risk assessments than that from animal studies. Unlike nonessential chemicals, tissue levels, and therefore biological effects, of essential metals are under homeostatic control. Also, tissue levels may be markedly altered by speciation and bioavailability from various dietary matrices, as well as nutrient-nutrient and nutrient-diet interactions that might occur. All of these facts present unique problems in the application of the risk assessment model for the development of AROIs for ETMs. It is beyond the scope of this chapter to describe in more detail the process of health risk assessment. It has been described in detail elsewhere (57–60).

The method, taking into account all data that are important for the dose-response curve for the critical effects, makes it possible to use relevant toxicokinetic and toxicodynamic information that may be available as well as all data describing the shape of the dose-response curve and related statistical uncertainty. The use of toxicokinetic and toxicodynamic modeling with estimated interindividual variability in computing the dose-response curve for the critical effect has been advanced by Nordberg and Strangert (61–63) and by Nordberg (25). These methods have been used in WHO/IPCS criteria documents for nonessential metals, e.g., methylmercury (64) and cadmium (65). The methods advanced by Nordberg and Strangert also allows for the statistical uncertainties in the data to be taken into account and safe levels of intake can be estimated at the safety level desired. The latter procedure, i.e., the derivation of the lower confidence limit of the dose that produces a small increase in the occurrence of critical effect is similar to the one advanced by Crump in 1984 (66) called benchmark dose approach. These methodologies should preferably be applied to data from humans.

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They should be employed as widely as is practical. As mentioned previously, some features of these methods are used in deriving the SRPMI (6).

In an effort to ensure that all available data on the effects of exposure to ETMs are evaluated in a consistent manner by all appropriate disciplines working in a collaborative manner, and considering the special properties of nutrients discussed above, a framework of seven steps for the determination of an AROI has been proposed (20,67). Depending on the data available for evaluation, the threshold + UF approach or the method using all available data for the dose-response curve for the respective critical effects can be used in relevant parts. The seven steps can be summarized as follows: (1) select data, evaluate adequacy of human and animal data, select biologically significant end points, and identify population groups of concern, (2) evaluate homeostatic mechanisms, nutrient and dietary interactions, and consider the impact these may have on the effects of concern (both deficiency and toxicity), (3) using comparable procedures, define the critical effect(s) from deficient and excess intakes, (4) dose-response analysis: qualitative and quantitative evaluation of the critical effects in population groups of concern (defined by gender and life stages), (5) balanced quantitative assessment of critical effects to derive, for population groups of concern, an AROI, the limits of which are not outside the customary range of intakes within healthy populations, (6) exposure assessment: determination of exposure pathways, exposure routes, bioavailability, and exposure patterns for subgroups in the population, (7) risk characterization: the integration of exposure information and the AROI, taking into account the variability in exposure and dose responses for multiple subpopulations and uncertainties within the database and population of concern. Risk characterization should recognize the unique characteristics of each specific situation and ETM. All of these steps are carried out in an iterative manner, and any summary prepared must describe the overall strengths and limitations (including uncertainties) of the assessment and conclusions. Detailed guidance on exposure assessment and risk characterization (steps 6 and 7) is available (68,69).

6. EXAMPLES OF ASSESSMENTS CONCERNING ACCEPTABLE RANGES OF INTAKE FOR ESSENTIAL TRACE METALS

6.1 Selenium

As mentioned earlier [cf. also Alexander and Aro (70)], severe selenium deficiency is considered to be a factor in the endemic, sometimes fatal Keshan disease (occurring in the Keshan region in China). This disease is characterized by myocardial abnormalities and occurs in persons with serum levels of Se lower than 0.13 micromol/L (10 μg/L), particularly in children and women of childbearing

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age. Daily intake of Se in the Keshan area has been calculated to range from 3 to 22 μg. It is possible that this disease occurs as a result of a combination of Se deficiency and a viral disease (or other additional factor), since myocardial abnormalities are not always seen in persons even with severe Se deficiency, but there is no clear evidence concerning such additional factors (37,38). There may also be an impairment of the thyroid in Keshan disease (71). Another disease considered to be related to Se deficiency is Kashin-Beck disease, a joint and muscle disease occurring in the Keshan area in China. In less severe selenium deficiency, as recorded in a study from Finland (serum Se less than 45 μg/L) a significant negative correlation between low selenium levels and cardiovascular death was reported (72).

In animals, Se deficiency has been shown to give rise to growth failure, reproductive failure, and degenerative changes in several organs including cardiomyopathies (70).

The essentiality of Se to humans and animals is believed to be related to the fact that it is a constituent of glutathione peroxidase. It is also a constituent of several other enzymes (e.g., tetraiodothyronine deiodinase).

Excessive intake of selenium can give rise to toxic effects, i.e., selenosis characterized by hair changes, thickened nails with spots, and skin changes. Higher intakes can give rise to neurological symptoms. An early indicator of toxicity is a prolonged prothrombin time, seen at dietary intakes exceeding approximately 800 μg/day (70).

For selenium the WHO/FAO/IAEA Expert Consultation (6) used the intake necessary to obtain two-thirds of maximum plasma glutathione peroxidase activity as the criterion for normative requirement. The 50% risk of minimal deficiency (using this criterion and an assumed interindividual variability of dietary selenium intake of 16%) corresponding to the lower limit for normative population mean intake was set at 40 μg/day for adult men. The 50% risk of toxicity was not possible to identify. The highest population mean intake was set at 400 μg/day, being 50% of the intake where biochemical signs of disturbances in selenium metabolism, in the form of a reduction of the ratio of selenium in plasma to that in erythrocytes, start to occur. Clinical signs of selenosis are observed at and above intakes of 900 μg/day. If the same interindividual variation in requirement as used for the zinc estimates (CV 12.5%) were applied an AROI for selenium would be 32–400 μg.

6.2 Iron

In contrast to many other essential trace elements the body has an ability to store excess iron to be utilized in periods of increased requirement or reduced intakes. The body homeostasis of iron is also strongly regulated through changes in efficiency of absorption while the possibility to get rid of excess iron through an

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increased excretion is limited. The requirement of iron is estimated by the factorial approach, i.e., the sum of obligatory losses with the addition of that needed to provide for normal body accretion rates during growth and pregnancy.

In adult men, iron loss is mainly due to the exfoliation of cells while losses in urine and sweat are negligible. These basal losses have been estimated at 14 μg/kg body weight (0.8–0.9 mg/day). The individual variation in these losses appears to be of the order of 15%. In women of childbearing age, menstrual losses are added to the basal losses. The frequency of distribution of menstrual blood losses is highly skewed with a proportion of women having high losses. The median menstrual iron loss corresponds to about 0.5 mg/day; 10% lose more than 1.3 mg/day and 5 percent more than 1.6 mg (73). Iron required for infant, child, and adolescent growth corresponds to 0.22–0.65 mg/day. The expansion of the red cell mass and the growth of the fetus and the placenta during pregnancy correspond to an increased iron need of 3.6 mg/day in the second trimester and 5.5 mg/day in the third trimester. These figures are all related to the amount of iron that has to be absorbed. Iron absorption is strongly influenced by the composition of the diet and in a similar way as for zinc, dietary iron requirements have been given for three types of diet characteristics: low (5% absorption), intermediate (10% absorption), and high (15% absorption). The median basal requirement of iron in a population consuming an intermediate bioavailability diet varies from 5 mg in children aged 1–2 years to 16 mg in children aged 12–16 years. If these figures were used to derive a SRPMI value, assuming the same variation in intakes as was used for zinc in the WHO/FAO/IAEA Expert Consultation (6), the lower range for the SRPMI would be 10–32 mg. The skewed requirements of menstruating women could, however, mean that at these mean intakes a substantial part of the women would not have covered their requirements. The same problem arises when trying to define an AROI for iron. Adopting a coefficient of variation of 15% would give AROIs of the order of 6–20 mg for population groups excluding menstruating and pregnant women. Taking the actual distribution of iron requirement in menstruating women into account would mean that $<$30 mg of dietary iron would be needed at intermediate bioavailability to cover the needs of 95% of the individuals in this population group. These levels cannot be reached by typical diets but require fortification or supplementation. The same is valid for the requirements in late pregnancy.

Few data are available to estimate the upper range of SRPMI or AROI for iron. The acute toxic dose in infants has been estimated to approximately 20 mg/kg and the lethal dose to about 200–300 mg/kg body weight. The long-term potential negative effects of a high iron intake are related to the prooxidative characteristics of iron and the potential interaction with other elements. Chronic iron overload as in primary hemochromatosis results in hepatomegaly, cardiac disease, and liver cirrhosis. Use of iron utensils for cooking and preparation of alcoholic beverages in urban Africans has been reported to result in excessive

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iron deposits in the liver and liver cirrhosis (74,75). Traditional beers can contain up to 75 mg iron/L (76) resulting in an exposure of 50–100 mg/day. A few cases of increased serum-ferritin levels after long-term intake of iron supplements have also been reported (3). At typical dietary iron intakes absorption is closely regulated and not likely to lead to iron overload (77). However, it can be estimated from the relation between efficiency of absorption and the impact of iron status that 5 years' intake of a supplement containing 60 mg of iron would increase serum-ferritin concentrations to 140 μg/L (3), i.e., close to suggested cutoff levels for iron overload (78).

Oxidative damage is involved in the pathogenesis of cardiovascular disease and cancer. It has been suggested that high iron stores could increase the risk for these diseases. The epidemiological evidence for this is, however, inconsistent and inconclusive.

Excessive iron intake could result in negative interactions with other trace elements. Addition of 50 mg of iron to an aqueous solution of zinc has been shown to reduce zinc absorption by 50%, while the same amount of iron added to a meal did not affect zinc absorption (79). Decreases in plasma zinc concentrations after iron supplementation have been observed at doses >60 mg/day (80–82), but occasionally also at lower doses (83).

These data suggest that the upper level of SRPMI and AROI would be <50 mg and consequently the range of safe or acceptable intakes would be narrow.

6.3 Manganese

Manganese shares many chemical similarities with iron and at least in the absorptive process the body appears not to be able to distinguish between iron and manganese (84,85). The biochemical function of manganese is well established and manganese deficiency has been produced in several animal species, but not so far in humans. The lack of clinical or biochemical signs of deficiency in humans has made it impossible to establish requirements for manganese. Manganese toxicity as a result of chronic inhalation of airborne manganese in mines and steel mills is well described resulting in neurological disorders similar to Parkinson's disease (86,87). A few reports of toxicity due to contaminated water have also been published (88). These studies do not, however, allow estimates of acceptable upper intake levels. Manganese absorption is increased in states of iron deficiency (13) and theoretically a low iron status could increase the risk for adverse effects of high manganese intakes. However, in contrast to iron, manganese is reexcreted via the bile, which should reduce the possibilities for body accumulation of manganese.

6.4 Zinc

There is a relatively large database that can be used to develop an AROI for the chronic oral intake of zinc from food, water, and supplements. The data on zinc

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as an essential nutrient, human intake, bioavailability, metabolism, and toxicity have been reviewed elsewhere (6,20,89–92).

Maintenance of the levels of zinc in the human body requires the daily absorption of about 5 mg zinc/day. Under controlled conditions approximately 33% of dietary zinc is absorbed; however, this can vary between 15 and 55% depending on the source of zinc and dietary-nutrient and nutrient-nutrient interactions. Diets high in unprocessed plant material are considered low in zinc content and of low bioavailability, whereas diets containing red meat and/or shellfish have the highest concentrations of zinc. Very little, if any, zinc in the diet is free and bioavailability depends on many factors, whereas zinc in supplements tends to be easily ionizable with bioavailabilities as high as 95% (e.g., zinc sulfate, zinc gluconate). Dietary zinc intakes worldwide reflect the great variations found in the dietary constituents between regions. Adult males consuming diets high in red meat and/or shellfish (e.g., Australia, Belgium) have intakes of 13–15 mg Zn/day, whereas those consuming vegetarian diets have intakes between 6 and 9 mg Zn/day. However, total zinc content of the diet provides only a gross estimate of zinc intake. In setting an AROI, it is the bioavailable zinc that is crucial. Homeostatic control of zinc metabolism takes place in the small intestine and involves a balance between the absorption of zinc from the diet and that from endogenous sources.

Growth retardation, a delay in sexual and skeletal maturation, development of orificial and acral dermatitis, diarrhea, alopecia, decreased appetite, and behavioral changes are the principal clinical features of severe zinc deficiency (6). Given the wide range of biological reactions for which zinc is essential, it is not surprising that the clinical and functional changes noted in mild zinc deficiency are extremely diverse, not necessarily specific for zinc deficiency, and often inconsistent between studies. Diagnosis of mild zinc deficiency in humans is hampered by the lack of a single, specific, and sensitive biochemical index. At present, the most reliable method for such diagnosis appears to be a positive response to zinc supplementation. Fortunately, there are many well-documented studies of the metabolic control of zinc homeostasis in humans that can be used to determine the lower boundary (nutritional needs) for an AROI, rather than relying on the available, but not well-validated, indices of zinc deficiency. That is, using a factorial analysis, the minimal quantity of absorbed zinc is determined that is necessary to match total daily losses of endogenous zinc (91), and this value used to calculate the lower boundary of an AROI for zinc.

There is no evidence of adverse health effects from the intake of zinc normally found in the various diets consumed worldwide. However, adverse effects associated with the chronic ingestion of zinc supplements (usually as the sulfate or gluconate) include suppression of immune response (300 mg Zn/day as the gluconate for 6 weeks), decreased high-density lipoprotein (HDL), and anemia from copper deficiency (90). Acute effects from the ingestion of high doses of soluble zinc salts have been reported and include epigastric pain, nausea, vom-

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iting, loss of appetite, abdominal cramps, diarrhea, and headaches. An emetic dose of 1–2 g zinc sulfate (225–450 mg zinc) was calculated by Fosmire (89). Such data are of little value in developing an AROI for chronic ingestion of zinc. The effects of zinc supplements on lipoprotein levels, and the effect of zinc on copper status, occurred at similar daily intakes (50–160 mg Zn/day). However, the effects on lipoproteins were less consistent between experiments. In fact, one well-conducted study failed to find a decrease in HDL at 150 mg Zn/day and found, in fact, a slight decrease in LDL in females under this regimen. On the other hand, in all studies where the interaction of excess zinc and copper was measured, there was a consistent decrease in the activity of the enzyme ESOD, an erythrocyte enzyme, which is an extremely sensitive indicator of copper status. Yadrik et al. (93) reported a decrease in ESOD activity following the intake of approximately 60 mg zinc/day (50 mg supplemental zinc plus 10 mg dietary zinc) for 10 weeks. The clinical significance of decreased ESOD activity is not known; however, it is recognized as an extremely sensitive indicator of the effect of excess zinc intake on copper homeostasis, which could result in copper-deficiency anemia if exposure to high levels of zinc continued.

Factorially based estimates of the basal and normative physiological requirements of individuals for absorbed zinc were determined by the WHO Expert Consultation (6). Taking into consideration the variables governing the efficiency with which zinc is absorbed, the Consultation developed a SRPMI for diets of low, moderate, and high bioavailability of zinc. Average individual normative requirements for adult males were calculated for low, moderate, and highly bioavailable diets to be 9.4, 4.7, and 2.8 mg Zn/day, respectively. Assuming a coefficient of variation for zinc intake of 25%, the SRPMI for adult males consuming these three classes of diets would be 18.7, 9.4, and 5.6 mg Zn/day, respectively. These values should be considered as the lower boundary of the AROI.

The lower activity of ESOD, a sensitive marker enzyme for copper status, after chronic intake of zinc from nondietary sources was taken as the adverse effect for the calculation of the upper boundary of the AROI. In calculating the upper level of zinc intake it is important to realize that there is still debate whether the intake of 60 mg Zn/day represented a LOAEL or a minimal NOAEL. Whether a NOAEL or a LOAEL, the effect measured is considered a minimal adverse effect. Based on the effect level of 60 mg Zn/day, and a variation in intakes of 20%, a population mean intake of 45 mg Zn/day was recommended (6). This upper limit should be considered as extremely conservative, since it is based on the effects of ingestion of an extremely bioavailable form of zinc, not normal dietary zinc levels. Intakes of 60 mg dietary zinc in a moderately bioavailable diet would not result in equivalent amounts of absorbed zinc as that from the zinc supplement administered.

In summary, the lower limit of AROI for zinc in adult males is 18.7, 9.4, and 5.6 Zn/day for diets of low, moderate, and high bioavailability, respectively.

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The upper limit is 45 mg Zn/day. Using the risk assessment model to determine the level of toxic risk for individuals within a defined population, based on the minimal effect noted on the activity of ESOD from 60 mg Zn/day, and applying an UF of 1.5 to take into consideration the variability in zinc intakes, an upper boundary for the AROI would be 40 mg Zn/day. Based on present daily intakes of zinc (6–15 mg/day), there appears to be more concern that populations are consuming diets suboptimal in zinc with little risk from toxicity from combined intakes from diet, water, and supplements.

It should be noted that in estimates of requirement and lower limits of populations mean intakes for zinc at three levels were defined corresponding to low, moderate, and high dietary availability while there were no data available to make a similar distinction for the upper limits of intakes. It is, however, reasonable to assume that dietary factors also will affect the fractional uptake and metabolic handling of toxic levels of intake.

6.5 Copper

In developing the AROI for copper it is essential to differentiate the bioavailability and adverse effects of highly ionized copper found in water and beverages, as well as supplements, from copper intake from a mixed diet where the copper is largely bound to organic ligands and is thus less available for absorption (6). It has been shown in humans that consumption of copper, as copper sulfate, in excess of 3 mg Cu/L of drinking water, results in nausea and other adverse effects on the gastrointestinal tract (94). The threshold for effects and the variability of effects within populations remain to be elucidated. There are no reports on similar gastrointestinal effects from copper in a mixed diet. Several reviews are available on the metabolism, nutritional needs, and toxicity of copper (6,19,40,95), which can be used to develop an AROI for copper for the chronic intake of copper from dietary sources, including fortified foods.

Dietary copper is well absorbed in the small intestine and varies with intake. At <1 mg copper/day absorption is $>50\%$ whereas at 5 mg Cu/day absorption is $<20\%$. Copper homeostasis is maintained by the intestine, with biliary excretion being adjusted to maintain copper balance (96). In general, the composition of the diet plays little role in affecting the availability of copper. However, probably mediated through the increased synthesis of metallothionein, high intakes of zinc and cadmium can decrease copper absorption. Also, diets high in iron and molybdenum have adverse effects on copper metabolism in laboratory animals and ruminants and may influence copper bioavailability in humans. Globally, daily dietary intakes of copper in healthy, nonoccupationally exposed populations vary between 0.9 and 2.2 mg.

Symptoms associated with copper deficiency in humans include hypochromic anemia, neutropenia, hypopigmentation of hair and skin, abnormal bone de-

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velopment, vascular abnormalities, and uncrimped or steely hair. Biomarkers of copper deficiency, with normal ranges in parentheses, include: serum copper (0.64–1.56 μg/ml), ceruloplasmin (0.18–0.4 mg/ml), urinary copper (32–64 μg/24 hr), hair copper (10–20 μg/g), decline in erythrocyte copper-zinc superoxide dismutase (0.47 ± 0.07 mg/g hemoglobin), and platelet cytochrome-C-oxidase activity (~1740 U/mg protein). Many of these markers may not be sensitive to marginal copper status and do not reflect dietary intakes of copper. At present, no single marker provides an adequate basis on which to estimate copper requirements.

In humans, acute copper poisoning is rare and is generally the result of ingestion of highly ionized copper in drinking water or beverages or the deliberate ingestion of copper salts such as copper sulfate. The symptoms have been well described (6,19); however, they are not relevant for the setting of an AROI for chronic copper ingestion from food. Chronic copper toxicity is also rare and appears to occur only in humans having defective homeostatic mechanism for copper (e.g., Wilson's disease, infant childhood cirrhosis, infant copper toxicosis). In such cases, when the capacity for protective hepatic sequestration of copper is exceeded, hepatitis, jaundice, and liver cirrhosis may results.

Based on the above indicators of copper deficiency, the WHO Expert Consultation (6), calculated the normative requirement in adults as 0.7 and 0.8 mg/day for women and men, respectively. Based on these data, and assuming a coefficient of variation for population intakes of 20%, the lower value for the SRPMI was calculated as 1.2 and 1.3 mg/day for women and men, respectively. Noting that the uncontrolled ingestion of milligram quantities of soluble salts should be avoided owing to gastric irritation, there was a lack of adverse effects in adults consuming around 10 mg dietary copper. The upper limit was therefore set at 12 mg/day for men and 10 mg for women. Given the poorly developed homeostatic mechanisms in infants and children, which might result in increased copper storage in liver and cellular damage, an upper limit of the SRPMI for infants was set at 150 μg Cu/kg body weight/day (6). The data supporting this value are not well described in the report.

The WHO Task Group on copper concluded that animal studies were ''less than useful'' in developing an AROI for copper (19). Although they were unable to identify with precision the upper limit of the AROI for copper from available human studies, on the basis of available data showing no effects from the consumption of about 10 mg of dietary copper, they suggested an upper limit for the AROI of several milligrams but not many (<10). Such a value is supported by the small double-blind study by Pratt et al. (97). In seven subjects given 10 mg Cu/day as the gluconate salt (a very soluble form of copper) for 12 weeks, liver function tests were normal and no change was measured in copper levels in serum, urine, or hair. Using the risk assessment model, the NOAEL from this human study could be divided by an UF of 1.2 to take into

account the variability in response and extrapolation from a short-term study to chronic exposure, to suggest an upper limit for the AROI of 8 mg/day. Although there is some uncertainty with this number, it is not markedly different than the upper limit for the SRPMI and is supported by the large international database in humans, which indicates no adverse effects from the chronic consumption of 10 mg dietary copper/day.

In summary, the SRPMI for adult males is 1.3–12 mg Cu/day. Using a model that determines the level of risk for individuals within the population and a small human study, the AROI for adult males would be 1.3–8 mg Cu/day. Based on present intakes of copper (0.9–2.2 mg) it would appear that there are populations worldwide consuming diets marginally deficient in copper.

7. CONCLUSIONS

A new set of principles and methods for assessment of risks from essential trace elements has been described. Nutritional and toxicological information is considered in a balanced approach based on adequate (human) data. Speciation and bioavailability are taken into account based on available information. Critical effects of deficiency and toxicity of similar clinical significance are identified and used in the evaluation.

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2

Analytical Methods for Heavy Metals in the Environment: Quantitative Determination, Speciation, and Microscopic Analysis

Richard Ortega
University of Bordeaux, Gradignan, France

1. INTRODUCTION

The aim of this chapter is to give an overview of the principal analytical methods used in environmental sciences for heavy metals determination. The monitoring of trace metals in the environment has been a subject of great concern over the last decade and will continue to be as there is an ever-increasing amount of metals that have to be found in the environment. Of the 92 naturally occurring elements, approximately 30 metals and metalloids are potentially toxic to humans, Be, B, Li, Al, Ti, V, Cr, Mn, Co, Ni, Cu, As, Se, Sr, Mo, Pd, Ag, Cd, Sn, Sb, Te, Cs, Ba, W, Pt, Au, Hg, Pb, and Bi.

Analytical measurements are an integral part of environmental management. Quantitative determination techniques, described below, are required and must provide valid and affordable element analysis. They are used to assess health effects, which are important in prioritizing contaminants for regulation. Routine monitoring of regulated contaminants ensures compliance with allowed levels and can indicate a hazardous situation. In addition, cleanups of contaminated sites are driven by measurements indicating the location and extent of contamina-

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TABLE 1 Some Useful Internet Links for Environmental Analysis

http://www.who.int/peh/site_map.htm	World Health Organization Protection of the Human Environment
http://www.eea.eu.int/	European Environment Agency (EEA)
http://www.epa.gov/	United States Environmental Protection Agency (USEPA)
http://www.epa.gov/epahome/index/sources.htm	Sources of USEPA test methods
http://clu-in.org/	The Hazardous Waste Clean-up Information Web Site. The site is managed by USEPA's Technology Innovation Office
http://www.osha.gov/	Occupational Safety and Health Administration (OSHA)
http://www.cdc.gov/niosh/homepage.html	The National Institute for Occupational Safety and Health (NIOSH)
http://nvl.nist.gov/	National Institute of Standards and Technology (NIST)

tion. Most of the new information about the environmental chemistry of heavy metals results from continuing improvements in trace element analytical research. This is particularly true in the fields of heavy metals speciation analysis and microscopic analysis, reviewed below.

The problems associated with the collection, preservation, and storage of samples as well as sample preparation and pretreatment will not be detailed in this chapter. The reader is referred to specialized textbooks and monographs for heavy metals water analysis (1), soils and sediments analysis (2), and dust sampling (3). Finally, some Internet links on environmental analysis are given in Table 1, they provide valuable complementary information to this chapter.

2. HEAVY METALS QUANTITATIVE ANALYSIS

This section will only focus on quantitative determination techniques. It could have been organized either by analytical methods, analytes, or matrices (air, water, soil, and sediments). In an effort of concision the first type of presentation has been selected as analytical methods used for heavy metals quantitative determination are often multielemental and applicable to various type of matrices. The element-specific methodologies for individual determination of metals and metalloids, from lithium to transuranium elements, have been recently reviewed in the excellent textbook of Lobinski and Marczenko (4). The detailed description

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for individual analysis of heavy metals can also be found in other books (5,6), or monographs, such as for mercury determination in the environment (7), or for lead analysis (8). On the other hand, the very useful articles of Clement and Yang (9,10) reviewed the developments in applied environmental analytical chemistry in recent years, including inorganic analysis, ordered by matrix type: air, water, soil, and sediments. Detailed protocols depending on matrix type are also given in a number of books, for the examination of heavy metals in soils (2), water and wastewater (1), or workplace atmosphere (11).

2.1 Atomic Absorption and Emission Spectrometry

Atomic absorption spectrometry (AAS) and atomic emission spectrometry (AES) are the most widely used techniques for heavy metals quantitative analysis in environmental samples. These two techniques, and their environmental applications, will be briefly described in this section. For greater depth description than is possible in this chapter, there are many books and articles on analytical atomic spectrometry and these should be consulted (12–14). AAS and AES are particularly applicable where the sample is in solution or readily solubilized. The U.S. EPA has published a sample preparation procedure for spectrochemical determination of total recoverable elements, method 200.2 (15). This method provides sample preparation procedures for the determination of total recoverable analytes in groundwaters, surface waters, drinking waters, wastewaters, and in solid-type samples such as sediments, sludge, and soils. This method is applicable for the following analytes: Li, Be, B, Na, Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sn, Sb, Ba, Hg, Tl, Pb, Th, and U. U.S. EPA method 200.3 describes sample preparation procedure for spectrochemical determination of total recoverable elements in biological tissues (16).

2.1.1 AAS

AAS is one of the most valuable technique for environmental heavy metals analysis (for review see ref. 13). AAS is very simple to use, reliable, and cost effective. Although flame AAS has now largely been superseded by inductively coupled AES (see below), electrothermal (ET) AAS, hydride generation (HG) AAS, and cold vapor (CV) AAS, still present very interesting features for heavy metals analysis.

Description. AAS involves the absorption of radiant energy produced by a special radiation source (lamp), by atoms in their electronic ground state. The lamp emits the atomic spectrum of the analyte elements, i.e., just the energy that can be absorbed in a resonance manner. The analyte elements are transformed in atoms in an atomizer. When light passes through the atom cloud, the atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. A monochromator is used for selecting only one of the characteristic wave-

lengths of the element being determined, and a detector, generally a photomultiplier tube, measures the amount of absorption. The amount of light absorbed indicates the amount of analyte initially present.

Since samples are usually liquids or solids, the analyte atoms or ions must be vaporized and atomized. Several AAS can be distinguished depending on the mode of sample introduction and atomization. Flame (FAAS), electrothermal atomizers (ETAAS), hydride generation (HGAAS), and cold vapor (CVAAS) systems have been described extensively (12,14,17). In FAAS, the liquid sample is pneumatically nebulized, the aerosol is mixed with acetylene, and then introduced in a flame atomizer. FAAS is applicable for quantitative analysis of nearly 70 elements. In ETAAS, which includes graphite furnace AAS (GFAAS), as the atoms are concentrated in a smaller volume than a flame, more light absorption takes place, resulting in detection limits approximately 100 times lower than those for FAAS. However, GFAAS generally requires time to heat the furnace, which makes it slower than flame AAS. ETAAS is applicable to nearly 60 elements. In HGAAS, the analyte is reduced to its volatile hydride (AsH_3, SeH_2, etc). The hydride is stripped-out from solution by an inert purge gas Ar and atomized in either a flame, an electrically heated tube, or a plasma. This technique is only applicable for the elements forming covalent gaseous hydrides, Ge, As, Se, Sn, Sb, Te, Bi, and Pb. Finally, CVAAS applies solely to Hg as it is the only analyte that has an appreciable atomic vapor pressure at room temperature.

Multielement Capability. AAS is predominantly a single-element technique. Although there is a potential for simultaneous multielement analysis (two to six elements), AAS is, however, seriously rivaled by other truly multielement techniques such as ICP-AES and ICP-MS.

Detection Limits.

FAAS <10 μg/L for Li, Be, Na, Mg, K, Ca, Mn, Cu, Zn, Ag, Cd 10–100 μg/L for Al, Ti, V, Fe, Co, Ni, As, Rb, Sr, Rh, Pd, Te, Cs, Au, Tl, Pb 100–1000 μg/L for Si, Sc, Cr, Ga, Ge, Se, Y, Ru, In, Sn, Sb, Ba, Ta, Os, Pt, Hg, Bi

GFAAS <0.01 μg/L for Be, Mg, K, Cr, Mn, Fe, Cu, Co, Zn, Sr, Ag, Cd 0.01–0.1 μg/L for Li, Na, Al, Ca, Sc, Ni, Ga, Rb, Mo, In, Cs, Ba, Au, Tl, Pb, Bi 0.1–0.5 μg/L for B, Si, Ti, V, Ge, As, Se, Y, Zr, Nb, Tc, Ru, Rh, Pd, Sn, Sb, Te, La, Hf, Ta, W, Re, Os, Ir, Pt, Hg

HGAAS <0.1 μg/L for As and Se

CVAAS ~0.02 μg/L for Hg

Environmental Applications. AAS can be applied to a wide range of elements, provided a suitable light source is available. In choosing among AAS techniques, FAAS should be considered first, or second if simultaneous ICP-AES is available, in the determination of Li, Na, Mg, Al, K, Ca, Mn, Fe, Ni, Cu, Zn,

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Cd, Ba, and Pb. FAAS has been widely used with adapters for flame gases and atom traps for the measurement of toxic metals such as Cd and Pb, with respective detection limits of 0.1 and 1 μg/L. U.S. EPA 7000 series methods detail FAAS protocols for 25 metals and metalloids (18). Owing to its better sensitivity, ETAAS is a technique of choice for the following elements: Be, Si, Cr, Co, Mo, Ag, In, Sn, Sb, Au, Tl, and Bi. It is probably the most commonly used technique for measuring ambient levels of chromium in environmental samples (19). U.S. EPA method 200.9 provides a procedure for the determination of dissolved and total recoverable elements by GFAAS in groundwater, surface water, drinking water, storm runoff, industrial and domestic wastewater, sludge, and soil (15). This method is applicable to the following elements: Be, Al, Cr, Mn, Fe, Co, Ni, Cu, As, Se, Ag, Cd, Sb, Sn, Tl, and Pb. HGAAS can be used to determine virtually all elements forming volatile hydrides, such as Ge, As, Se, Sn, Sb, Te, Bi, and Pb, to overcome problems associated with flame AAS determinations. CVAAS is the technique of choice for mercury with limits of detection down to 0.02 μg/L. U.S. EPA method 245.1 describes the determination of total mercury in drinking, surface, ground, sea, brackish waters, and industrial and domestic wastewater by CVAAS (15). U.S. EPA methods 245.5 and 245.6 describe the determination of mercury by CVAAS, respectively, in sediments, and tissues (16).

2.1.2 Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

Flame AAS was until recently the most widely used method for environmental trace metal analysis. However, it has now largely been superseded by ICP-AES (for review see ref. 20).

Description. AES measures the optical emission from excited atoms to determine analyte concentration. High-temperature atomization sources are used to promote the atoms into high energy levels causing them to decay back to lower levels by emitting light. Inductively coupled plasma is a very high excitation source (7000–8000 K) that efficiently desolvates, vaporizes, excites, and ionizes atoms (21). The wavelengths of photons emitted are element specific. The intensity of emission is generally linearly proportional to the number of atoms of that element in the original sample. ICP-AES and the other atomic emission techniques simultaneously or sequentially measure the concentrations of 20 elements or more at sensitivities equivalent to those of AAS. A second advantage of ICP-AES is its broad dynamic range; ICP-AES calibration curves can be linear over several orders of magnitude. In addition, ICP-AES quantifies some nonmetals; phosphorus in particular is an example.

Multielement Capability. Since all atoms in a sample are excited, they can be detected simultaneously, which is the major advantage of AES compared to AAS.

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Detection Limits. 0.1–10 μg/g (solids); 1–50 μg/L (aqueous).

Environmental Applications. Environmental applications utilizing ICP-AES for metal determination encompass a wide range of materials, such as natural waters, seawater, soils, sediments, biological tissues, and air particulate. Waters, wastewaters, and solid samples should be prepared as described in U.S. EPA method 200.2 (15). U.S. EPA method 200.7 describes ICP-AES measurement of metals and some nonmetals (15). This method is applicable to the following analytes: Li, Be, B, Na, Mg, Al, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sn, Sb, Ba, Ce, Hg, Tl, and Pb. OSHA method ID-125G (11) describes ICP-AES analysis procedure for metal and metalloid particulate in workplace atmospheres. It is applicable for the quantitative analysis of 13 elements found in welding fume: Be, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, Sb, Pb, and Bi.

2.2 Inductively Coupled Plasma–Mass Spectrometry (ICP-MS)

Description. ICP-MS is the marriage of two well-established techniques, namely the inductively coupled plasma and mass spectrometry (for review see ref. 22). An ICP argon plasma is used as ion source, ensuring almost complete decomposition of the sample into its constituent atoms (21). The ionization conditions within ICP result in highly efficient ionization and importantly, these ions are almost exclusively singly charged. Mass analysis is simply a method of separating ions depending on their mass-to-charge ratio (m/z). Two types of mass analyzers are commonly employed for ICP-MS: the quadrupole and the magnetic sector. Quadrupoles are comprised of four metal rods, ideally hyperbolic cross section. A combination of radiofrequency (RF) and direct-current (DC) voltages are applied to each rod, which creates an electric field within the region bounded by the rods. Depending on the RF/DC ratio, the electric field between the rods will allow ions in a narrow m/z range to pass, typically 0.8 m/z. Hence by changing the RF/DC ratio in a controlled manner, the quadrupole can be scanned through the range allowing ions of consecutively higher m/z to pass through. Therefore, the quadrupole mass analyzer can only be operated in sequential mode, although the speed with which this can be achieved makes it seem almost like simultaneous mass analysis. The quadrupole mass analyzer has the advantage of being cheap, reliable, and compact, with mass resolution that is sufficient for elemental analysis. It is the most commonly used mass analyzer. However, if an extremely high degree of resolution or true simultaneous mass analysis is required, then a magnetic sector must be used. Magnetic sector mass analyzers rely on the fact that ions are deflected by a magnetic field. In typical commercial instruments, the ions are accelerated after they are skimmed from the plasma, then travel through an electric sector, which acts as an energy filter. The ions

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are then deflected in a single plane by the magnetic field, with the degree of deflection increasing with increasing m/z. A mass spectrum can be generated by scanning the magnetic field. Alternatively, the magnetic and electric fields can be held constant and several detectors arranged in an array, thereby allowing truly simultaneous mass analysis. Magnetic mass analyzers are more expensive, less common, and less easy to operate than quadrupoles. Magnetic sectors cannot be scanned as rapidly as quadrupoles, and they are also capable of simultaneous operation for a limited number of masses. The main advantage of a magnetic sector is the high degree of resolution obtainable ($R = M/\Delta M$). The resolution obtainable with quadrupoles used in ICP-MS is typically between 12 and 350, depending on m/z, which corresponds to peak width between 0.7 and 0.8. In comparison, magnetic sectors are capable of resolution exceeding 10,000. For most applications the resolution provided by quadrupoles is sufficient; however, for applications where spectroscopic interferences cause a major problem, the resolution afforded by magnetic sector may be desirable. For example, a particular problem is the determination of arsenic, $m/z = 75$, in a matrix that contains chloride because of interference with $^{40}Ar^{35}Cl^{+}$.

Multielement Capability. If many elements must be determined in a sample, ICP-MS is fast, many times faster than GFAAS and comparable to ICP-AES. A major advantage over any other spectrometric technique is the access to isotope determination. ICP-MS offers rapid multielement capability but suffers from a number of interferences. Spectroscopic interferences arise when an interfering species has the same nominal m/z as the analyte of interest.

Detection Limits. Quadrupole 1–10 ng/L. Magnetic sector 0.01–0.1 ng/L.

ICP-MS is more sensitive than the GFAAS by more than one order of magnitude. By comparison with ICP-AES, it is more sensitive by almost three orders of magnitude.

Environmental Applications. The applications of ICP-MS are broadly similar to those for ICP-AES, although the better sensitivity of the former has resulted in applications such as the determination of ultralow levels of trace elements (23). ICP-MS technique has been employed to determine a large number of elements in environmental samples (for review see refs. 20,24), and it is especially suited for heavy metals analysis in groundwater samples (25). U.S. EPA method 200.8 provides procedures for determination of dissolved elements in groundwaters, surface waters, and drinking water using a quadrupole mass analyzer in scanning mode (15). It may also be used for determination of total recoverable element concentrations in these waters as well as wastewaters, sludge, and soil samples. This method is applicable to the following elements: Be, Al, V, Cr,

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Mn, Co, Ni, Cu, Zn, As, Se, Mo, Ag, Cd, Sb, Ba, Tl, Pb, Th, and U. The major drawback of ICP-MS is its expense, and that is gradually reducing.

2.3 X-Ray Fluorescence Methods

The English physicist H. G. J. Moseley discovered, in 1914, that the elements in any solid sample could be identified by measuring the spectrum of the secondary X-ray they emit when excited with a X-ray source. This result was of the utmost significance because it gave the periodic classification of elements its definite form; moreover, this technique is now widely used as a method of nondestructive analysis. When atoms are subjected to radiation of appropriate energy, provided by electrons, ions, or photons bombardment, electrons from the inner orbital shells are removed. The orbital vacancies formed are filled with outer orbital electrons producing X-ray radiation. The measurement of their energy and intensity forms the basis of X-ray fluorescence spectrometry.

2.3.1 X-Ray Fluorescence (XRF)

Description. XRF spectrometry uses X-rays as primary excitation source, usually provided by X-ray tubes, or radioisotopes, which cause elements in the sample to emit secondary X-rays of a characteristic wavelength. The elements in the sample are identified by the wavelength/energy of the emitted X-rays while the concentrations are determined by the intensity of the X-rays. Two basic types of detectors are used to detect and analyze the secondary radiation. Wavelength-dispersive XRF spectrometry uses a crystal to diffract the X-rays, as the ranges of angular positions are scanned using a proportional detector. Energy-dispersive XRF spectrometry uses a solid-state detector from which peaks representing pulse-height distributions of the X-ray spectra can be analyzed. Usually, sample preparation required for XRF analysis is minimal compared to conventional analytical techniques. However, for solid samples, since particle size, composition, and element form may affect the analysis, a homogeneous sample is usually prepared for quantitative analysis by fusion with a borate flux (2).

Multielement Capability. XRF spectrometry allows simultaneous determination of most elements with the exception of those with atomic number below 8.

Detection Limits. 10–100 μg/g (soil); 0.5–10 mg/L (water).

Environmental Applications. Energy-dispersive XRF has been successfully applied to determine the major constituents of soils but its poor sensitivity makes it less suitable for analysis of minor and trace elements. Wavelength-dispersive XRF is therefore the technique most used in soil analysis (2). XRF can be applied for elemental and trace metals analysis of ambient air particles. OSHA method ID-185 describes a protocol for vanadium pentoxide determina-

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tion in workplace atmosphere using XRF analysis of PVC filters (26). Portable field X-ray fluorescence spectrometry is becoming a common analytical technique for on-site screening and fast analysis of elements in hazardous waste samples (for review see ref. 27). U.S. EPA has published a standard operating procedure for elemental analysis using a field X-ray fluorescence analyzer (28). Applications include the *in situ* analysis of metals in soil, sediments, air monitoring filters, and lead in paint. The portable energy-dispersive XRF instruments can be used for scanning the ground surface to determine the presence of metals without collecting a sample for analysis. However, portable XRF instruments are relatively limited in sensitivity and accuracy.

2.3.2 Particle-Induced X-Ray Emission (PIXE)

Description. PIXE is a variant of the broad family of X-ray emission techniques; heavy charged particles, typically protons of 1–4 MeV, are used to produce the generated X-rays of the analyte in the sample. The emitted X-rays are virtually always measured with an energy-dispersive detector. For detailed information on the technique, the book of Johansson *et al.* (29) is highly recommended. An excellent review compares PIXE spectrometry to the other atomic and nuclear spectrometric techniques (30). Compared to conventional energy-dispersive XRF, PIXE offers detection limits that are often one order of magnitude better, it is faster, and also allows analysis of a smaller sample mass. The microbeam variant of PIXE, the micro-PIXE, offers the possibility of spatially resolved analysis with micrometer resolution (cf. section 4.2). The major drawbacks of PIXE are that it requires a MeV particle accelerator and that commercial PIXE apparatus are not readily available.

Multielement Capability. All elements from Na to U can in principle be measured simultaneously.

Detection Limits. 0.1–10 μg/g.

Environmental Applications. The major part of PIXE applications in environmental sciences are related to heavy metals measurement in aerosols and in biological samples (31–33).

2.3.3 Total-Reflection X-Ray Fluorescence (TXRF)

Description. The principle of TXRF is the use of the total reflection of the exciting beam from conventional radiation sources at a flat support (for review see ref. 34). TXRF analysis requires excitation with a very narrow beam at an angular divergence of less than 1 mrad. Due to a remarkable improvement of the signal-to-background ratio, absolute detection limits can be two or three orders of magnitude lower than that of conventional X-ray fluorescence techniques. For liquid samples, the classical sample preparation technique consists in deposition

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of a droplet of solution on a pure substrate, after evaporation of the solvent, the residue can be irradiated and analyzed.

Multielement Capability. Yes.

Detection Limits. 0.1 μg/L (water).

Environmental Applications. TXRF has been increasingly applied for multielement analysis in a wide range of environmental and biological materials. These specimens ranged from river water, seawater, rain, ice, and to a number of solid materials such as airborne particles, aerosols, biopsy samples, food, and humic substances (for review see refs. 34,35).

2.4 Neutron Activation Analysis (NAA)

Description. NAA is a highly sensitive procedure for determining the concentrations of chemical elements in the most varied substances (for review see ref. 36). NAA is based on conversion of stable nuclei of atoms into radioactive ones and subsequent measurement of characteristic nuclear radiation emitted by the radioactive nuclei. When a nuclear reaction results at a radioactive nucleus, the process is denoted as activation. The incident neutrons required for activation can be obtained by various means; fast neutrons with energies of several MeV can be produced with a neutron generator or in an isotopic neutron source. The produced radionuclide decays to a stable atomic nucleus under emission of characteristic radiation (often gamma-radiation). By determining the energy of the gamma-radiation and using the decay schemes, the emitting radionuclide can be identified as well as the nature of the activated element. Quantitative activation analysis is based on measurement of the intensity of the radiation. The radioactivity is proportional to the number of target nuclei in the irradiated sample. NAA has the advantage of requiring little, if any, pretreatment of the sample. The main drawback of NAA is probably the high cost and limited access to the facilities.

When the sensitivity of instrumental activation analysis is insufficient, radiochemical neutron activation analysis may be used. In this case, the radionuclides corresponding to the elements of interest are chemically separated post-irradiation. Various separation techniques can be used including ion exchange, chromatography, precipitation, electrolysis, and distillation. The separation schemes are specific not only for the elements to be measured, but also for the matrix composition of the material. Generally, the schemes cover a few elements in which the radionuclides are grouped in such a way that they can be determined without mutual interference (37).

Multielement Capability. Most elements can be determined with some limitations such as for Pb. Interferences occur when radionuclides emit gamma-rays of similar energy.

Detection Limits. Depending upon the kind of material under investigation the limits of detection may be as low as 0.1 ng/g.

Environmental Applications. NAA can be performed for a number of heavy metals by measuring the gamma activities of their activated radioisotopes such as: ^{51}Cr (but Hg might interfere); ^{52}V; ^{56}Mn; ^{60}Co (after ^{59}Fe separation); ^{65}Ni (after separation from other short-lived nuclides); ^{65}Zn; ^{75}Se; ^{76}As; ^{99m}Tc (for Mo determination from activation of ^{99}Mo); ^{110m}Ag (radiochemical separation required); ^{115}Cd; ^{122}Sb (interference with ^{76}As) or ^{124}Sb; ^{131}Ba (low sensitivity); ^{198}Au (high sensitivity); ^{199}Au (for Pt determination from activation of ^{198}Pt); and ^{203}Hg (4). The sensitivity for chromium is, for example, 0.3 μg/L by instrumental NAA on an interference-free basis, and a 100-fold enhancement with radiochemical NAA is possible (19). Typical applications of radiochemical NAA are often related with trace elements in human or animal tissues and body fluids, foodstuffs and other material of nutritional interest, and waters. The radiochemical NAA is less popular but produces very interesting data on less documented elements such as V and Mo for example.

2.5 Miscellaneous Measurement Techniques

2.5.1 Atomic Fluorescence Spectrometry (AFS)

Description. AFS is a complementary technique to AAS in that it measures the light that is reemitted after absorption (for details see refs. 4,14). The fluorescence is normally measured at a 90° angle to the exciting line source to keep transmitted line source radiation out of the detector. Air/acetylene and nitrous oxide/acetylene flames are the most commonly used atomizers but suffer from chemical interferences. ICP plasma is an efficient atomizer offering minimum light scatter and chemical interferences. The intensity of the fluorescence is directly related to the intensity of the light source, so high-intensity sources are sought and used. These include electrodeless discharge lamps, tuneable dye lasers, and pulsed hollow cathode lamps.

Multielement Capability. AFS is essentially a single-element technique but multielement analysis can be achieved using a continuum source, or a laser sequentially tuned to different wavelengths.

Detection Limits. 0.5 μg/L.

Environmental Applications. The recent availability of a commercial AFS instrument with hydride generation enables the analysis of some environmentally important elements, including mercury, arsenic, and selenium (38).

2.5.2 Ion Chromatography (IC)

Description. IC is a form of liquid chromatography that uses ion-exchange resins to separate atomic and molecular ions based on their interaction

in the resin. It is the most convenient analytical approach for the determination of environmentally important inorganic anions such as NO^{3-}, NO^{2-}, PO_4^{3-}, etc. However, it has gained in importance for metal determination (for review see ref. 39). For cation separation, the cation exchange resin is usually a sulfonic or carboxylic acid. For anion separation, the anion exchange resin is usually a quaternary ammonium group. Metals in solution are generally detected by measuring the conductivity of the solution. Postcolumn reactions can be employed to enhance the specificity and selectivity of the detection, 4-(2-pyridyzalo)resorcinol (PAR) being the preferred reagent for most metal ions (40).

The lack of selectivity control limits the versatility of IC methods, particularly if there is interest in trace metals eluting in the presence of massive amounts of other metals, e.g., seawater samples. However, there is a solution to this problem, that is, to use a metal chelating ion-exchange rather than a simple ion-exchange substrate. High-performance substrates can be used in analytical separation columns in a IC system just like ion-exchange columns, but have the added advantages of selectivity control and insensitivity to changes in ionic strength (41).

Multielement Capability. Yes.

Detection Limits. 0.1–1 μg/g (soil); 1–50 μg/L (water).

Environmental Applications. Although IC can be used for the direct determination of some heavy metals, it is more often combined with atomic spectrometries for metal speciation analysis (cf. Section 3). U.S. EPA method 218.6 describes the procedure for Cr(VI) determination in water (15).

2.5.3 Electrochemical Methods

Description. Electroanalysis is a broad spectrum of techniques that can be distinguished by the variable that is controlled: voltage or current. The usual practice is to apply one of these variables to a solution containing the analyte species and measure one of the other variables. From a plot of the measured variable versus the applied variable, information regarding the concentration and identity of electroactive species in solution is determined. Of the many electrochemical techniques, only a few are routinely used for environmental analysis: voltammetry, direct-current DC, polarography, and potentiometry.

Voltammetry is the name usually given to the family of techniques in which current is measured in function of applied potential. Anodic stripping voltammetry (ASV) is used for the determination of metals in soils and water. The measurement is performed in an electrochemical cell under polarizing conditions on a working electrode. Analysis involves a two-step process consisting of electrolysis and stripping. The analyte of interest is reduced and collected at the working electrode, then stripped off and measured. Electroanalytical techniques can pro-

vide quantitative and qualitative information. They are also among the most cost-effective methods to do environmental analysis.

Multielement Capability. Only consecutive analysis of distinct metal ions is possible. However, the advantage of ASV is the ability to distinguish between different oxidation states of the same metal.

Detection Limits. ASV 100–1000 μg/g (soil); 1–50 μg/L (water).

Environmental Applications. Electrochemical determination of metal ions has been widely applied to seawater, natural and potable water, wastewater, air, soil, sewage, sediment, dust, ash, and many other matrices. Electrochemical techniques are able to determine most transition metals and metalloids, from Ti to Bi (for review see ref. 42). Seawater, as a matrix, may cause problems for many analytical techniques because of its high salt content and corrosivity. The determination of metal ions in seawater is done almost exclusively with stripping techniques. ASV was the first stripping technique used extensively to analyze seawater for metal ions such as Cu, Zn, Cd, and Pb. ASV is sensitive enough to determine these elements at their natural concentrations, typically <20 μg/L in unpolluted seawater, without any sample pretreatment. U.S. EPA methods 7063 and 7472 describe a protocol for, respectively, arsenic, and mercury, determination in aqueous samples and extracts using ASV (18).

2.5.4 Spectrophotometry

Description. Spectrophotometry is based on the simple relationship between the molecular absorption of UV-VIS radiation by a solution and the concentration of the colored species in solution. A good theoretical and technical description of spectrophotometry with special reference to environmental analysis is given by Gauglitz (43). The basic components of a spectrophotometer include a light source, a monochromator, which isolates the desired source emission line, a sample cell, a detector-readout system, and a data-processing unit. Spectrophotometric measurements are based on the Beer-Lambert law, which describes a linear dependence of absorbance on the concentration. Heavy metals spectrophotometric methods rely on reactions of analytes with color-forming reagents such as dithizone (for Co, Ni, Cu, Zn, Ga, Pd, Ag, Cd, In, Pt, Au, Hg, Tl, Pb, Bi) and thiocyanates (for Ti, Fe(III), Co, Mo, W, Re, U).

Multielement Capability. Spectrophotometry is not a multielement technique. Moreover, it often suffers from a poor selectivity and requires a prior separation of the element to be determined.

Detection Limits. About 1 μg/L after preconcentration.

Environmental Applications. Antimony can be determined by extraction of $SbCl_6^-$ with a basic dye, e.g., Rhodamine B or Crystal Violet. For arsenic,

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the arsenomolybenum blue method is the most widely used. Bismuth forms an orange-brown dithizonate with dithizone; cadmium forms a pink dithizonate. Mercury, lead, and zinc are also determined by the dithizone method. Reaction of Cr(VI) with 1,5-diphenylcarbazide at pH 1 is the basis of a sensitive and fairly selective method. Tin(IV) reacts with phenylfluorone at pH 1 to form an orange-red solution. The excellent book of Lobinski and Marczenko (4) gives a brief individual protocol for spectrophotometric determination of most metal ions, as well as complementary references.

2.5.5 Biomethods

Description. Immunoassay technology relies on an antibody that is developed to have a high degree of sensitivity to the target compound. This antibody's high specificity is coupled within a sensitive colorimetric reaction that provides a visual result. Immunoassays offer significant advantages over more traditional methods of metal detection; they are quick, inexpensive, simple to perform, and can be both highly sensitive and selective.

Detection Limits. 0.1–1 μg/g (soil); 1–50 μg/L (water).

Environmental Applications. Although most environmental immunoassays are directed toward halogenated aromatic compounds and pesticides, the technique is theoretically applicable to any pollutant for which a suitable antibody can be generated. Antibodies that recognize chelated forms of metal ions have been used to construct immunoassays for Ni(II), Cd(II), Hg(II), and Pb(II) (44).

3. SPECIATION ANALYSIS

The IUPAC definition of the speciation of an element is the distribution of defined chemical species of an element in a system, speciation analysis being the analytical activity of identifying and measuring the quantities of one or more individual chemical species in a sample. The chemical species of an element are the specific forms of an element defined as to molecular, complex, or nuclear structure, or oxidation state. The chemical and physical associations of toxic elements with their environment can strongly influence their distribution, mobility, and biological availability; therefore, there is an increasing need for metal speciation analysis in environmental samples (for review see refs. 45–50). The main environmental applications involve speciation analysis of redox and organometallic forms of antimony and arsenic, redox forms of chromium, protein-bound cadmium, organic forms of lead such as alkyllead compounds, organomercury compounds, inorganic platinum compounds, inorganic and organometallic compounds of selenium, organometallic forms of tin, and redox states of vanadium. Appropriate methods for speciation analysis of individual elements will be reviewed later.

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Species stability in samples is an important issue, since natural environmental samples are not usually analyzed immediately after sampling and long-term storage can produce a significant alteration of the chemical species. The use of different preservation treatments such as acidification, low temperature, drying, freezing, pasteurization, freeze-drying, adsorption on cartridges or solid-phase microcolumns, and storage in the dark makes the species stabilization in some environmental samples possible. The stabilization methods have been recently reviewed for Cr, As, Se, Sn, Sb, Hg, and Pb species in environmental samples (51). The atomic and nuclear spectrometric methods (AAS, ICP-AES, XRF, PIXE, MS, NAA) are techniques for elemental analysis independent of the chemical form. Without selective chemical pretreatments, the results obtained do not provide information about the chemical speciation of the elements. Some methods, however, are able to provide direct and selective determination of chemical species of an element; they are discussed in the next section. Coupled methods for speciation analysis, which generally involve a separation method and an element-specific analytical technique, are presented below.

3.1 Direct Methods for Speciation Analysis

3.1.1 Spectrophotometry and Colorimetry

Colorimetry and spectrophotometry determination originate with changes in molecular rather than atomic or nuclear energy levels and therefore depend on the chemical form of the element providing information about its speciation. Oxidation state determination using colored reagents has been reported for a number of metals and metalloids, such as Al(III), V(V), Cr(VI), Fe(II), Se(IV), Sn(IV), Pt(II), Pt(IV), Tl(III) (for review see ref. 4). The best-known example is the determination of hexavalent chromium by reaction with diphenylcarbazide in acid solution. Spectrophotometric determination of V(IV) in the presence of V(V), based on the catalytic oxidation of aniline blue by bromate, is a more recently studied example (52). In addition, these procedures frequently offer the advantage of speed, simplicity, and low cost of instrumentation. These advantages are sometimes compromised by a lack of specificity and sensitivity.

3.1.2 Electrochemical Methods

Electrochemical techniques can be used to study speciation of metal ions in natural, drinking, and seawater. They are useful for the measurement of the effect of ionic species and oxidation states on bioaccumulation and geochemical cycling. The ability to provide speciation information in seawater is an important area in which electrochemistry is used. Adsorptive stripping voltammetry is used in seawater analysis for the measurement of metal ions such as Al^{3+}, Ti^{4+}, V^{5+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Se^{4+}, Se^{6+}, and Mo^{6+}; adsorptive stripping potentiometry for Co^{2+}, Ni^{2+}, and Zn^{2+}; potentiometric stripping analysis for Cd^{2+} and

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Pb^{2+}; anodic stripping voltammetry for Hg^{2+}; and differential pulse polarography for Cr^{6+} (for review see ref. 42). The determination of metal ions in seawater is done almost exclusively with stripping methods because of their appropriate sensitivity. Natural concentrations of metal ions in seawater are typically <20 μg/L and the in situ preconcentration, deposition step of stripping techniques makes these techniques fast, sensitive, inexpensive, and portable.

3.1.3 X-Ray Absorption Fine-Structure Spectroscopy (XAFS)

In photoelectric interaction, X-ray photons are absorbed, and their energy is expended in kinetic energy to orbital electrons of absorber atoms. As the energy of the original photon must be equal to or higher than the binding energy of the ejected electron, the photoelectric cross-section features abrupt discontinuities, called absorption edges, at photon energies corresponding to those of various electronic levels in the atom. In condensed matter, oscillations on the absorption profiles can be observed close to absorption edges. These features are called the X-ray absorption fine structure (XAFS). The region from 0 to 40 eV above the actual edge is called the XANES (X-ray absorption near-edge structure) or NEXAFS (near-edge XAFS). Also preedge features may be observed. Above 40 eV, the EXAFS (extended XAFS) region commences. The fine structure is caused by the interference of the outgoing photoelectric wavefront with the waves backscattered from neighboring atoms. From the fine structure, the interatomic distances and coordination numbers around the absorbing atom can be determined. As such, the XAFS is a very important structural investigation method for studying noncrystalline materials. Because of the requirement for a highly monoenergetic X-ray beam, XAFS measurements are almost exclusively performed with synchrotron radiation sources.

XANES can be applied to the determination of the oxidation state of heavy metals in solid samples. For example, the ratios between the different oxidation sates of chromium and arsenic during the deposition of fly ash could be determined quantitatively using XANES spectrometry (53), as well as Cr(VI)/Cr(III) ratios in chromium-contaminated soils (54). On the other hand, EXAFS spectra contain structural information such as the central atom-neighbors' atom distance, the nature of the neighbors, the local disorder, and the number of neighbors. EXAFS have been applied to direct determination of heavy metals speciation in various type of matrices. The molecular-level speciation analysis of arsenic and lead in mine tailings, and selenium in contaminated soils, has been recently reported (49); EXAFS was proposed as an efficient tool for evaluating chemical remediation strategies in chromium-contaminated soils (55); lead speciation analysis in contaminated soils enabled the differentiation between sources of lead pollution (56,57); chemical speciation analysis of lead and copper in potable water was also investigated (58). EXAFS is also a useful tool for metal cations

complexation studies with natural molecules such as for the structure determination of metal ions complexes with humic substances (59,60).

3.1.4 Other Techniques for Direct Metal Speciation Analysis

Direct methods of metal speciation have been reviewed by Glidewell and Goodman (61). They include most spectroscopic techniques applicable to metals such as circular dichroism for spectroscopic studies of Cr(III), Co(III), Rh(III), Ir(III), and Pt(IV) compounds; magnetic susceptibility, Fourier transform infrared spectroscopy, and Raman spectroscopy for structural information on the nature and geometry of the metal's ligands; and specific metal magnetic resonance spectroscopy to perform structure analysis on Al, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, Hg, and Pb compounds.

Direct speciation analysis can also be carried out using separation and preconcentration of particular metal species by either chromatographic methods, coprecipitation, ion exchange, separation with chelating resins, or solvent extraction. A comprehensive review of these methods has been recently accomplished by Lobinski and Marczenko (4) for each metals and metalloids. These separation and preconcentration protocols are usually poorly sensitive and/or specific, by themselves. To gain in specificity and sensitivity, the chemical or chromatographic separation of metal species is often coupled, off-line, or on-line, to element-specific analytical methods such as AAS, ICP-AES, ICP-MS, XRF, PIXE, and NAA. For example, As(III), As(V), Se(IV), Se(VI), Sb(III), and Sb(V) were simultaneously determined in natural water by coprecipitation and neutron activation analysis (62). The detection limits were 1 ng/L for arsenic and selenium and 0.1 ng/L for antimony. On-line chromatographic combinations will be described in the next section.

Finally, biological substrates such as algae, plant-derived materials, bacteria, yeast, fungi, and erythrocytes can be used for metal preconcentration and direct speciation analysis (63). The sorption properties of living or dead organisms can be used to differentiate metal species: red blood cells for specific sampling of chromate even at high Cr(III) levels; baker's yeast cells to separate, respectively, Hg(II) from CH_3Hg, Sb(III) from Sb(V), and Se(IV) from Se(VI).

3.2 On-Line Coupled Methods for Metal Speciation Analysis

Directly coupled systems that utilize the separatory powers of chromatography and the sensitive detection of atomic spectroscopy are increasingly used for environmental speciation studies. In a hybrid technique the separation process and elemental detection occurs on-line (for review see refs. 64,65). The state of development and various problems that occur in determination of organometallic com-

pounds using hyphenated techniques have been recently reviewed (66). This article also gives an overview of the most available reference material for the determination of selenium, tin, mercury, and lead species of environmental interest. Gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) coupled to sensitive element-selective detectors are the main hyphenated techniques developed in a number of laboratories. With the exception of one instrument, a GC-microwave-induced plasma (MIP)-AES, there are no commercial instruments available for on-line speciation analysis of organometallic compounds. Hyphenated techniques are therefore usually developed in experienced analytical laboratories. Finally, if the sensitivity achieved by direct detection using GC or LC or CE, or using coupled methods, does not meet the requirement in real-sample analysis, chemical modification of the target metal, which transforms the existing states of the metal to another by derivatization or complexation, can be applied to facilitate their analysis (for review see ref. 67).

3.2.1 Gas Chromatography (GC)

Gas chromatography is a powerful separation technique and it is relatively easy to couple the gas effluent to an element-specific determination method such as AAS, AES, or MS without any sample loss. A number of hyphenated techniques based on the combination of GC with AAS, MIP-AES, and ICP-MS have been used in many environmental studies (for review see ref. 65). GC-AAS is normally used for the investigation of volatile or thermally stable compounds such as mercury, tin, and lead alkyl compounds. MIP-AES is an excellent detector for GC capable of detecting virtually all metals and metalloids. Absolute detection limits offered reach the subpicogram level for many elements including Hg, Sn, and Pb and picogram levels are found for most of the others. On the other hand, GC-MS is potentially a highly sensitive and selective technique. GC system hyphenated to ICP-MS has been applied to the investigation of environmental volatile compounds of selenium, tin, mercury, and lead with respective detection limits for water sample as low as 0.8, 0.2, 0.05, and 0.08 pg/L (68). Finally, a GC-AFS system has been developed for organomercury compounds determination in various environmental samples (38).

3.2.2 Liquid Chromatography (LC)

Most hyphenated techniques for environmental metal speciation rely on liquid chromatography for the separation stage. Current literature provides a wide number of applications for Cr, As, Se, Sn, Hg, and Pb speciation by hyphenated LC methods. It appears that numerous combinations of ion chromatography (IC) and high-performance liquid chromatography (HPLC) with atomic spectrometry techniques have been described for environmental metal speciation such as: IC-FAAS, IC-ICP-AES, IC-ICP-MS, HPLC-HGAAS, HPLC-MIP-AES, HPLC-ICP-AES, and HPLC-ICP-MS (for review see refs. 39,64,65,69). Since liquid

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chromatography is suitable for the separation of ionic, polar, and nonpolar compounds, it is recognized to be especially efficient in the separation of organometallics. Very low detection limits (0.03–0.05 μg/L) can be achieved using HPLC with ICP-MS. Other systems such as HPLC-HG-AFS reached detection limits down to 0.2 μg/L for As(III), As(V), and organoarsenic compounds (38).

3.2.3 Capillary Electrophoresis (CE)

CE has recently been found to be a powerful technique for the rapid and highly efficient separation of a variety of compounds, including metals species. While CE supplements GC and LC methods, it shows unique promise for speciation purposes by exerting only minor disturbance on the existing equilibrium between different species. Separation by CE is based on differential migration of charged analytes along a capillary filled with a suitable conducting electrolyte. Although CE is less indicated for oxidation states metal speciation than LC complexation methods, CE clearly holds great promise to separate free metal ions from complexed metal ions, different organic and inorganic compounds of the same metal, and for the analysis of anionic metalloid analytes, such as oxoanions of arsenic and selenium. An overview of the state of the art of CE for metal speciation analysis is presented by Dabek-Zlotorzynska *et al.* (70). This review also describes CE individual metal speciation methodologies for selenium, arsenic, tin, mercury, lead, and chromium. Detection sensitivity is limited by the small detection volumes inherent in CE analysis. The concentration sensitivity is generally one or two orders of magnitude lower than that for HPLC. On-line detectors based on UV absorbance, electrochemistry, or fluorescence have been utilized in metal speciation by CE. Only hyphenated methods with ICP-AES or ICP-MS are able to provide detection limits below the μg/L with multielement capability. The potential and challenges of elemental speciation by CE-ICP-MS and other mass spectrometries have been recently reviewed (71). The combination of CE with PIXE has also been reported by Vogt *et al.* (72).

3.3 Speciation Analysis of Individual Elements

3.3.1 Chromium

The accurate differentiated quantitative determination of chromium in each species is important when investigating its environmental analysis as chromium is a redox active metal with two commonly encountered valence states, trivalent and hexavalent, that are the most stable forms found in the environment, but exhibiting markedly different physicochemical behavior and toxicological properties. For a complete review on environmental chromium speciation analysis the reader is referred to Kotas and Stasicka (73) and Katz (19), for specific Cr(VI) analysis in soils to Petura *et al.* (74). Spectrophotometry of the diphenylcarbazide chromophore is widely used to quantify hexavalent chromium (19). The different

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behavior of Cr(III) and Cr(VI) on ion exchangers is also a common method for their separation. The U.S. EPA method 218.6 (or method 7199) provides a procedure for determination of dissolved hexavalent chromium (as CrO_4^{2-}) in drinking water, groundwater, and industrial wastewater effluents by ion chromatography (15,18). Cr(VI) is separated on a high-capacity anion exchange separator column. Postcolumn derivatization of the Cr(VI) with diphenylcarbazide is followed by detection of the colored complex at 530 nm. The method detection limit obtained in the above-listed matrices was determined to be 0.4 μg/L. Other analytical procedures have also been reported by U.S. EPA. Methods 7195, 7196A, 7197, and 7198 present Cr(VI) determination, respectively, by coprecipitation, colorimetry, chelation/extraction, and differential pulse polarography (18).

3.3.2 Arsenic

Both inorganic and organic compounds of arsenic are found in environmental samples. The various arsenic compounds differ with respect to their biological properties; some organic arsenic compounds are nontoxic, whereas most inorganic arsenic compounds cause detrimental effects. For these reasons, analytical methods that identify and quantify distinct arsenic species are needed. The determination of arsenic compounds has been reviewed by Irgolic (75). Are reported in this excellent review, the determination of arsenite and arsenate by electrochemical methods (see also ref. 76), colorimetry, and HG-AAS, as well as arsenite and arsenate separation by extraction, precipitation, and chromatography, are discussed. Also discussed are the identification and determination of organic forms of arsenic such as methylarsenic compounds using IC, HG, GC, HPLC, thin-layer chromatography (TLC), and pyrolysis mass spectrometry. The analysis of organoarsenic compounds in natural and potable waters, seawater, sediments, soils, industrial effluents, and wastewaters has also been recently reviewed by Crompton (46). HG-AAS is a widely used method for arsenic speciation analysis. The optimization of analytical parameters and application to environmental samples for arsenic determination by HG-AAS has recently been studied by Molénat *et al.* (77). Absolute detection limits in the range of 0.1–0.5 ng for As(III), As(V), monomethylarsonic acid, dimethyl arsinic acid, and trimethylarsine oxide were obtained. However, as pointed out by the EPA in its research plan for arsenic in drinking water (78), these hyphenated methods represent the state of the art in chromatographic and detection technology but considerable research is still required before they could be considered as routine methodologies for environmental arsenic speciation analysis.

3.3.3 Mercury

To understand mercury distribution pattern in nature, and the effect of anthropogenic mercury additions to the environment, it is important to discriminate between the different mercury species. The two basic types of methods include

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operational discrimination between inorganic and organic mercury and chromatographic separation of individual species followed by their on-line detection (for review see refs. 4,46). Nonchromatographic methods are based on dividing the total mercury in inorganic and organic mercury fractions using chemical methods followed by a traditional detection technique (CVAAS, GFAAS, ICP-MS). On the other hand, mercury speciation using chromatographic separation by HPLC and GC followed by CVAAS, ICP-MS, ICP-AES, or MIP-AES have been developed. Reported detection limits may vary between 1.5 and 40 ng/g depending on the method. Examples of other chromatographic techniques for methylmercury analysis are TLC of dithizonates extracts combined with AAS and HPLC of dithizonates combined with spectrophotometry (79).

3.3.4 Lead

Tetraalkylead compounds (PbR_4) in gasoline degrade during combustion and decompose to inorganic lead in the environment via a series of ionic compounds (PbR_3^+, PbR_2^{2+}). Speciation analysis of organolead compounds is based on GC or HPLC with spectrometric detection such as AAS (for review see refs. 4,46,80). The most widely used technique is GC quartz furnace AAS, which offers absolute detection limits down to a few pg and relative ease of operation. Liquid chromatography–direct injection nebulization ICP-MS is probably the most efficient method with absolute detection limits down to 0.2 pg.

3.3.5 Other Elements (V, Se, Cd, Sn, Pt)

Vanadium. It is recognized that V(V) as vanadate is more toxic that V(IV) present as vanadyl ions. These two oxidation states are the most common in environmental samples. The redox method using the Fe(II)/Fe(III) system in the presence of 1,10-phenanthroline and diphosphate in a flow-injection manifold offers a simple approach to the determination of V(V) and V(IV). However, methods based on colorimetry often suffer from a lack of sensitivity when applied to environmental samples where detection limits below 1 mg/L are required. Sensitive analytical procedures based on selective separation (high-temperature capillary GC, reverse-phase HPLC, size exclusion chromatography) followed by off-line detection (ICP-MS, ICP-AES, GF-AAS) have been developed. Taylor (81) and Lobinski and Marczenko (4) reviewed the analytical methods for vanadium speciation with regard to its oxidation state.

Selenium. Speciation is fundamental for understanding selenium biogeochemical processes such as biological uptake, adsorption, precipitation, mobility, and remediation. Selenium speciation has been reviewed by Lobinski and Marczenko (4) and Reddy (82). Different analytical approaches are available. The HGAAS and ion chromatography are the most commonly used methods to analyze the speciation of dissolved selenium. The HGAAS method measures total

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dissolved selenium as Se(IV) from which Se(VI) and organic carbon complexes of selenium can be calculated from the difference using specific chemical pretreatment. The detection limit can be as low as 1 μg/L selenium in aqueous solution. The ion chromatography method can measure the concentration of SeO_4^{2-} and SeO_3^{2-} simultaneously in aqueous solution. However, SO_4^{2-}, a common anion of natural waters, interferes in SeO_4^{2-} and SeO_3^{2-} separation with ion chromatography. Limit of the ion chromatography quantification for Se analysis is in the 100 μg/L range.

Cadmium. Speciation studies for cadmium in liquids and solids have to consider the differentiation between the liquid phase, dissolved complexes, and the metal adsorbed on colloidal particles and on distinct solid phases in soils and sediments that determine bioavailability (2). Several operational methods have been developed for available cadmium from soils using IC usually followed by FAAS or ICP-AES (83). Protein-bound cadmium speciation must also be assured such as in metallothioneins. Size-exclusion chromatography is the most widely used separation technique whereas ICP-MS the most popular determination method (4).

Tin. As the toxicity of tin is strongly species dependent, speciation of tin in environmental matrices is essential. Compounds with short organotin alkyl chains or phenyl substituents generally exhibit considerable toxicity toward both aquatic organisms and mammals. Organotin compounds may accumulate in sediments and aquatic organisms. Valid methods for tin speciation should allow the determination or the target compound without any interference from other organotin species at levels of <0.1 ng/L for water and <1 ng/g for dry solid materials. This is obtained with combination of separation techniques such as GC, HPLC, or supercritical fluid chromatography, with AAS, ICP-AES, or MS detection (4,46).

Platinum. The use of platinum group elements (Pt, Rh, and Pd) as components of autocatalytic converters attached to motor vehicles has resulted in serious contamination of the environment. Studies of the speciation of platinum in plants is required in understanding the potential risks to the health human populations. Platinum compounds can be separated by ultrafitration and gel permeation chromatography, then detected with highly sensitive methods such as ASV or ICP-MS (84).

4. MICROSCOPIC ELEMENT ANALYSIS

A variety of techniques exist that provide chemical information in the form of a spatially resolved image (for review see ref. 85–87). These methods provide information on the spatial distribution of trace elements within solid samples with

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a micrometer resolution. The following section gives an overview of the analytical principle, performance, and environmental applications for the main microanalytical techniques that can be used for heavy metals determination (summarized in Table 2). Microbeam techniques have been used in environmental studies essentially for single-particle analysis. These techniques are very valuable as a complement to the more conventional bulk analysis, because they provide detailed information concerning the origin, formation, transport, reactivity, transformation reactions, and environmental impact of particulate matter (for review see ref. 88).

4.1 Electron Probe X-Ray Microanalysis (EPXMA)

Description. EPXMA is a technique with which it is possible to determine the elemental composition of a specimen in the electron microscope. When an electron beam irradiates a specimen a number of different interactions occur, and particularly, X-rays are generated as a consequence of the rearrangement of outer-shell electrons after an inner-shell electron has been ejected from the atom (cf. X-ray fluorescence methods, Section 2.3). Emitted X-rays carry information about the elemental composition of the specimen in the region that is being irradiated. An excellent and comprehensive description of scanning electron microscopy and EPXMA is given in the textbook of Goldstein *et al.* (89).

Multielement Capability. Simultaneous detection of all elements of $Z > 10$ with usual energy-dispersive X-ray spectrometers (EDS); consecutive detection of elements of $Z > 5$ with wavelength-dispersive X-ray spectrometers (WDS).

Detection Limits. 100–1000 μg/g with EDS; 10–100 μg/g with WDS.

Spatial Resolution. 0.1–1 μm.

Environmental Applications. EPXMA is a well-established technique for the study of particulate materials. Microscopic examination and energy dispersive X-ray analysis of individual particles in aerosols and sediments enables the morphological and chemical characterization of samples that can be compared to those observed in the particulate matter of suspect sources (for review see ref. 88). Associations of elements in specific particles may suggest the origin of the particle; for example, particles containing lead and bromine were linked to car exhaust emission, lead-to-silicon ratios were linked to soil, and lead-to-potassium ratios were linked to refuse burning (3). To ensure the statistical relevance of the results, a large number of particles need to be analyzed and this makes individual particle analysis time consuming. Computer-controlled EPXMA is the most advanced example of automation and computerization that reduce the long analysis times. EPXMA has been also widely applied to analysis of biological samples, essentially to study lung particulate mineral composition and its relationship with

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TABLE 2 Comparison of the Main Techniques Used for Heavy Metals Microanalysis

Elemental Microanalysis	Analytical depth	Limit of detection	Spatial resolution	Multielement capability	Specificities	Sample nature
Electron Probe X-ray Microanalysis (EPXMA)	1–15 μm	EDS 100 to 1000 μg/g WDS 10 to 100 μg/g	0.1–1 μm	EDS multielement ($Z > 10$) WDS monoelement ($Z > 5$)	Quantitative elemental imaging	Solid
Micro-PIXE Analysis	100 μm	1–10 μg/g	0.3–3 μm	Multielement ($Z > 10$).	Quantitative elemental imaging	Solid
Synchrotron Radiation μ-XRF and μ-XAFS	>100 μm	μ-XRF 1–10 μg/g μ-XAFS 100–1000 μg/g	1–100 μm	Multielement ($Z > 10$). Chemical Species	Quantitative elemental imaging	Solid or liquid
Laser Microprobe Mass Spectrometry (LMMS)	1 μm	0.01–1 μg/g	1–10 μm	Multielement (isotopes). Chemical Species	Semiquantitative	Solid
Secondary Ion Mass Spectrometry (SIMS)	0.01 μm	1 μg/g	0.1–1 μm	Multielement (isotopes)	Semiquantitative Elemental imaging	Solid

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lung diseases (90), and to study the uptake and tissue and cell distribution of pollutant heavy metals by animals and plants (for review see ref. 91).

4.2 Micro-PIXE Analysis

Description. The focusing of ion beams down to small areal dimensions, typically a few μm^2 cross-section, enables the application of PIXE analysis to microscopic regions of a sample (87,92). The ion microbeam can be scanned over the surface of the specimen and thus provide information on the lateral distribution of elemental species, in much the same way as is done in EPXMA. However, by using a proton beam instead of an electron beam, microanalytical determination can be performed with a sensitivity that is 100 times greater than that of EPXMA. Ion microprobes are often called nuclear microprobes, which involve the distinct microspectrometries that can be performed depending on the ion beam effect of interest, such as PIXE, but also elastic recoil detection analysis (ERDA), Rutherford backscattering spectromety (RBS), ionoluminescence, or particle induced gamma emission (PIGE), and other nuclear reaction spectrometries. These techniques are complementary to micro-PIXE analysis as they can be applied to light elements determination.

Multielement Capability. Micro-PIXE allows simultaneous detection of all elements of $Z > 10$ with usual energy-dispersive X-ray detectors. Light elements can be determined simultaneously or consecutively with ERDA, RBS, or PIGE.

Detection Limits. 1–10 μg/g.

Spatial Resolution. 0.3–3 μm.

Environmental Applications. Micro-PIXE has been applied to metal analysis of individual particles of atmospheric aerosol samples (93) and medium-size fly ash (94,95). It was also applied to airborne particulate matter localization in the human respiratory system (96). The microscopic distribution of heavy metals in various tissues, or cells, as markers of exposure to these metals, has been extensively studied using micro-PIXE analysis (87,97,98). Figure 1 illustrates an example of intracellular elemental distributions, obtained with the micro-PIXE setup of Bordeaux-Gradignan (Fig. 2), which shows chromium and iron perinuclear localization in tissue culture cells exposed in vitro to Fe/Cr insoluble particles.

4.3 Microscopic XRF and XAFS with Synchrotron Radiation (SR)

Description. The recent development of microprobe beamlines on third-generation synchrotrons enables the conduction of spatially resolved XRF and XAFS down to microscopic levels (99). Compared to ion and electron microbeams, SR X-ray microbeams possess a higher penetration depth, up to 1000

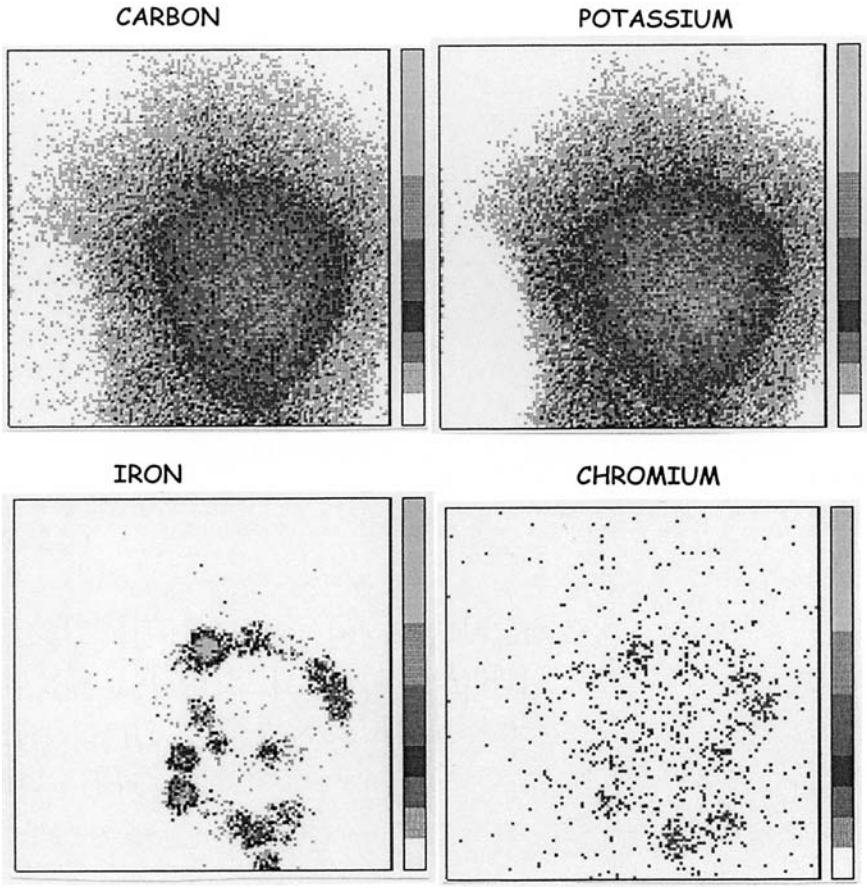

FIGURE 1 Intracellular elemental distributions obtained by μ-PIXE (potassium, iron, and chromium) and μ-RBS (carbon), within a single human ovarian cell exposed *in vitro* to Fe/Cr insoluble particles, and processed as described in Ortega *et al.* (97). The nuclear region of the cell is depicted by the high carbon and potassium levels. Note the perinuclear localization of Fe/Cr particles. Element concentration increases according to the color code from white to yellow. Scan dimension: 80 μm × 80 μm.

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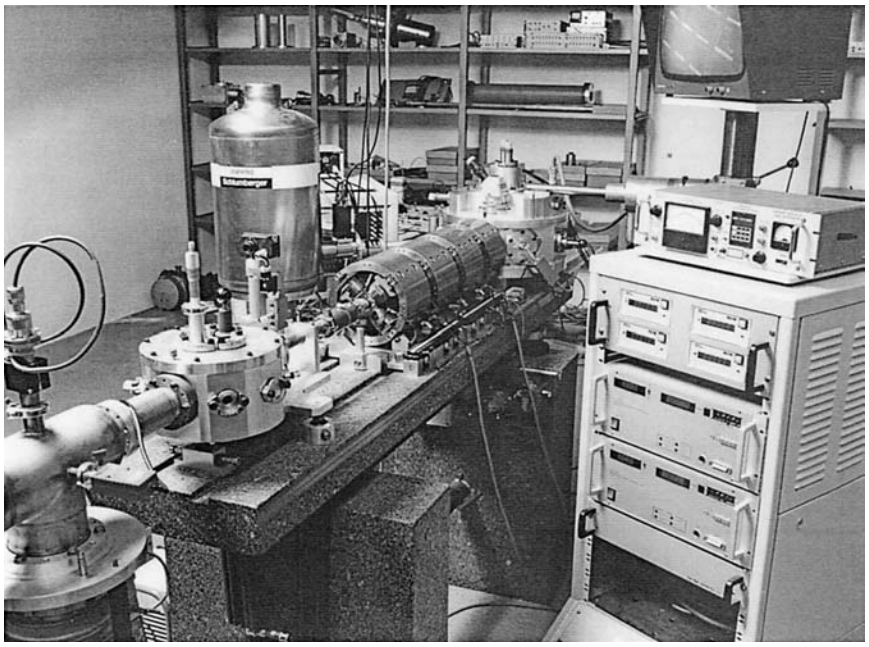

FIGURE 2 Nuclear microprobe of Bordeau Gradignan. View of beamline, magnetic lenses, and analytical chamber.

μm versus 100 μm with μ-PIXE, and 5 μm with EPXMA, and the ability to operate spatially resolved speciation analysis thanks to SR μ-XAFS. Moreover, samples can be irradiated in air contrary to μ-PIXE or EPXMA, which require vacuum analysis and relevant sample processing.

Multielement Capability. Simultaneous detection of all elements of $Z > 10$ with usual energy-dispersive X-ray detectors is possible.

Detection Limits. 1–10 μg/g for μ-XRF; 100–1000 μg/g for μ-XAFS.

Spatial Resolution. 1–100 μm.

Environmental Applications. SR μ-XRF and μ-XAFS have been applied to a variety of environmental samples (100). μ-XANES is a powerful technique for the determination of metals and metalloids oxidation states within microscopic areas and with minimal sample manipulation (101). The synchrotron X-ray microprobe at the National Synchrotron Light Source, Brookhaven, was used to determine hexavalent chromium content in oxides, silicates, glasses, and cementitious wastes forms using μ-XANES (102). Distinct regions within sediments as small as 50 μm were irradiated for U oxidation state analysis in uranium-contaminated

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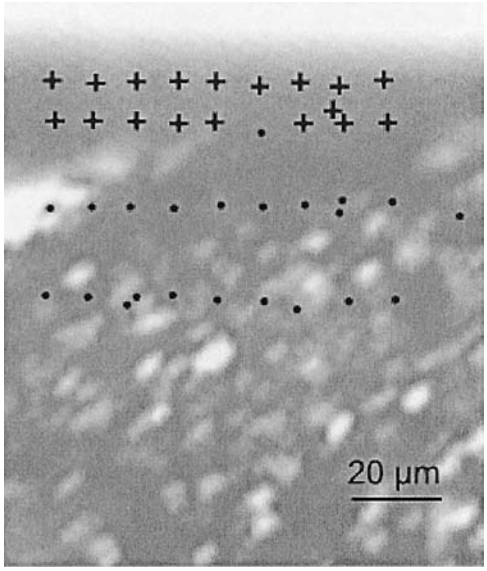

FIGURE 3 SR μ-XRF analysis of chromium in tissue sections of male mice reproductive glands after i.p. exposure to 1 mmol/kg $CrCl_3$ [Ortega *et al.* (106)]. Point microanalyses were performed with a 1 μm × 3 μm beam spot at 6.13 keV. Chromium (crosses) was preferentially localized within the tunica albuginea, but was not found within the seminiferous tubule (points).

soils using μ-XANES at 10 μm spatial resolution (103). Complementary X-ray techniques were used to identify, at the tissue and cell levels, chemical species of Cr and Se in biota collected from contaminated environments (104). SR μ-XRF with 100 μm resolution was performed at the Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (LURE) in Orsay, France, on teeth to evaluate the influence of living habits in dental elemental composition and environmental contamination (105). More recently, SR μ-XRF with 1 μm X-ray beam at the European Synchrotron Radiation Facility allowed the identification of chromium distribution (Fig. 3) in animal tissues at the cellular level (106).

4.4 Laser Microprobe Mass Spectrometry (LMMS)

Description. The LMMS technique is based on the ionic emission induced by a pulsed focused laser beam. The microplasma created consists of neutral fragments as well as elementary, molecular, and fragmentary ions with a predominant unit charge. Emitted ions are then injected into a mass spectrometer. Isotopic and semiquantitative information can thus be obtained using mass spec-

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tra of either positive or negative ions. Absolute quantification is still difficult to achieve owing to a lack of knowledge of laser-matter interaction. However, LMMS can be a very useful technique, even as a semiquantitative method, if a series of similar samples are compared or if the spatial distribution pattern of trace elements in a sample is determined. The combination of laser ablation (LA) for solid sample introduction into an ICP-MS provides a highly sensitive method for spatially resolved analysis with minimal sample preparation prior to analysis, which saves time and reduces the problem of sample contamination.

Multielement Capability. Yes, with isotopic capability (cf. ICP-MS, section 2.2).

Detection Limits. 0.01–1 μg/g.

Spatial Resolution. 1–10 μm.

Environmental Applications. LMMS was used in a number of studies to determine heavy metals occurrence in single particle aerosols (for review see ref. 88). LA-ICP-MS has been applied to multielemental analysis of environmental matrices, particularly to the determination of up to 40 metals and metalloids in reference sediments (107); to copper, cadmium, and lead analysis in teeth from walrus and beluga whales, and in a fin ray from a sturgeon fish (108); and to zinc, tin, and lead microanalysis in teeth slices to provide element-versus-time profiles of metal exposure (109). Speciation analysis of chromium, lead and nickel oxides, and salts in solid samples with a lateral resolution in the μm range using time-of-flight LMMS has recently been evaluated (110,111).

4.5 Secondary Ion Mass Spectrometry (SIMS)

Description. The SIMS technique is based on the interaction of primary accelerated ions (Ar^+, Xe^+, O_2^+, Cs^+) in the keV range with the surface components of solids. The ion beam is scanned over the sample surface to sputter the first external atomic layers. Therefore, SIMS is a method of surface analysis. Atoms, or clusters of atoms, can be emitted in either a neutral or charged state. The secondary ions are then directed toward a mass spectrometer and analyzed according to their m/z ratio. SIMS has been used with different types of mass analyzers such as quadrupoles or time-of-flight analyzers. Similarly to LMMS, SIMS is a destructive method, but contrary to this late technique, which only displays point analysis, SIMS provides imaging capabilities of element distributions (112).

Multielement Capability. Yes, with isotopic capability.

Detection Limits. 1 μg/g in element imaging mode.

Spatial Resolution. 0.1–1 μm.

Environmental Applications. SIMS element distribution imaging was used for chemical inventory at the surface of airborne particles with submicron spatial resolution (113–115). Moreover, the history reconstruction of ambient levels of metals, such as Cr, As, Cd, and Pb, by using tree-ring chemistry can be resolved in part through the use of SIMS-selective microanalysis in individual wood rings and wood cells (116,117).

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3

In Vitro Toxicological Assessment of Heavy Metals and Intracellular Mechanisms of Toxicity

Wendy E. Parris and Khosrow Adeli
Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada

1. INTRODUCTION

There is an urgent need to develop and establish new toxicological approaches to assess the potential cytotoxic and genotoxic effects of heavy metals found in the environment. In the past several decades numerous in vitro and in vivo assays have been utilized to assess the effects of environmental pollutants on their cellular targets. Increasing public interest in these issues has created a demand for alternatives to using animals in such testing. Bacterial assays are used both for fundamental studies of mutagenesis and for screening of environmental samples as potential genotoxins. Mammalian cell culture systems have also been used in risk evaluation, both for investigating mechanisms of chemical carcinogenesis and as bioassay systems for monitoring environmental genotoxins. Isolated cells have been extensively used in toxicological studies in vitro. One organ of particular importance to toxicological research is the liver. The use of in vitro hepatic systems for heavy metal toxicity studies has received increasing attention in recent years. These have been used advantageously in hepatocyte-based cytotoxic-

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ity and genotoxicity assays in vitro. DNA damage in hepatocytes is often measured as covalent DNA adducts or as strand breaks that occur as a result of the DNA repair process. Assessment of DNA damage induced by heavy metals can employ either primary hepatocyte cultures or established hepatic cell lines such as HepG2. The latter cell model provides a convenient and sensitive tool for rapid screening of environmental samples for potential genotoxic and cytotoxic effects. Other recently developed methods for assessing genotoxic effects include use of microarrays that express multiple genes and from which large amounts of screening data can be obtained. More recently, human cells have been used to investigate the mechanisms by which certain heavy metals such as cadmium interact with intracellular regulatory systems that control expression of genes and intracellular stability of newly synthesized proteins. An interesting new finding is the linkage between heavy metal–induced toxicity and the function of the ubiquitin-proteasome system in the cell. The ubiquitin-proteasome system is involved in regulating protein stability for a wide array of important proteins involved in control of cell cycle, cell division, gene transcription, protein secretion, and many other vital cell functions. It was recently shown that expression of this ubiquitin-dependent proteolysis pathway in yeast is activated in response to cadmium exposure and that mutants deficient in specific ubiquitin-conjugating enzymes are hypersensitive to cadmium. This indicates that a major reason for cadmium toxicity may be cadmium-induced formation of abnormal proteins. This may be a common mechanism by which heavy metals induce cytotoxicity. Furthermore, inhibition of proteasome activity may either directly or indirectly trigger apoptosis and cell death as shown for synthetic inhibitors of this multicatalytic protease system.

This chapter focuses on a variety of in vitro toxicological screening methods for the biomonitoring of heavy metals, discusses some of the mechanisms of heavy metal toxicity, and suggests where the area of heavy metal biomarker research may proceed in the future.

When studying environmental change and its consequences, it is important to establish cause-and-effect relationships between the biological systems and the toxicant in the environment to which they are exposed. This is a challenging task when examining potential adverse effects on the human population since epidemiological data do not readily reveal such relationships and only suggest these effects by circumstantial evidence. For this reason, the evaluation of pollutant effects has usually been performed using such organisms as rats, mice, rabbits, and other experimental animals, and trying to interpret these results in the context of the human. Although this approach has been valuable in providing some predictive information, it has obvious disadvantages. For example, in addition to a variety of differences between the species, the genetic variability among such alternative organisms also interferes with the consistency of the results. This issue has been addressed to a certain degree by developing inbred strains of test ani-

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mals. However, there is an increasing demand by society to find alternatives to the use of animals in traditional in vivo toxicological testing as the use of experimental animals is not only regarded as expensive but also highly controversial.

In response to such growing demand, the development of rapid, simple, and sensitive toxicological screening methods for biomonitoring of environmental pollutants that affect human health is a universal goal. This chapter presents a review of the current application of in vitro mammalian systems for monitoring the biological effects of heavy metals.

The current philosophies of present use and future development focus on biomarkers that measure cell death mechanisms (necrosis and apoptosis), those of cell growth, regeneration, and proliferation, including cell cycle control, gene expression effects, and nucleic acid synthesis, and genetic and preexisting disease that increase susceptibility (1–4).

Some of the methods discussed in this chapter include those that measure cytotoxicity and the effects on cell cycle and apoptosis, assays for the induction of xenobiotic-metabolizing enzymes and genotoxicity, the application of DNA expression arrays, and direct techniques for monitoring damage to DNA and DNA-repair activity.

2. IN VITRO ASSESSMENT OF HEAVY METAL–INDUCED CYTOTOXICITY

2.1 General Considerations

A number of important general considerations must be taken into account when choosing a system and method by which to measure in vitro toxic effects. These have been recently discussed by Tiffany et al. (5). If permanent cell lines are used (which have both technical and economic advantages), the observations and conclusions made may differ greatly from what actually occurs in vivo after toxicant exposure. Many continuous cell lines are hardy, and may not show realistic exposure effects unless they are subjected to unusually high toxicant concentrations. Continuous lines do not exhibit the usual cellular stages of development. When primary cell cultures are used, batch-to-batch cellular variety may influence observed toxicant responses. If tissue slices are used, it is important to consider the method by which they are prepared. Cell-cell interactions may also be crucial to toxic effects, and should be taken into consideration when a test system is selected. Cell-cell interactions between different cell types may be implicated in toxic effects. Concentrations of toxicants that are effective in vivo may be very different than those relevant in vitro. If the results obtained from in vitro studies are to be meaningful, they must mimic as closely as possible those conditions present in vivo. It is important to generate both time and dose-response curves to cover a variety of scenarios and gain meaningful information. It is also impor-

tant to note that in vitro systems allow monitoring of only short-term effects and that a clear understanding of the advantages and limitations of such in vitro systems will need to be considered when interpreting data generated from in vitro toxicological assessments.

2.2 Cell Systems

The cellular toxic effects resulting from exposure to heavy metals manifest themselves in conditions and processes involving cellular oxidation state, lipid peroxidation, DNA breakage, protein expression and folding, proteasome-mediated degradation, protein-protein interactions, cell cycle, and apoptosis. Many in vitro assays for heavy metal cytotoxicity are those that measure one or more of the above end points. The types of organ and cell systems currently available to perform in vitro tests for metal toxicity have been extensively reviewed (6–8) and include that of the liver, kidney, neural tissue, the hematopoetic system, the immune system, reproductive organs, and the endocrine system. Perfused organs such as the liver and kidney, brain, lung, etc. are examples of one such in vitro system. The prime advantage of using entire organs lies in the fact that general morphology and cell-cell interactions are preserved. Precision-cut organ tissue slices also retain the general morphology and cell-cell interactions. Studies on a variety of metals or toxicants at a variety of concentrations and times can be easily performed. However, such studies can only be short term (few hours to a few days) and have the disadvantage that animal material is still required. Another option is the use of suspended cells from either blood or isolated cells from tissue. This provides the opportunity for toxicity assays of several agents at different concentrations, but only for short terms. It is possible to cryopreserve such cell preparations for further investigations. Interpretation of data from these assays requires consideration since the organ of cell source is no longer intact, and crucial processes that require cell-cell contact, such as intercellular signaling, may no longer be functioning.

Primary cell cultures from organs of interest (liver, kidney, etc.) may also be prepared. Their use permits longer-term studies of from a few days to a few passages. A large selection of toxic agents at several concentrations may be examined. Some differentiated functions may be retained, and coculture is possible with other cellular types. On the contrary, such cultures have unstable phenotypes and may quickly lose many differentiated functions.

The use of immortalized cell lines offers ease of propagation and the ability to generate unlimited numbers of cells for testing. Such lines are useful for specific mechanistic studies and may be cocultured. They may also be genetically manipulated to express proteins of interest, and can be cryopreserved. Their disadvantage is that they may have lost a variety of specific cell functions, and have an unstable genotype.

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One cell line, the human hepatocyte HepG2, retains many functions of the normal hepatocyte (liver) including the synthesis and secretion of hepatic-specific proteins (9) and expression of xenobiotic-metabolizing enzymes (10) and has been used extensively. Cell lines used to monitor nephrotoxicity include continuous renal epithelial cell lines: LLC-PK_1 cells (Yorkshire pig, proximal nephron), OK (North American oppossum, proximal nephron), JTC12 (monkey proximal nephron), MDCK (dog collecting duct), and A6 (*Xenopus*, distal tubule/collecting duct) (11). Neurotoxicity [reviewed by Costa (12)] can be monitored using neuroblastoma or glioma cell lines or PC12 cells (11), HT4 cells (mouse neuronal cell line), or astroglial cells (13). For reproductive and developmental toxicity (reviewed in ref. 14), ovarian somatic cells (granulosa, thecal, and stromal cells) (15), testicular cell types (Sertoli–germ cell cocultures, Seroli-cell-enriched cultures, germ-cell-enriched cultures, Leydig cell cultures, and Leydig–Sertoli cell cocultures) (16) have been used. Other cell types that have been used in toxicity studies include embryonic stem cells (14), as well as primary cultures of human lymphocytes, and rat chondrocytes and human amniotic cell lines (WISH) (17).

It is also possible to use a variety of subcellular fractions such as microsomes, mitochondria, or various vesicles. Major disadvantages are that they are only useful for very-short-term studies and are technically demanding to prepare (18).

Genetically engineered bacteria, yeast, insect cells, and mammalian cells that express one or more genes of interest offer good potential in the future for toxicity studies. In the future, artificial tissue material such as reconstructed skin models will continue to evolve and be useful as tissue models to assess some types of toxicity and provide an in vitro system that substitutes for animal use (19).

2.3 Membrane Integrity

One perspective by which to assess the overt toxic effects of metals in cultured cells and other cell types has been to examine cell-membrane integrity. Such methods include detecting enzyme leakage from cells or measuring the uptake of dye compounds into cells. Assessing cell viability (for example, primary hepatocytes) involves monitoring the leakage of lactate dehydrogenase (20) or aspartate aminotransferase (21). Alternatives include techniques that are based on the uptake of a dye, such as trypan blue, by nonviable cells and its active exclusion by viable cells (22). This method requires visually examining the cells by light microscopy and then scoring the cells for percent survival.

A similar procedure involves the uptake of dye by viable cells (for example, in attachment cultures) and quantitation of the incorporated dye by spectrophotometry. This process is the basis for the Neutral Red uptake (NRU) assay (23). In this procedure, after being treated with a test compound, cells are incubated in the presence of NR, which is endocytosed and sequestered into the lysosomes

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of viable cells. The cells are washed with a mild fixative, and the NR is then extracted and quantified spectrophotometrically.

The development of many fluorochromes and the increasing availability and expertise in flow cytometry have led to increasing use of this technology in cytotoxicity assays. For example, very subtle changes in membrane integrity can be visualized by increased staining with 7-aminoactinomycin D. Levels intermediate between healthy and necrotic cells can be analyzed by flow cytometry (24). A variation on this method is to test for viability by the uptake of propidium iodide. Its uptake occurs during the later stages of apoptosis (programmed cell death), and indicates secondary necrosis of dying cells. The propidium iodide interchelates the DNA. It has been suggested that in the early stages of apoptosis during DNA condensation, uptake is decreased since the DNA is less accessible. Then as the DNA becomes fragmented, it becomes more accessible and more propidium iodide binding occurs. This results in increased DNA stainability (25) and red fluorescence, which may be detected by flow cytometry.

2.4 Oxidation State

Many metals alter the oxidation state of cells owing to the production of free radicals. An altered oxidation state in turn causes multiple cellular effects. The level of reactive oxygen species (ROS) in cells is often determined by monitoring the oxidation of a fluorescent probe such as 2′7′ dichlorfluorescin diacetate (DCFH-DA). DCFH is converted to DCF in the presence of H_2O_2 and is measured by flow cytometry (26,27). However, recent studies outline some difficulties in interpretation of these results in cells since the deacetylation of DCFH-DA, even by esterases, may produce peroxides that could interfere with accurate measurements of the oxidation state of the cell (28).

Intracellular redox status may also be deduced from the glutathione (GSH) concentration in the cells. The induction of HSP70 (heat shock protein 70) and metallothionein (MT) (both as a result of heavy metal exposure) is considered to be associated with the intracellular glutathione metabolism in the cellular protection mechanism against metal (cadmium)-induced injury (29,30). GSH content of cultured cells exposed to heavy metals may be determined by a fluorometric assay using *o*-phthalaldehyde (31). Lysed cells are incubated with the flourescent compound and fluorescence changes are related to the protein concentration and GSH content.

The oxidative state of the cell can also be determined by examining the concentration of the malondialdehyde product of lipid peroxidation as measured by the colorimetric thiobarbituric acid assay using exposed and control homogenized tissue culture cells (32).

Measurement of mitochondrial activity is another method of assessing cytotoxicity. One such assay measures the reduction of the tetrazolium dye substrate

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MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide, by active mitochondria, to a visible product (33). The color change may be quantitated and used as a measurement of cell viability, and comparisons made between analyses using both untreated and toxicant-treated cells.

The mitochondrial transmembrane potential decreases in injured cells (34,35) and the use of fluorochromes allows its measurement by flow cytometry (36,37). Measurement is made of the accumulation of mitochondrial specific membrane-permeable cationic fluorescent compounds such as DiOC6 (green fluorescence) (26,38,39) and JC-1 (34,40), a fluorochrome that changes from green (monomeric form) to red (aggregated form) at high membrane potentials. A similar method measures the retention of Rh123, which is readily sequestered by active mitochondria again depending on their membrane potential (41).

There are intracellular changes in Ca^{2+} concentration in response to toxic insult as heavy metals are postulated to interfere with the Ca influx to cells. The Ca-selective sensitive dye Indo-1 (indo-1 acetoxymethyl ester) may be used to assess the Ca^{2+} concentration variation in cells. The fluorescent emission spectrum of Indo 1 shifts after calcium binding. The dye has two different emission wavelengths, 395 nm and 525 nm. Their ratios are altered depending on the amount of Indo-1 calcium binding and hence measures the concentration of free Ca^{2+} in the cell. This measurement of intracellular Ca provides a means of quantitating cellular insult in comparison to untreated cells (42).

2.5 Presence of Specific Marker Proteins

Other methods more directly measure proteins specifically involved in programmed cell death (apoptosis) or necrosis both of which may result from heavy metal exposure. One such example is a recently developed fluorescence energy transfer assay, for caspase-3, an important member of the caspase isoform family of proteases that cleave after aspartate residues and are activated during apoptosis. Evidence exists that toxicants are able to induce apoptosis by activating caspase-3 (43–45). The consensus recognition and cleavage site of caspase-3, DEVD (43), has been identified. A hybrid green fluorescent protein (GFP) and blue fluorescent protein (BFP) have been constructed and linked with the caspase proteolytic recognition site. Cleavage of this protein by caspase-3 causes a change in UV excitation and emission characteristics of the labeled protein (fluorescence energy transfer, FRET). This may be detected by FACS analysis (46). In a modification of this assay that has been sucessfully used in thymus tissue in vivo, the peptide DEVD coupled to the fluorophore MCA is used as the caspase substrate. Cleavage of the peptide releases MCA, which can be determined fluorimetrically (47).

Another technique for analyzing caspase activity looks for the processing of caspase substrates such as the nuclear substrate poly-ADPribose polymerase (PARP). Exposed tissue culture cell lysates are electrophoresed and Western blot-

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ted with PARP antibodies. The appearance of an 89-kDa cleavage product in addition to the 116-kDa native PARP band indicates the level of caspase activity (37).

Fluoro-Jade and its second-generation compound Fluoro-Jade B are fluorochromes recently used in the detection of neuronal degeneration by well-characterized toxicants, specifically the detection of apoptosis, amyloid plaques, astrocytes, and dead cells in tissue culture. Development of this method will add to the repertoire of cytochemical techniques available for detecting toxicity (48).

Recently a system of radionuclide imaging of apoptosis has been reviewed by Blankenberg et al. (49). One of the cellular effects after caspase activation is the expression of phosphatidylserine on the external surface of the cell membrane. It acts as a signal to adjacent cells that it is undergoing apoptosis. This expression of phosphatidlyserine is a molecular target that can be used to image apoptosis. Annexin V lipocortin, which binds strongly to membrane-bound phosphatidylserine, has been radiolabeled through its sulfhydryl groups with technetium-99m. This procedure has permitted the imaging of apoptosis in animal models and may be an important diagnostic tool in the future.

3. IN VITRO ASSESSMENT OF HEAVY METAL–INDUCED GENOTOXICITY

3.1 DNA Strand Breaks

Genetic approaches to measuring toxicological effects are becoming increasingly popular as our expertise in this area of technology quickly advances. Over the past several decades, many in vitro assays have been used to assess the genotoxic effects of xenobiotics, such as heavy metals, on target organisms. For example, bacterial assays, such as the *Salmonella* mutagenicity assay (50), have been used not only for fundamental studies of mutagenesis but also for the screening of environmental samples for potential genotoxicity. The methods used in this test system have been extensively reviewed elsewhere (51). Several mammalian cell lines have also been used for investigating the mechanisms of chemical carcinogenesis (52) and as bioassay systems for monitoring environmental genotoxins (53). Of the various end points that have been used as indices of genotoxic insult, the formation of DNA single-strand breaks (SSB) has experienced increasing use. This trend may be attributed to the relatively high sensitivity of the SSB response to xenobiotic exposure, as well as to the toxicological sequelae that are associated with the SSB response, including clastogenesis, heritable mutations, and cancer. This type of DNA lesion may be brought about in one of two general ways. The first is the direct cleavage of the DNA strand by the ionizing radiation or free radicals (54), and the second is through faulty repair (misrepair) of nucleotides whose nitrogenous bases have been damaged. Briefly, the DNA repair pro-

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cess involves several enzyme-mediated events, including the following: (a) cleavage of the phosphodiester bond that is adjacent to the damaged base, (b) removal of the damaged base, (c) replacement with an undamaged base, and (d) ligation of the DNA strand (55). Should step (b), (c), or (d) be interrupted, a strand break may remain. The misrepair-mediated formation of SSB can result from various forms of base damage, including covalent adduct formation or oxidation. Formation of SSB may result from exposure to a wide variety of genotoxic heavy metals that increase the production of reactive oxygen species.

The methods of quantitating single-stranded breaks are generally based on exposing the DNA strand to alkaline conditions (pH $>$ 11.5), so that unwinding of the helix occurs at the single-stranded break sites. If an appropriate, fixed period of unwinding is used, the formation of single-stranded DNA will be proportional to the number of ''alkali-labile'' break sites present. Several procedures exist for facilitating the unwinding and for quantifying the single-stranded (SS) and double-stranded (DS) DNA fractions. One of the simplest procedures involves an alkaline unwinding step and then DNA quantification using a fluorescent DNA-binding stain (Hoechst 33258) in the samples, which contain both SS and DS fractions (56). A second procedure, known as alkaline elution, involves loading the cells onto a porous membrane (for example, a polycarbonate filter) and eluting the SS DNA from the filter with an alkaline buffer (57). The DNA is quantified radiometrically, using cells that are prelabeled in culture with [^{3}H]thymidine. A third, recently developed method for quantifying single-stranded breaks is the single-cell gel electrophoresis assay (58), in which individual cells are embedded in agarose gel on microscope slides and then subjected to an electrophoretic field under alkaline conditions to facilitate unwinding. The cellular DNA is then stained with ethidium bromide and visualized under a fluorescence microscope. The DNA ''comets'' that form as a result of the electrophoretic migration of SS DNA from the nucleus are then scored. The ratio of tail length (SS DNA) to head (nuclear) diameter is determined and may be interpreted as the extent of SSB formation. Theodorakis et al. (59) described a similar method, in which fish DNA was subjected to electrophoresis in a batchwise manner under neutral and alkaline conditions, revealing the respective double- and total strand breakage.

A procedure using hydroxylapatite DNA chromatography has been developed (60) and optimized for use with human cells in culture in our laboratory. Briefly the first step involves alkaline unwinding of [^{3}H]thymidine-labeled DNA, which is carried out directly in a culture dish (such as a 24-well plate), and then loading the contents of the dish onto a hydroxylapatite column. The respective SS and DS DNA fractions are eluted separately with low- and high-phosphate buffers. The radioactivity in each [^{3}H]thymidine-labeled DNA fraction is then quantified in a liquid-scintillation counter, and the ratio of SS to DS DNA is determined.

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TUNEL [terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labeling method] (61) may also be used to determine the percentage of cells with DNA strand breaks. TdT labels the 3′OH end of DNA fragments with deoxy-UTP. Approaches include direct labeling (with FITC-dUTP, BODIPY-dUTP, CY2-cUTP) and indirect labeling (with digoxigenin-conjugated dUTP, biotin-conjugated dUTP followed by secondary detection systems based on fluorescein, peroxidase, or alkaline phosphatase). Cells may then be scored by microscopy (61,62). In early stages of DNA damage, when only single-stranded breaks exist, in situ nick translation (INST) or in situ end-labeling (ISEL) using DNA polymerase and the above-mentioned labels may be a more useful tool (63,64). The advantage of TUNEL in comparison to conventional immunohistochemical methods is that cells with minimal DNA damage are detectable at an earlier stage, before the appearance of major nuclear changes (25).

In addition, DNA degradation may be measured quantitatively by a commercial ELISA method specific for histone-bound DNA fragments in the cytosol (62). Cells of interest are cultured in 24-well plates to near confluence, and then treated with the test metal (toxicant) at various concentrations and for various time periods. Following treatment the plate is centrifuged to collect both attached and unattached cells on the plate surface. After careful removal of the medium, cells are lysed and placed in wells of an ELISA streptavidin-coated microtiter plate. Both antihistone biotin (which binds the histone component of the nucleosome) and anti-DNA conjugated to horseradish peroxidase (which binds the DNA component of the nucleosome) are subsequently added. Peroxidase activity is detected after addition of a colorimetric substrate and the product quantitated with a microplate reader. By comparing the product formation of the experimental sample cells and the control cells, the level of cell and DNA breakage due to the toxicant exposure can be determined.

The micronucleus technique is another technique for assessing DNA damage. Micronuclei originate from chromosome fragments or whole chromosomes not included in the main daughter nuclei during nuclear division. When kinetochore or centromeric antibodies are used in conjunction with FISH (fluorescence in situ hybridization) staining with adjacent chromosomal probes, it is possible to distinguish between chromosomal breakage and alteration of chromosomal number (65–67). Since this method facilitates the examination of large numbers of cells, it has a statistical advantage. Attempts are being made to standardize and collect international data obtained by this procedure by the Human MicroNucleus Project, and determine its efficacy as a biomarker of human toxicant exposure (68).

A method that indicates oxidative stress in response to toxic environmental exposure is one that quantitates the modified DNA base, 8-hydroxy-2′-deoxyguanosine (8OH2′dG). It is regarded as the principal stable marker of hydroxyl radical damage to DNA. It can be measured in a variety of biological matrices by

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a liquid chromatography electrochemical column switching method (HPLC-ECD) (69) and will likely become a more common marker in the future.

The use of DNA fingerprinting to detect genotoxic effects has been suggested by some to be a very sensitive procedure that may reveal damage not presently detected by other methods (70). DNA is isolated from the source of interest (for both control and exposed), and subjected to AP-PCR (arbitrarily primed-PCR) at less stringent reaction conditions. From this method a number of PCR products of a variety of lengths are obtained. The products of these PCR reactions will have DNA fingerprints affected by the loss or gain of priming sites due to mutations or DNA breaks. Changes observable in control and exposed subjects may be due to the presence of DNA adducts (not all of which will result in mutations), or mutations or DNA strand breaks. The sensitivity of PCR means that only very small quantities of DNA are required; however, it is essential to ensure that no contaminating DNA is present. AP-PCR has a sensitivity that should enable it to be an early warning of toxicity, since it can detect changes in advance of other methods that rely on chromosomal abnormalities or mutations.

3.2 DNA-Protein Cross-Links

Cell exposure to heavy metals has been shown to result in DNA-protein cross-links (DPC). DPCs of a high molecular weight have been shown to have a significant mutagenic effect (71). They may be detected in two ways, as follows. Cell nuclei are prepared from tissue culture cells after incubation with and without toxicants. After treatment, DPCs are purified and the DNA released by treatment with DNase. The remaining proteins are electrophoresed and transferred onto nitrocellulose. Specific proteins of interest, such as actin, may be detected by immunoblotting (72). Another method for detecting DPCs on a more general basis is described by Zhitkovich et al. (71) and involves the selective precipitation of proteins and protein-linked DNA in the presence of sodium dodecyl sulfate and K^+. DPC is quantitated as the percentage of total cellular DNA precipitable by K-SDS treatment, and its detection limit is estimated at 1 adduct per $1–2 \times 10^7$ bases.

3.3 DNA Repair Activity

Another approach to assessing genotoxicity is monitoring DNA repair activity in cells after genotoxic insult. The most widely used approach involves quantifying unscheduled DNA synthesis (UDS), which indicates the repair of DNA lesions. UDS assays are based on the incorporation of radiolabeled nucleotides (commonly [^{3}H]thymidine) into the DNA of cells that are not undergoing replicative (scheduled) DNA synthesis. The two general methods for quantifying [^{3}H]thymidine incorporation are (a) autoradiography and (b) liquid scintillation counting (LSC). In both procedures, the cells are exposed to the test compound in the

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presence of a radiolabeled nucleotide. For autoradiographic UDS detection, cells grown on microscope slides are first exposed to the test compound and then fixed, dried, and coated with a nuclear tracking emulsion (73). After an exposure period requiring several days to several weeks, depending on the level of radioactivity in the cells, the emulsion is developed, and the nonreplicating cells are scored for nuclear grain densities that are proportional to the magnitude of the UDS response.

In the interest of developing methods to assess genotoxicity in ways that are both sensitive and rapid, our laboratory has optimized the LSC-based UDS assay for use in human cultured cell lines, following the procedures described by Martin et al. (74). This technique differs from the autoradiography assay in that the cellular DNA is assessed in a batchwise manner rather than by the visual examination of individual cells. After treatment with a test compound in the presence of a radioactive label, the cells are collected onto a porous membrane, lysed, rinsed, and the nuclear material is analyzed by LSC. To ensure that replicative DNA synthesis does not interfere with or obscure the UDS response, the cells should be pretreated with hydroxyurea to inhibit replicative synthesis without significantly affecting UDS-mediated incorporation of [^{3}H]thymidine (74).

3.4 Gene Induction, Toxicogenomics, and Microarrays

Some genotoxic metals may alter the expression of several inducible genes. Such alterations are useful to monitor since altered expression often significantly precedes detectable effects on the organism as a whole. One such gene is phosphoenolpyruvate carboxykinase (PEPCK) (75). Cell lines with PEPCK promoter-luciferase reporter genes have been constructed to examine the effects of heavy metals on promotor function. The use of reporter genes will provide a system by which to identify DNA and protein cellular targets of heavy metal exposure leading to changes in expression of specific genes. This will provide sensitive biomarkers as well as help understanding mechanisms of damage for heavy metal exposure (75). Other gene expression biomarkers might include ''stress proteins'' such as the human metal inducible genes for the metallothionein isoforms, Bcl-2 family members [Bcl-2, Bcl-X, Bax, and hsp70 (70-kD heat shock protein)], hsp90, hsp60 (chaperonin), caspase activation, c-fos genes, and other genes involved in cell cycle events (76). The use of gene expression markers will be discussed further below.

The impact of the human genome project and its related technologies is also applicable to the science of toxicology. High-throughput screening procedures and the use of DNA expression array technology are beginning to play a significant role in determination of chemical toxicity. Readers are referred to the review article by Farr and Dunn (77), which discusses monitoring changing gene expression patterns in response to chemical toxins (stress), in depth. They catego-

rize four types of ''stress genes'': those that respond to the presence of a compound, those that are damaged by a compound, those that are affected by altered levels of crucial metabolites such as ATP and NAD(P)H, and those that are sensitive to changes in the cellular redox status or its pH. Of these, heavy metals induce ''sensor''-type genes. For example, metallothionein I and II, metal-binding proteins, are induced by the presence of cadmium or zinc (78). The activation of genes at inappropriate times is responsible for much of the toxicity mediated by receptors. Genes may respond to damage to DNA, proteins, membranes, endoplasmic reticulum, cytoskeleton, and/or mitochondria. RNA damage may also induce genes (79). This could affect a multitude of normal cellular processes including cell cycle, microtubule assembly, ATP synthesis, etc. (5). Metabolic genes can respond to varying levels of molecules required for normal cell function. The amount and redox status of iron, for example, influences the expression of a number of genes (80).

Although Farr and Dunn (77) elaborate on the need for careful interpretation of toxicity data obtained from the use of array technology, they point out that aside from the obvious ability to look at multiple genes and multiple potential toxicants, other advantages of this methodology are that it is possible to identify genes related to toxic effects that are poorly understood, and gene-gene associations that would be difficult to elucidate from knowledge of their function alone. This technology may also facilitate the identification of people at toxicological risk given their individual genetic makeup. A disadvantage of this approach is that not all changes in gene expression are toxicologically relevant. Undoubtedly this area has the greatest potential for specific determinations of heavy metal and other chemical toxicity and is already in extensive use in the pharmacological setting.

4. MOLECULAR AND CELLULAR MECHANISMS OF HEAVY METAL–INDUCED TOXICITY

4.1 Bioavailability

Some reference has been made in previous sections to mechanisms mediating the toxicology of heavy metals on cellular processes and metabolism as well as gene expression. Important factors impacting effects and mechanism of action are the type of tissues exposed and method of heavy metal exposure, and whether the toxicant is ingested in food or water, or by surface contact or inhalation. Such considerations have been recently reviewed by O'Flahery (81). Properties of a heavy metal such as its solubility, or particle size, will influence its toxicity potential and bioavailability. The status of the gastrointestinal tract of the exposed subject, for example the dietary habits and age, and the dosage of the metal will influence its fate. With respiratory exposure, particle size and solubility will

determine whether the metal accumulates in the lymph nodes of the lung (insoluble) or whether it appears in the blood, urine, bone, or other tissues.

Metals may form oxyanions in biological systems. Some, such as vanadate and arsenate, resemble phosphate structurally and use the phosphate transport system to penetrate cells (82). Others that resemble sulfate (chromate, molybdate, selenate) use the sulfate transport system (83,84). Uptake into cells may be mediated by such mechanisms as ion channels [calcium uptake channels for lead and cadmium (85)] or by simple diffusion in the case of uncharged lipid-soluble complexes, such as Cr^{3+} and As^{3+} (82,86). Some metals, such as cadmium, enter the cell using carrier transport mechanisms (the protein metal conjugate of glutathione). Once the metal has entered the cell, its toxicity is related to whether it accumulates in the cytoplasm or is further taken into the nucleus or other cell organelles.

4.2 Genotoxicity

For an extensive review of mechanisms of heavy metal toxicity, in particular cadmium, the reader is referred to Beyersmann and Hechtenberg (87). Heavy metals and other toxins generate free radicals in the cell and increase the concentration of reactive oxygen species (such as hydrogen peroxide or the peroxide radical, superoxides, and nitric oxide). This can result in both single- and double-stranded DNA breaks, DNA and chromatin conformational changes, and chromosomal aberrations. There may be a decreased fidelity of base pairing by DNA polymerase, for example, by cadmium (88), and inhibition of DNA repair (89–91). In some cases DNA synthesis is enhanced, but this is thought to be a nonspecific consequence of cell injury (92). Metals (as in the case of cadmium) may also impact the synthesis of RNA, inhibiting synthesis in some cell types or stimulating it as in liver cells. Cadmium also inhibits protein biosynthesis. Thus cadmium (and other metals) may inhibit all processes of information transfer from DNA to RNA to protein.

As already mentioned, the expression of a number of proteins may be altered. Cadmium and other metals can induce the expression of metallothionein (a low-molecular-weight Zn-binding protein that also binds Cd^{2+}) and glutathione both of which aid in the protection of the cell by maintaining its oxidation state. GST(glutathione-S transferases) catalyze the nucleophilic attack of glutathione on electrophilic substrates, thus decreasing their reactivity with cellular macromolecules (85). They are a multigene family of enzymes that have been shown to be involved in detoxification by removal of free radicals, and in some cases activation of a variety of chemicals (93). Other candidate genes for induction are those for a number of stress proteins also known as heat-shock proteins or chaperones, which bind to and stabilize labile protein conformations, as well as proteins involved in the apoptotic or necrotic process of cell death. Redox-sensitive transcription factors (AP-1 and NF-κB) are activated during oxidative stress

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(94,95) and have been shown to have increased DNA binding activity in lung epithelial cells during cadmium-induced apoptosis (92). Acute-phase reactants (APR) usually induced during inflammation, such as alphal-acid glycoprotein and serum amyloid, may be also induced by metal ions (96). The protooncogenes c-jun, c-fos, and c-myc are induced by cadmium (97). Their expression seems to involve the activity of protein kinase C (39). c-fos induction by cadmium in rat kidney LLC-PK1 cells has been shown to be related to mobilization of intracellular Ca^{2+} ions and the activation of protein kinase C. p53 expression is also stimulated by cadmium (98). The inflammatory cytokines IL-1 α, IL-1β, ICAM-1, MIP-2, and TNF-α are also transcribed and secreted in response to cadmium injection in mice (99). One steroid receptor, progesterone, is induced by cadmium, whereas that for estrogen is decreased (100). As discussed by Beyersmann and Hechtenberg (87), there does not seem to be a single reason as to why cadmium affects the expression of a particular gene and is likely to depend on the signaling pathway affected by exposure.

There are a number of ways in which cadmium and other heavy metals may interfere with major signaling pathways. Heavy metals may: (a) interact with cell surface receptors, (b) interfere with the uptake and intracellular distribution of Ca^{2+}, by altering function of several enzymes and regulatory proteins involved in intracellular signaling (Ca^{2+} ATPases), (c) substitute for Zn in cellular proteins, (d) interfere with normal protein kinase C activity, MAP kinase activity (still uncertain), or calmodulin-dependent protein kinase (not yet described), (e) affect the activity of transcription factors and other regulatory proteins (not yet demonstrated).

4.3 Protein Conformation and Folding

Metal ions form an integral part of some protein domains such as Zn fingers. The interruption of normal Zn metallothionein binding by other metal ions such as Cd^{2+}, Co^{2+}, and Ni^{2+} may in turn change the amount of Zn available to be a part of Zn fingers. This may result in a change in protein conformation, and lead to variation or loss of the protein's activity or enable its recognition for degradation by the proteasome. This could be especially disruptive if many Zn-finger-containing transcription factors are affected (101). This phenomenon has not yet been demonstrated in vivo.

4.4 Protein-Protein Interactions

Heavy metals have been shown to influence protein-protein interactions. One example is the disruption of the E-cadherin/catenin cell adhesion complex via the displacement of extracellular calcium by cadmium (102,103) and by oxidative stress (104). This may be mediated by changes in tyrosine (105–107) or serine phosphorylation (108) of β-catenin. Similar effects on phosphorylation of other

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proteins may impact a multitude of protein-interactions and signaling events (104).

4.5 The Role of the Proteasome in The Response to Heavy Metal Exposure

Proteins that are damaged by oxidative stress or that are incorrectly folded or localized due to disruption of cell processes caused by metal toxicity are candidates for degradation by the ubiquitin proteasomal system. This system has been the subject of many investigators and has been reviewed by Hershko (109), Hershko and Ciechanover (110), Baumeister et al. (111), Kornitzer and Ciechanover (112), Ciechanover et al. (113), Wilkinson (114), and Brodsky and McCracken (115). This system is important not only in normal cell growth and development, but also in defending the cell against environmental insults that cause disruptions in normal cellular state. It regulates the entry and exit into mitosis through the coordinated degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (116,117). It is important for the degradation of transcription factors, regulatory proteins, antigen processing, and angiogenesis (118–124). Mutations in genes encoding the inner proteolytic core of the proteosome are lethal (125). Degradation of proteins by the proteasome is dependent on prior ubiquitination. Ubiquitination is a complex process by which the cell selects targets and, by the addition of amino acid chains of the protein ubiquitin to the protein, may target the protein for degradation by the proteosome, a form of intracellular ''quality control.'' The ubiquitin-proteosomal degradation pathway consists of a sequence of events that is initiated by the covalent addition of several 76-residue ubiquitin amino acid chains to the substrate protein molecule, and the subsequent degradation of the ubiquitinated protein by the 26S proteosome. The ubiquitination process has three distinct steps. The ubiquitin protein C-terminal is activated by the enzyme E1. Subsequently several ubiquitin carrier proteins (E2 enzymes) or ubiquitin-conjugating enzymes (Ubc) transfer ubiquitin from E1 and specifically bind it to E3, a member of the ubiquitin-protein ligase family. E3 in turn catalyzes the covalent attachment of ubiquitin to the protein substrate. The mechanism by which E3 forms the polyubiquitin chain is not yet understood. In special situations, E4 may be another component of the system required to elongate the ubiquitin chain attached to the target protein. The covalent addition of this polyubiquitin chain to the protein molecule targets it for degradation by the 26S proteasome. The specificity of E3 ubiquitination may be determined by primary protein structural motifs, by post-translational modifications of the target protein by phosphorylation, or by association with other proteins such as chaperones. This is an area of very active research.

The 26S proteasome is a large multisubunit, multicatalytic protease that degrades the polyubiquinated proteins into peptides 3–22 amino acid residues

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long. It appears to have a variety of cleavage pattern characteristics (111). It consists of two smaller complexes, a 20S and a 19S particle. The 20S particle is the catalytic subunit and consists of two α subunits and two β subunits. The 19S particle has two subunits, a ''base'' and a ''lid.'' The base contains six ATPases as well as Rpn1, Rpn2, and Rpn 10, and the lid contains eight additional subunits. The 19S complex may be involved in recognition of ubiquitinated proteins and others destined to be substrates. Its second function may be to alter the conformation of the 20S particle to facilitate substrate accessibility to the proteolytic machinery.

The proteasome can be found in both the nucleus and cytoplasm and has multiple nuclear localization signals located on four of the human α-type subunits. Changes in cellular localization of the proteasome add another level of complexity to its proteolytic activity. Regulation of the proteosome could occur at the level of ubiquitination or level of proteosomal activity. It appears that the proteasome and the ubiquitin-activating enzyme are constitutively active, a feature that is expected since it is required for many cellular functions. However, components of the pathway can be up-regulated by specific pathological conditions (126,127) and the specificity of the proteasome can also be changed, as reviewed by Rock and Goldberg (128).

As already discussed, heavy metal exposure has been shown to produce a variety of reactive oxygen species and change the redox status of the affected cells. This phenomenon has multiple cellular effects, some of which include free radical damage to DNA and proteins, induction and protein activation, incorrect protein folding, or cellular localization. The ubiquitination reaction and the proteasome are an important component of the cell's reaction to all of these effects. It was recently shown that expression of this ubiquitin-dependent proteolysis pathway in yeast is activated in response to cadmium exposure and that mutants deficient in specific ubiquitin-conjugating enzymes are hypersensitive to cadmium (129). This indicates that a major reason for cadmium toxicity may be cadmium-induced formation of abnormal proteins. This may be a common mechanism by which heavy metals induce cytotoxicity. Furthermore, inhibition of proteasome activity may either directly or indirectly trigger apoptosis and cell death as shown for synthetic inhibitors (e.g., lactacystin) of this multicatalytic protease system. Thus inhibition of the proteasome by environmental chemicals that mimic proteasomal inhibitors can potentially lead the cell to an apoptotic state (130).

4.6 Effects on Cell Cycle and Cell Death

A result of the oxidative stress induced by heavy metal exposure is genotoxic stress, which triggers cell cycle checkpoint responses. Readers are referred to a recent review article by Shackelford et al. (131), which describes in detail the

ramifications of genotoxic stress on cell cycle control and checkpoint mechanisms. As discussed previously, DNA modifications induced by reactive oxygen species such as hydrogen peroxide or the peroxide radical, superoxides, and nitric oxide include single-stranded and double-stranded DNA breaks, DNA-protein cross-links, and a variety of base and sugar modifications. Cells are very sensitive to DNA damage, and the point in the cell cycle during which DNA is damaged determines the cell cycle and checkpoint response. Most cells have the ability to arrest cell cycle in G_1, S, and G_2 phases, and then resume after DNA damage is repaired. Otherwise, cells may undergo apoptosis or enter into a permanent G_0-like state. The exposure of cells to genotoxic agents during early to mid-G_1 may delay proliferation in G_1 at the G_1 checkpoint (132). The p53 gene product plays a major role in G_1 cell cycle arrest in response to DNA damage (133), which under normal circumstances is a short-lived protein, but is induced in response to DNA damage by posttranscriptional stabilization (133,134). p53 is a transcription regulatory factor that binds to regulatory sequences that can both activate and represses a variety of genes (135–139). The G_2 checkpoint response is a function of the accumulation of phophorylated $p34^{cdc2}$ molecules. This results in the inhibition of kinase activity of cyclinB/Cdc2, which in turn has been shown to be important in inhibition of the G_2 checkpoint. The spindle checkpoint functions to stop cells in mitosis until all the chromosomes are attached appropriately to the spindle (140–142). To proceed to metaphase and anaphase the proteasome must degrade a number of the proteins previously essential for entry into mitosis (143–145). DNA-damaging agents and spindle-damaging agents can activate the spindle checkpoint mechanism by affecting a number of signaling proteins such as Cdc 20, Mad (146,147), Mec 1, Psd1p (148), and the polo-like kinase (plk) proteins (149–152). For further details on checkpoint intermediates and mechanisms the reader is referred to Shackelford et al. (131) and Lipton (153).

After it has been initiated, the complex apoptotic process involves the coordinated action of several different proteases, nucleases, membrane-associated ion channels, and phospholipid translocases (154). Reactive oxygen species (ROS) play an important role in apoptosis initiation (156). A detailed study by Hart et al. (62) has shown that cadmium-induced apoptosis in rat lung epithelial cells (as evaluated by cell morphological features, DNA degradation, and TUNEL) was preceded by indications of oxidative stress. There was an up-regulation of glutathione-S-transferase, γ-glutamylcysteine synthetase, and metallothionein-1. In addition, they showed that the transcription factors AF-1 and NFκB, which are redox sensitive, are activated, and there are changes in the species of glutathione (reduced, oxidized, and protein bound) in the stressed cells.

The caspase family of proteases, with their specificity for aspartate residues, are a significant component of the apoptotic cycle. Caspases are present in the cell in a number of forms, and need to be proteolytically processed to become active. There appear to be two subsets of caspases, those involved in cell death

(caspase 2, 3, 6, 7, 8, 9, 10) and those related to caspase-1 (caspase 1, 4, 5, 11), and have a role in cytokine processing. Their role in the apoptotic process has been recently reviewed by Slee et al. (156). They describe apoptosis as consisting of four phases: initiation, when signaling events trigger the cell death process; commitment, after which the cell cannot return to normal cell cycle; amplification, when multiple caspases (from the first category) are recruited to destroy the cell; and demolition, when active caspases destroy cell structures directly or indirectly by activating other enzymes. The initiation phase involves the activation of cell receptors containing the death domain on their cytoplasmic tails (157–158). These domains bind adaptor molecules that in turn bring caspases to the receptor complex. The caspase activation is enabled by this process, since the proximity of several molecules predisposes their activation by each other. When the stimulus on the cell to enter apoptosis is from toxicants such as heavy metals, the death pathway seems to focus on the mitochondria (159). A variety of stimuli cause changes in the membrane permeability of the mitochondria outer membrane and permit the escape of certain proteins normally found only inside the mitochondria. This escape appears to be in both a caspase-dependent and independent method, depending on the apoptotic initiating event. The consequence of the release of apoptosis-inducing factor (AIF) is its translocation to the nucleus, and the chromatin collapse and DNA fragmentation that has been discussed earlier. Cytochrome c regulates the activity of apoptotic protease activating factor (Apaf-1), a molecule that promotes caspase-9 clustering. The clustering process is similar to that which occurs when the caspases bind to the cell surface death receptors and become activated by cleaving each other. The death signal is further amplified by this caspase 9 cleaving other caspases, caspase 3, followed by 2 and 6. For the details of this process the reader is referred to Slee et al. (156), the important concept here being the activation of many caspases that will take the cell farther into the apoptotic process. The final destruction of the cell begins once all of the necessary caspases have been activated. Although caspases are contained in the cytoplasm, many of their substrates are in the nucleus or other organelles. How the caspases move from compartment to compartment in the cell is an area of active research.

The ubiquitin proteasome pathway also has a major role to play in cell cycle and apoptosis and this subject has been the topic of a recent review article by Orlowski (160). A number of studies have shown the correlation of apoptosis with ubiquitination, in terms of increased polyubiquitin expression, increased levels of proteasomal subunits, ATPase regulatory subunits, and migration of proteasomes from the nucleus to apoptotic sites after apoptotic induction by p53 (reviewed in ref. 160). Although overall there seems to be a direct role for the ubiquitination and proteasomal activity in apoptosis, interference with proteasomal activities induces apoptosis. The effects of inhibition of the proteasome at various points in the cell cycle progression have been reviewed by Hershko (116)

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and King et al. (161). It has been speculated that apoptosis is initiated when conflicting signals for cell growth and cell cycle arrest can be resolved only by self-destruction of the cell. Undoubtedly as the functions and mechanisms of the proteasome and cell cycle are better understood, the role of heavy metals and their subsequent effect on oxidation state, gene expression, etc. will become more clear.

5. CONCLUDING REMARKS

The rapid development of new technology that facilitates the analysis of DNA damage, signaling systems, and cellular processes, such as flow cytometry and fluorescence labeling techniques, imaging techniques, and the development of DNA microarrays for expression analyses, will accelerate the development of heavy metal and other toxicological markers, some of which may be incorporated into direct monitoring of heavy metal–induced human and environmental toxicity. As our understanding of the mechanisms of toxicity continues to increase, more apparent markers and means of measurement will become available. As suggested by Mueller et al. (4), examples of future biomarkers might include growth factors, transcription factors and protooncogenes, cytokines, lipid mediators, extracellular matrix components such as collagen, glycoproteins, and proteoglycans, and cell adhesion molecules.

Metals have the ability to induce gene transcription of detoxifying proteins (metallothioneins and glutathione), protective proteins (chaperones), and proteins involved in cell cycle and proliferation and apoptosis. They have the potential to interfere with DNA synthesis and repair, the activities of Zn-containing proteins, the correct folding of protein molecules and the elimination of incorrectly folded molecules, Ca^{2+} signaling, protein kinase signaling pathways, and inflammatory responses. These effects have broad-reaching implications for cellular functions. The understanding of these mechanisms will enable us to more carefully determine the early physiological effects of heavy metals in our environment and how they can be more carefully monitored to protect human and environmental health.

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4

Radionuclides in the Environment

David M. Taylor
Cardiff University, Cardiff, Wales

1. INTRODUCTION

A broad spectrum of radionuclides was produced following the creation of the cosmos and those whose radioactive half-lives are long compared to the age of the earth remain as ubiquitous components of today's environment. These primeval radionuclides include those of the uranium and thorium series, and their daughter products, and ^{40}K (1). Another radioelement, plutonium, was formed in large quantities in early supernova explosions, but because of the relatively short radioactive half-lives of its principal isotopes, it is virtually extinct today; however, some natural ^{239}Pu is present in the earth's crust due to continuous production by spontaneous neutron capture in ^{238}U (2,3). In addition, largely as a result of the development of nuclear weapons and nuclear technology, a number of artificial radionuclides, especially ^{134,137}Cs, ^{90}Sr, and ^{239}Pu, have been released to become part of the human environment. This chapter discusses the concentrations of the primeval radionuclides, especially those of the actinide elements and their radioactive daughter products, and the nature of the radioactive environment in which life developed on earth. The present distribution and concentrations of both natural and manmade radionuclides in the earth's crust and the processes underlying their transfer to plants animals and human beings are considered. The concentrations of radionuclides that occur in human tissues are considered and discussed

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in terms of their possible long-term effects on human health. Although the emphasis is on radionuclides of heavy metals, it is also necessary to consider other radioelements, metallic and nonmetallic, particularly those that are members of the uranium and thorium decay chains, or are components of the fallout from nuclear weapon testing.

2. RADIONUCLIDES IN THE ENVIRONMENT

Table 1 lists the known primeval radionuclides, together with their radioactive half-lives and estimates of their present concentrations in the earth's crust and of their residual global radioactivity. Only two of the 17 elements listed in Table 1, ^{40}K and ^{82}Se, are known, or suspected, to be biologically essential. The alkali metal potassium is, of course, an essential component of the human body and of all other living organisms. The normal human body contains ~140 g of potassium (4); of this only ~17 mg (~480 mBq) is present as ^{40}K but this is sufficient to deliver a radiation dose of ~150 μSv a^{-1} to the average person, about half the total annual dose from natural radionuclides incorporated into the body tissues (5). Since the alkali metals ^{40}K and ^{87}Rb, together with ^{82}Se and ^{128,130}Te, cannot

TABLE 1 Concentrations and Residual Global Radioactivity of the Primeval Radionuclides in the Earth's Crust (1,6)

Radionuclide	Z	Half-life (a)	Principal radiation	Isotopic abundance (%)	Elemental concentration		Residual global radioactivity (Bq)
					g/kg	Bq/kg	
^{40}K	19	1.2E+09	β^-	0.01167	2.1E+01	6.9E−02	1.6E+21
^{82}Se	34	1.4E+20	β^-	9.2	5E−05	5.1E−13	1.2E+10
^{87}Rb	37	4.9E+10	β^-	27.83	9.0E−02	2.2E+01	5.2E+23
^{113}Cd	48	9E+15	β^-	12.2	1.5E−04	2.9E−08	6.9E+14
^{115}In	49	5.1E+14	β^-	95.7	2.5E−04	5.3E−05	1.2E+18
^{128}Te	52	1.5E+24	β^-	31.7	1E−06	7.4E−18	1.5E+08
^{130}Te	52	2E+21	β^-	34.5	1E−06	6.9E−15	1.8E+05
^{138}La	57	1.1E+11	β^-	0.089	3.9E−02	3.5E−05	8.2E+17
^{144}Nd	60	2.1E+15	α	23.8	4.1E−02	9.8E−05	2.3E+18
^{147}Sm	62	1.1E+11	α	15.1	7.0E−03	1.3E−01	3.1E+21
^{148}Sm	62	8E+15	α	11.3	7.0E−03	9.6E−07	2.3E+16
^{152}Gd	64	1.1E+14	α	0.21	6.2E−03	2.1E−08	4.9E+14
^{176}Lu	71	3.6E+10	α	2.61	8E−04	1.1E−04	2.7E+18
^{174}Hf	72	2.0E+15	α	0.16	3E−03	2.9E−10	6.8E+12
^{187}Re	75	4E+10	β^-	62.60	7E−07	4.8E−04	1.1E+19
^{190}Pt	78	6E+11	α	0.013	5E−06	9.7E−10	2.3E+13
^{232}Th	90	1.5E+10	α	100	9.6E−03	3.9E+01	9.2E+23
^{235}U	92	7.0E+08	α	0.720	2.7E−03	1.1E−02	2.6E+20
^{238}U	92	4.5E+09	α	99.27	2.7E−03	3.3E+01	7.8E+23
^{239}Pu	94	2.4E+04	α	100	2.4E−14	4.6E−08	1.1E+15
^{244}Pu	94	8.3E+7	α		3E−25	2E−22	5E+00

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be classified as heavy metals, these primeval radionuclides will not be discussed further in this chapter.

All the primeval radionuclides are ubiquitous components of the earth's crust, oceans, and other natural waters. Table 1 shows that, except for ^{235}U, ^{238}U, ^{239}Pu, and ^{244}Pu, their radioactive half-lives are so long compared to the age of the earth, ~4.5E + 09 a, that their concentrations will have remained virtually unchanged throughout the evolution of life on the planet. Because of the presence of these primeval radionuclides in the earth's crust and oceans all forms of life evolved in an environment of ionizing radiation. Adding up the figures in the last column indicates that the global residual radioactivity from the primeval radionuclides in the earth's crust amounts to ~2 million EBq (~2.10^{24} Bq); this is an enormous amount of radioactivity, many orders of magnitude greater than the manmade radioactivity produced since the beginning of the nuclear age in the 1940s.

Varying fractions of the primeval radionuclides enter the atmosphere in the form of fine dust particles or aerosols that may be deposited directly on growing vegetation or be inhaled directly by humans and other animals. Transfer within the biosphere depends on many factors, chemical, biochemical, and physical, and an important question is how large are the quantities of these natural radionuclides that enter the human food chain and are incorporated into the human body? Environmental radionuclides can enter the human body by two routes, inhalation of respirable dust particles or aerosols, and through food and water. The relative importance of these two uptake routes will vary with the element, but for radioelements such as thorium and plutonium, whose absorption from the human gastrointestinal tract is very low, inhalation may in fact become the major entry pathway. This will be discussed later as the specific elements are discussed.

Figure 1 shows the remaining primeval radionuclides with their position in the periodic table. It can be seen that 10 of the total of 21 radionuclides are members of the lanthanide and actinide series of elements whose geo- and bioinorganic chemistry exhibits a number of similarities. The information on the occurrence of each of the radionuclides in the environment and in humans will now be reviewed.

3. THE BIOINORGANIC CHEMISTRY OF THE RESIDUAL PRIMEVAL RADIONUCLIDES

3.1 Cadmium

Cadmium is the 64th most abundant element in the earth's crust (6). Cadmium minerals are rare and the element occurs by isomorphous displacement in almost all zinc ores, the most common of which is sphalerite, (ZnFe)S (7). The predominant oxidation state is Cd(II) and this is the oxidation state to be expected in all environmental situations. The cadmium concentration in the earth's crust is ~150

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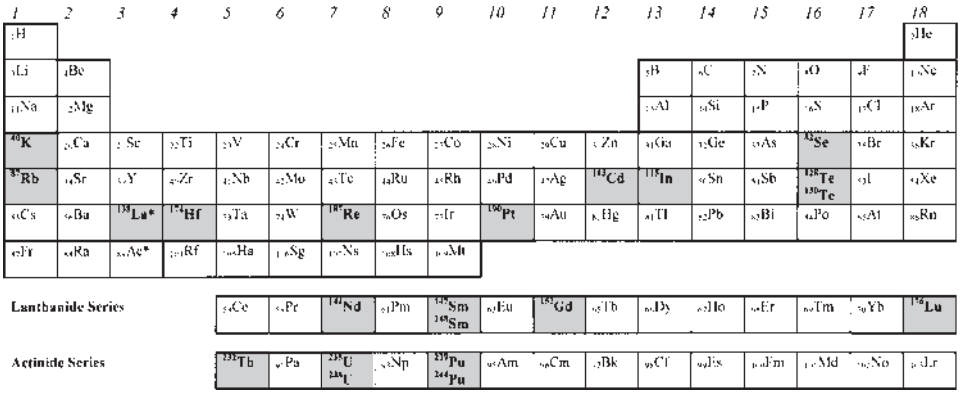

FIGURE 1 The periodic table of the elements indicating the remaining primeval radioelements.

μg kg^{-1} (6) and that in seawater is ~3 orders of magnitude lower at ~110 ng dm^{-3}. The fraction of the radioactive isotope ^{113}Cd in the total cadmium is 12.2% (Table 1). The zinc concentrations in both the earth's crust and the oceans are about 100-fold greater than those of cadmium, and a similar Zn/Cd ratio is also found in biological materials, including human and animal tissues. Cadmium is taken up readily from the soil and water by many plants, and in edible fungi such as mushrooms levels may reach mg kg^{-1} fresh weight. The daily intake of cadmium in the human diet and drinking water is ~150 μg d^{-1} (8); of this ~5% may be expected to be absorbed from the gastrointestinal tract (8,9). Cadmium in tobacco leaves contributes to increased levels of the metal in the bodies of smokers. Because cadmium is a potentially highly toxic metal, its levels in human tissues have been widely studied (8–10). The whole-body content of cadmium ranges from ~30 to 50 mg, of which ~15%, 35%, and 35%, respectively, are located in the liver, kidneys, and skeleton. The whole-body content of ^{113}Cd is calculated to be ~50–80 μBq; this means that on average 1 atom will disintegrate somewhere in the human body about every 4 h, thereby releasing a β^- particle with an energy of 91 keV. This amount of energy, when deposited in the human body, will deliver a lifetime radiation dose, a committed effective dose (CED) (9), of ~10 pSv, or about 9 orders of magnitude less than that from the primeval ^{40}K.

3.2 Indium

Indium, with a concentration of ~250 μg kg^{-1} in the earth's crust, has a slightly greater abundance than that of cadmium (6). Indium is assigned, together with

aluminum and gallium, to Group 13 of the periodic table (Fig. 1), and in common with these latter metals the predominant oxidation state is In(III) (7). In the earth's crust traces of indium, $\ll 1\%$, occur in aluminum and zinc ores. In contrast to cadmium, indium has few industrial or medical applications and, in consequence, it has attracted little environmental or toxicological interest and its concentrations in natural waters, or in plant, animal, or human tissues have been little studied. Consequently there is virtually no direct information on which an assessment of the indium content of the human body can be made. Experimental studies in animals suggest that the absorption of indium from the gastrointestinal tract is about 2% (9). Since, like aluminum, indium occurs in the earth's crust in silicates, such as micas and feldspars, and in minerals like bauxite (a hydroxo oxide) and cryolite ($NaAlF_6$), which are not very soluble, its transfer from the soil into the food chain and thence into the human body is likely to be very low. A rough assessment of the indium content of the human body can be made from the aluminum content and the relative abundance of the two elements in the earth's crust. The aluminum content of the human body is ~60–100 mg, or a concentration of ~1.2 mg kg^{-1} (4,10); the aluminum content of the earth's crust is 82.3 g kg^{-1} (6), suggesting a concentration factor (CF) of ~7E−04. Assuming that this factor would also apply to the intake of indium and allowing for a fivefold lower absorption from the gastrointestinal tract, its concentration in the human body might be ~100 ng. Studies with ^{111}In in animals and humans show that ~30% of the nuclide deposits in bone and ~20% in liver (11). In the blood plasma, indium is transported on the iron-transport protein transferrin, to which it binds very strongly (12). Assuming that the human body contains 100 ng indium, the radioactivity of the ^{115}In would be ~20 nBq. These estimated body contents of both total indium and ^{115}In must be recognized as having large uncertainties and it would be wise to assume that the actual levels that might be measured in individual members of the population would lie in the range 10–1000 ng (2–1000 nBq). The presence of 20 nBq of primeval ^{115}In in the human body would correspond to the decay of 1 atom, with the emission of a β^- particle of 153 keV every 250 days, or a lifetime CED of ~5 nSv.

3.3 Hafnium

The chemistry of hafnium is almost identical to that of its companion Group 4 element zirconium; thus hafnium, as Hf(IV), occurs in all zirconium minerals (7). These minerals are widely distributed in the earth's crust and are not concentrated into major deposits (7). The average concentration of hafnium in the earth's crust has been estimated to be 3.0 mg kg^{-1} (6), making it of comparable abundance to uranium and many of the lanthanide elements; in contrast zirconium is present at 165 mg kg^{-1}. The microchemical analysis of hafnium is difficult and this difficulty is reflected by the paucity of information on its concentrations in

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natural waters or in plant, animal, or human tissues. The daily intake of zirconium in the human diet and drinking water is estimated to be 4.2 mg d^{-1} (8); thus, on the basis of their relative abundances, that of hafnium might be ~0.1 mg d^{-1}. Experimental studies in animals indicate that the absorption of hafnium from the gastrointestinal tract is very low, ~0.05% (9), and that the major sites of deposition are the skeleton (~25%) and liver (~5%) (13,14). Like indium, Hf(IV) is also associated with transferrin in the blood plasma (12). The zirconium content of the human body has been estimated to be 420 mg (8); this implies a concentration factor of ~4E–02; thus by simple analogy based on the close chemical similarities between hafnium and zirconium, the body content of hafnium might be of the order of 100 μg. A body content of 100 μg hafnium would correspond to ~10 pBq of ^{174}Hf. These estimated body contents of both total hafnium and ^{174}Hf must be recognized as having large uncertainties and it would be wise to assume that the actual levels that might be measured in individual members of the population would lie in the range 1–1000 μg (1–100 pBq ^{174}Hf). A body content of 10 pBq ^{174}Hf would result in less than 1 α-particle of 2.5 MeV being emitted in a human lifetime.

3.4 Rhenium

Rhenium lies in Group 7 of the periodic table, together with manganese and technetium (Fig. 1). The abundance of rhenium in the earth's crust is ~700 μg kg^{-1} (6). The element appears together with molybdenum in various ores as the sulphide ReS_2 or as the oxide Re_2O_7. Rhenium can exist in various oxidation states between −1 and +7 and the Re(IV) and Re(VII) states are probably the most important from the environmental point of view (7). In seawater the element believed to be present in very low concentrations is the perrhenate ion, ReO_4^-. Rhenium is produced and purified industrially for use as an oxidation catalyst, or as filaments and coatings in electronic and electrical equipment. However, the rarity and the high cost of the pure metal combine to prevent widespread environmental contamination or toxicological concern; thus there is little or no information on the concentrations of rhenium in vegetation or in animal and human tissues. Recent interest in the use of ^{188}Re for the treatment of cancer has prompted some studies of the biodistribution of this radionuclide in experimental animals (15), but these cannot yield any information on the normal concentrations of the element in the tissues or whole body. Radionuclide studies with [^{188}Re]-ReO_4^- in animals indicate that there is virtually complete absorption from the gastrointestinal tract and that of the absorbed radionuclide; ~30% is deposited in the liver, 4% in the thyroid, and 1% in the stomach wall; the remainder is assumed to divide equally among all other tissues (11).

The whole-body content of rhenium has not been measured; assuming a fairly conservative CF of 1E–04, it could be predicted that the rhenium content

of the whole body might be of the order of 100 pg, of which ~20 pg might be in the liver. This latter value would correspond to the presence of ~50 nBq of primeval ^{187}Re in the human body and to the emission of a single 0.66-keV β-particle about every one and a half years.

3.5 Platinum

Platinum, like rhenium, is a rare element with a concentration of only ~5 μg kg^{-1} in the earth's crust (6). The metal has no known essential physiological role, although in recent years *cis*-diaminodichloro-platinum and other platinum complexes have become first-line drugs in the treatment of certain types of cancer. Studies with radioactive *cis*-diaminodichloro-platinum indicate that about 10% of the radionuclide deposits in the liver and a further 10% in the kidney, the remainder being more or less equally distributed in the other tissues (16). No information on the natural concentrations of platinum in biological materials, including human tissues, appears to be available; however, it seems unlikely that the tissue concentrations will be markedly different from those of gold, which has a similar abundance in the earth's crust (6). Gold concentrations in human liver, lungs, and skeleton have been measured (17,18) and these indicate a total body content of ~1–30 μg. A whole-body platinum content of 30 μg would include ~60 pBq ^{190}Pt; this would correspond to the emission of less than 1 α-particle in a human lifetime.

3.6 The Primeval Lanthanides

The primeval radionuclides ^{138}La, ^{144}Nd, ^{147}Sm, ^{148}Sm, ^{152}Gd, and ^{176}Lu (Fig. 1) are members of the lanthanide series of elements. The natural abundance of these elements in the earth's crust ranges from ~40 mg kg^{-1} for lanthanum and neodymium to 0.8 mg kg^{-1} for lutetium; concentrations in seawater are 6 or 7 orders of magnitude lower than those in the earth's crust (6). Although the lanthanides have no known essential or potentially beneficial biological function, they are of biochemical and medical interest and their biodistribution and biokinetic behavior in animals and plants has been quite widely studied (19). The analysis of lanthanides at levels of <1 μg kg^{-1} is very difficult, and even with the best modern analytical methods, such as ICP-MS, ICP-AES, or neutron activation analysis, the published results show very large standard deviations, and the data are not always consistent, either from sample to sample or from element to element (19). In human organs there is also evidence that diseases such as cancer, cirrhosis of the liver, and myocardial infarction may increase lanthanide levels in some tissues (19).

Radionuclide studies in experimental animals indicate that the liver and skeleton are the major sites of deposition, accounting for 80% of the lanthanide that enters the systemic circulation (20,21); Durbin (20) has pointed out that liver

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deposition appears to decrease approximately linearly with increasing atomic radius of the lanthanide, while the skeletal content increases. The available data are far from complete and present only a general picture of the behavior of lanthanide elements in plants and tissues.

There are no comprehensive reports of measurements of lanthanides in food crops or animals and human tissues. The principal uptake route into plants and animals is by leaching of lanthanides from minerals into the groundwater, and also by the formation of respirable aerosols. Measurement of lanthanide concentrations in crops taken from a high background region of Brazil indicated levels ranging from <1 to ~ 700 μg kg^{-1} in vegetables (19). Comparing the lanthanide concentration in foodstuffs with those in the earth's crust led Evans to suggest a concentration ratio for lanthanides ranging from 1E–03 to 1E–05 (19). Since the fractional absorption of lanthanides from the human gastrointestinal tract appears to be ~5E–04 (7), the overall concentration ratio for humans might be expected to lie in the range 1E–07 to 1E–09.

If this assumption were true, the lanthanide concentrations in human tissues would be expected to lie in the ng-pg range. However, the sparse measurements of human tissues suggest higher concentrations; measurements of lanthanide concentrations in human spleen ranged from ~3 to ~900 μg La kg^{-1} fresh weight to 0–40 μg kg^{-1} for Sm (19). Neutron activation analysis of nonexposed human lung revealed mean values of 16.6, 46.2, 2.5, and 0.46 μg kg^{-1} fresh weight for La, Nd, Sm, and Lu, respectively (19). Lanthanum concentrations of 4.5 and 5.5 μg kg^{-1}, respectively, were reported in the lungs and liver of deceased smelter workers (19). Hamilton et al. (23), using mass spectrometry, reported lanthanum concentrations of 80 and 10 μg kg^{-1}, respectively, in liver and lung. McAughey (24), using ICP-AES, found that the daily urinary excretion of La, Sm, Gd, and Nd lay in the range 0 to ~150 ng d^{-1}. These liver and urinary values would be consistent with a total body content of ~200–1000 μg. However, even assuming a body content of 1 mg for each of the lanthanides of interest, the radioactivity would correspond to 0.5 μBq ^{138}La, 2.8 μBq ^{144}Nd, 19 mBq ^{147}Sm, 0.1 μBq ^{148}Sm, 3.4 μBq ^{152}Gd, and 143 μBq ^{176}Lu; in no case would this result in a CED > 1 μSv.

4. THE BIOINORGANIC CHEMISTRY OF THE PRIMEVAL ACTINIDES

4.1 Thorium and Uranium

After ^{40}K, the primeval actinides and their daughter products are the largest source of the natural radioactivity of mankind and the human environment. Of all the primeval actinides, ^{232}Th is the most abundant with an average concentration of 9.6 mg (39 Bq) kg^{-1} in the earth's crust (6). However, concentrations may vary from region to region and a realistic range might be $<0.5–>20$ mg

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kg^{-1}. Concentrations in seawater, at ~1 ng kg^{-1}, are, however, about 7 orders of magnitude lower, reflecting both the poor solubility of Th(IV), the predominant oxidation state, and its lower concentration in the mafic rocks of the ocean crust. The concentration of ^{238}U, the longest-lived uranium isotope, in the earth's crust is 2.7 mg kg^{-1} (6), about 4 times lower than that of ^{232}Th; however, the radioactivity in the earth's crust due to ^{235}U is 33 mBq kg^{-1}, only slightly less than that of ^{232}Th. The concentration of ^{238}U in seawater is 3.2 μg kg^{-1}, some 3000 times greater than that of thorium, largely reflecting the greater solubility of uranium minerals as compared to those of thorium. The second primeval isotope of uranium, ^{235}U ($T_{1/2}$ $7.038.10^4$ a), has an isotopic abundance of only 0.72%, but its radioactivity is 11 mBq kg^{-1} in the earth's crust.

Thorium-232 and ^{238}U, as well as most of their daughter products, emit α-particles, which, if they are emitted within the human or animal body, may be highly radiotoxic (5). There has, therefore, been considerable interest in the concentrations of the isotopes of the thorium and uranium decay series that are present in the human diet and in the bodies of humans and animals.

4.1.1 The Radioactive Decay of ^{232}Th and ^{238}U

Thorium-232 decays by α-particle emission to ^{228}Ra ($T_{1/2}$ 5.76 a) and thence to ^{228}Th ($T_{1/2}$ 1.913 a), ^{228}Ra ($T_{1/2}$ 6.7 a), ^{224}Ra ($T_{1/2}$ 3.64 d), ^{220}Rn (thoron) ($T_{1/2}$ 54.5 s), and, finally, through further emission of α-particles, to stable ^{208}Pb (Fig. 2). All the daughters of ^{232}Th have physical half-lives of <6 a; thus, even geologically young thorium-containing minerals and rocks will contain the whole radioactive series in equilibrium (1). Primeval ^{238}U also decays by α-particle emission to ^{234}Th ($T_{1/2}$ 24 days) and thence by β-particle emission to ^{234}Pa ($T_{1/2}$ 1.1 min) and through successive α-particle decays to ^{234}U, ^{230}Th, and ^{226}Ra to stable ^{210}Pb. Uranium-235 decays by α-particle emission to ^{231}Pa ($T_{1/2}$ 3.43 10^4 a) and thence by emission of a β-particle to ^{231}Th ($T_{1/2}$ 25.6 h) and through further α-particle emissions to stable ^{207}Pb. Thus the radiochemistry of both ^{235}U and ^{238}U also involves that of thorium.

There are two important daughter products of ^{226}Ra and ^{228}Ra, the gaseous radionuclides ^{222}Rn and ^{220}Rn, which diffuse out of the minerals into groundwater and to the atmosphere and add radioactivity to each through both themselves and their radioactive daughters (3). Since both ^{226}Ra, ^{228}Ra, ^{222}Rn, and ^{220}Rn are highly radiotoxic nuclides, capable of causing cancers of lung and bone, their behavior in the environment and in humans is considered below, even though they are not heavy metals.

4.1.2 Thorium and Uranium Isotopes in the Human Food Chain

Thorium-232, ^{238}U, and their decay products are present in at least trace concentrations in virtually all terrestrial and marine biota, and their concentrations in various types of foodstuff and drinking waters have been quite widely studied.

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$$^{232}\mathrm{Th} - \alpha,\ 1.39.10^{10}\ \mathrm{a} \rightarrow {}^{228}\mathrm{Ac} - \beta,\ 6.13\ \mathrm{h} \rightarrow {}^{228}\mathrm{Ra} - \alpha,\ 6.7\ \mathrm{a} \rightarrow$$

$$^{224}\mathrm{Ra} - \alpha,\ 3.64\ \mathrm{d} \rightarrow {}^{220}\mathrm{Rn}^{*} - \alpha,\ 54.5\ \mathrm{s} \rightarrow {}^{216}\mathrm{At} - \alpha,\ 3.10^{-4}\ \mathrm{s} \rightarrow$$

$$^{216}\mathrm{Po} - \alpha, \beta, .0.158\ \mathrm{s} \rightarrow {}^{212}\mathrm{Po} - \alpha,\ 3.0\ 10^{-7}\ \mathrm{s} \rightarrow {}^{212}\mathrm{Bi} - \alpha, \beta\ 60.5\ \mathrm{min} \rightarrow$$

$$^{212}\mathrm{Pb} - \alpha,\beta,\ 10.6\ \mathrm{h.} \rightarrow {}^{208}\mathrm{Pb}\ (\mathrm{stable})$$

$$^{238}\mathrm{U} - \alpha,\ 4.5.10^{9}\ \mathrm{a} \rightarrow {}^{234}\mathrm{Th} - \beta,\ 24\ \mathrm{d} \rightarrow {}^{234}\mathrm{Pa} - \beta,\ 1.1\ \mathrm{min} \rightarrow$$

$$^{234}\mathrm{U} - \alpha,\ 2.5.10^{5}\ \mathrm{a} \rightarrow {}^{230}\mathrm{Th} - \alpha,\ 8.10^{4}\ \mathrm{a} \rightarrow {}^{226}\mathrm{Ra} - \alpha,\ 1622\ \mathrm{a} \rightarrow$$

$$^{222}\mathrm{Rn} - \alpha,\ 3.824\ \mathrm{d} \rightarrow {}^{218}\mathrm{Po} - \alpha,\ 3.04\ \mathrm{min} \rightarrow {}^{214}\mathrm{Pb} - \beta,\gamma\ 26.8\ \mathrm{min} \rightarrow$$

$$^{214}\mathrm{Bi} - \beta,\gamma,\ 19.7\ \mathrm{min.} \rightarrow {}^{214}\mathrm{Po} - \alpha,\ 163.7\ \mu\mathrm{sec} \rightarrow {}^{210}\mathrm{Pb}\ (\mathrm{stable})$$

FIGURE 2 The radioactive decay of ^{232}Th and ^{238}U.

Table 2 lists some illustrative, and rounded, values for the concentrations of ^{230}Th, ^{232}Th, ^{234}U, ^{235}U, ^{238}U, and ^{226}Ra in some of the most important foodstuffs. These values are derived from the studies of Fisenne et al. (25), Shiraishi et al. (26) and Yu and Mao (27) in the New York City, Ukrainian, and Japanese diets; the values are also comparable with those of other studies (25–30). The highest concentrations listed in Table 2 are for shellfish. There are, however, variations that may reflect regional differences; for example, Yu and Mao (27) reported that in six varieties of fish obtained from the Hong Kong fish market the concentrations of ^{232}Th and ^{238}U were below the detection limits. Pronounced regional differences in the ^{238}U concentrations in drinking water between New York City, Salt Lake City, Utah, and Hong Kong are evident from Table 2.

Comparison of the estimated daily dietary intakes of thorium and uranium in various countries across the Northern Hemisphere indicates that average intake may range from ~2 to 10 μBq (0.5–2.5 μg) for ^{232}Th and from ~7 to 60 μBq (~0.5–5 mg) for ^{238}U. In thorium and uranium mineral-rich regions, intakes may

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TABLE 2 Illustrative Values for the Concentrations of Primeval Actinides and Their Decay Products in Some Foodstuffs

	mBq kg^{-1}					
Foodstuff	^{226}Ra	^{230}Th	^{232}Th	^{234}U	^{235}U	^{238}U
Dairy products	6	0.4	0.3	1	0.05	0.7
Fresh vegetables	60	20	18	23	1	25
Root vegetables	15	1	1	12	0.1	8
Fresh fruit	50	0.1	0.1	2		2
Meat	2	3	2	2	0.02	2
Fish	30	1	1	20	0.4	15
Shellfish	60	30	30	2200	90	1900
Bread and grain products	100	10	3	30	1	23
Drinking water, NYC	0.4	0.1	0.05	1	0.03	0.9
Drinking water, SLC						18
Drinking water, HK	4		7			79

NYC = New York City; SLC = Salt Lake City; HK = Hong Kong. The values are derived from refs. 25–30. The coefficients of variation on the reported values range from ~4 to >40%, but a realistic concentration range probably lies between 0.1 and 10 times the values shown.

be orders of magnitude higher (28–30). Table 3, which is recalculated from the data of Fisenne et al. (25) and Yu and Mao (27), compares the fractions of the daily intakes of ^{230,232}Th, ^{235,238}U, and ^{226}Ra due to diet, drinking water, and inhalation for New York City and Hong Kong residents.

The data in Table 3 indicate that for New York City, ~98% of the daily intake of ^{226}Ra and ^{230,232}Th was derived from the diet, 1–2% from the drinking water, and <0.15% by inhalation; the corresponding figures for ^{234,235,238}U were ~92% from the diet, ~8% from drinking water, and ~0.1% by inhalation. However, the thorium and uranium concentrations in New York City drinking water are low and the data of Yu and Mao (27) indicate that in Hong Kong, where the drinking water concentration of uranium is 80 times greater, ~22% of the daily intake of ^{226}Ra and ~40% of the ^{238}U are derived from drinking water.

4.1.3 Thorium and Uranium in the Human Body

Wrenn et al. (31,32) have provided the most comprehensive set of data on thorium isotopes in human tissues taken at autopsy from cases of sudden accidental death. Some further data are given for the concentrations of ^{232}Th, ^{230}Th, and ^{228}Th in the lungs and bones of persons living in high and normal radiation background regions of China (33). Figure 3 shows the whole-body contents of ^{228}Th, ^{230}Th, and ^{232}Th (Fig. 3a), and for total thorium (Fig. 3b), calculated from these data.

TABLE 3 Average Daily Intake of ^{230,232}Th, ^{234,235,238}U, and ^{226}Ra by Ingestion in Food and Water and by Inhalation in the United States and China (31,33)

	mBq Person^{-1} d^{-1}					
	^{226}Ra	^{230}Th	^{232}Th	^{234}U	^{235}U	^{238}U
New York City						
Food	51.2	6.06	4.07	16.8	0.7	14.7
Water	0.6	0.18	0.07	1.5	0.05	1.2
Air	0.01	0.01	0.01	0.02	0.0007	0.02
Total	51.81	6.25	4.15	18.3	0.751	15.9
Hong Kong						
Food	7.8					43
Water	2.2					26
Air	—					—
Total	10.0					69

There were no clear differences in the body content of residents of the mining area of Grand Junction and urban Washington, DC, and the calculated body contents are about an order of magnitude lower than those for residents of Beijing. However, as can be seen from Figure 3a, the body contents of all the thorium radionuclides in the residents of the high natural radiation background areas of China are 10–100 times larger than those observed in Beijing or the United States. The concentrations of thorium and uranium in the surface soil of the high background areas are 60.4 $\pm$ 28.6 and 7.7 $\pm$ 1.7 mg kg^{-1}, respectively, compared to 7.9 $\pm$ 3.2 mg Th and 1.7 $\pm$ 0.7 mg U kg^{-1} in the control area (33). Figure 3a also shows that, for each location, the radionuclides ^{232}Th, ^{230}Th, and ^{228}Th contribute broadly similar numbers of mBq to the total-body radioactivity; however, owing to their much higher specific activities, the contribution of ^{230}Th and ^{228}Th to the total mass of thorium in the body is less than 1 ng. There are differences in the ratios of ^{232}Th, ^{230}Th, and ^{228}Th at the different locations, and these may reflect past or present mining and other civilization-related activities. Wrenn et al. (31) suggest that the ^{230}Th and ^{232}Th in the human body is derived largely by inhalation of suspended particulates, while the ^{228}Th arises from ingestion in the diet and by ''ingrowth'' from the decay of ^{228}Ra. The presence of ~100 mBq ^{232}Th in the human body would result in the emission of ~9000 α-particles d^{-1}.

Within the body, thorium exists as Th(IV); about 60% deposits in bone, partly in the hydroxyapatite matrix, but predominantly on bone surfaces within α-particle range of radiosensitive cells, which could give rise to radiation-induced bone cancer (34–36). The liver contains ~4% of the body thorium, mainly depos-

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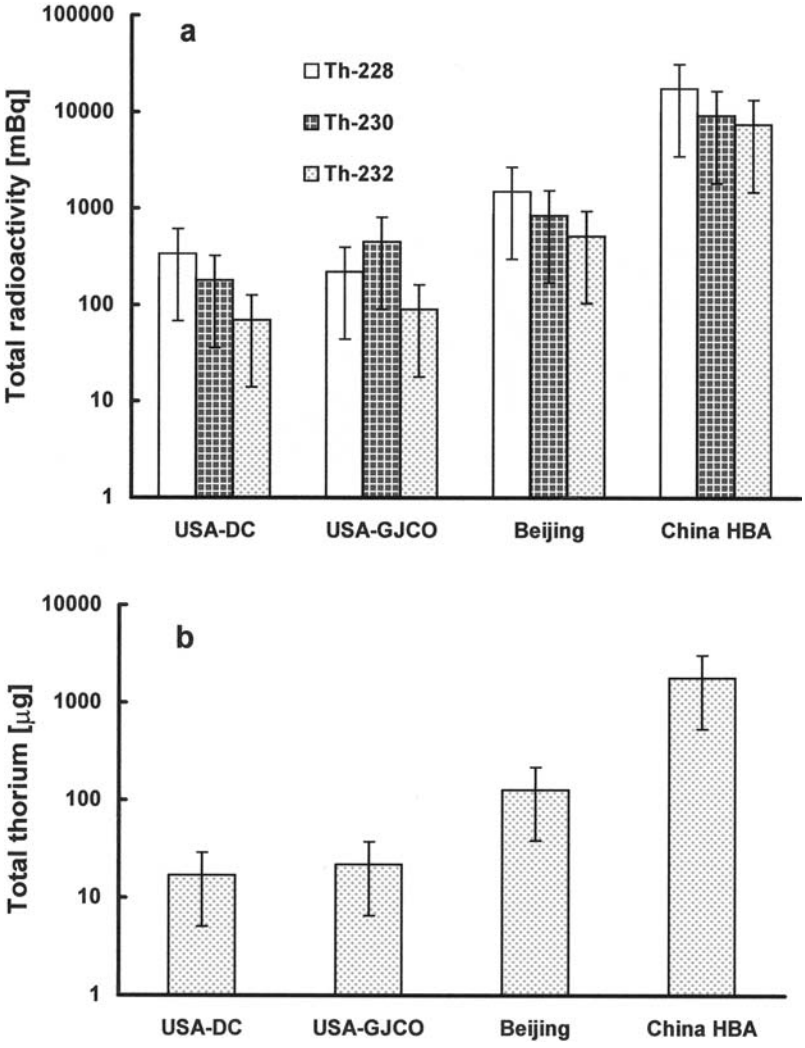

FIGURE 3 The total-body content of thorium in the human body in different regions of the world. (a) The mean body contents, measured in mBq, in former residents of Washington, DC (USA-DC), Grand Junction, Colorado (USA-GJCO) (31,32), Beijing, China, and the high background radiation areas of the Guangdong Province of China (33). (b) The same data for the total mass of thorium, which is essentially all contributed by ^{232}Th.

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ited in lysosomal structures, frequently in association with the iron storage protein ferritin (35,36). In the blood plasma thorium appears to be transported on the iron-transport protein transferrin (12,37).

Review of the information on the uranium content of the human body (38) indicates a total-body content of ~20 μg; this is illustrated in Figure 4, which also shows similar data for plutonium and radium. The total uranium content of the body is similar to that of thorium (Figs. 3 and 4). Since the natural abundance of thorium in the earth's crust is about four times greater than that of uranium (Table 1), the similarity in the total body content of the two elements probably reflects the greater mobility of uranium. Limited data from seven countries across the world indicate that the total uranium content of the human skeleton, the organ in which ~95% of the body content is located (8), may range from <1 to ~770 μg (31). A total-body content of 20 μg ^{238}U would correspond to a radioactivity of ~250 mBq, or the emission of ~20000 α-particles d^{-1}.

The uranium in the body is most probably in the hexavalent form, $[UO_2]^{2+}$,

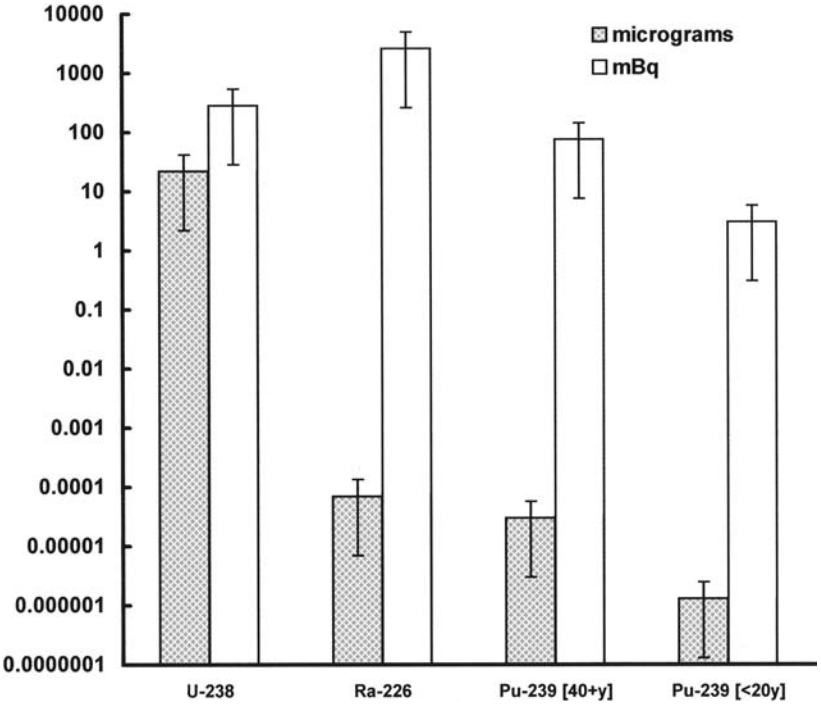

FIGURE 4 The estimated total-body contents of uranium, ^{226}Ra, and plutonium in the adult human body (2,38,40); the data are given as both mBq and μg.

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which in bone exchanges with Ca^{2+} ions on the surface of the hydroxyapatite crystals of the bone mineral (34). The uranium in bone is fairly rapidly lost to the plasma, with a half-life of ~150 days (34). In the blood plasma uranium has also been shown to be associated with transferrin (12,37).

4.2 Plutonium

Both ^{244}Pu ($T_{1/2}$ 8.3. 10^7 a) and ^{239}Pu ($T_{1/2}$ 2.4.10^4 a) were primeval radionuclides, but because of their short half-lives on a cosmic scale only minute traces of ^{244}Pu survive today. The present-day abundance of ^{244}Pu in the earth's crust has been estimated to range from $\sim 7.10^{-24}$ to $\sim 3.10^{-22}$ g kg^{-1} (34). Assuming that all this plutonium was primeval, and that the earth's crust has a mass of $2.367.10^{22}$ kg (6), the total residual cosmogenic ^{244}Pu today might range from ~0.2 g to ~7 g (2,40). However, there has been continuous, low-level production of ^{239}Pu from ^{238}U by spontaneous fission since the formation of the earth, according to the reaction:

$$^{238}U(n,\gamma)^{239}U \xrightarrow[23\ \text{min}]{\beta} {}^{239}Np \xrightarrow[2.3\ \text{days}]{\beta} {}^{239}Pu$$

Assuming secular equilibrium and a $^{239}Pu/^{238}U$ ratio of $(1.5 \pm 0.2) \times 10^{-11}$, the rate of formation of ^{239}Pu corresponds to a total annual production of ~28 kg in the entire earth's crust (40,41).

On the basis of an average uranium concentration of $2.7.10^{-3}$ g kg^{-1} in the earth's crust (6), the average ^{239}Pu concentration would be ~40 fg kg^{-1}, and this is, presumably, the plutonium concentration that has been present throughout the evolution of life. This is a very low concentration compared with that of thorium, ~9.6 mg kg^{-1} Th kg^{-1} (6), and since the chemistry of tetravalent plutonium, its most stable oxidation state, and tetravalent thorium resemble each other very closely, the geochemistry of the natural ^{244}Pu may well have followed that of thorium, rather than pursuing its own specific chemistry.

Since the birth of the nuclear age in 1945, some 6 tons of ^{239}Pu have been released into the earth's atmosphere, predominantly by the atmospheric nuclear weapons testing carried out in the 1950s and 1960s (42). The fallout plutonium from nuclear weapons testing was distributed unevenly between the Northern and Southern Hemispheres, with the deposition in the Northern Hemisphere being more than 3 times greater than that in the Southern Hemisphere (43). The concentrations of fallout $^{239,240}Pu$ in the upper layers of the earth's crust in 1970–71 were 3 ng kg^{-1} (~7 Bq kg^{-1}) in the Northern, and 0.6 ng kg^{-1} (~1 Bq kg^{-1}) in the Southern Hemisphere. Orders of magnitude higher levels of soil contamination with ^{239}Pu may be found in the region of nuclear test sites or nuclear-fuel-processing sites (38). This plutonium is almost certainly relatively immobile and the ratios of the amount of plutonium in the earth's crust to that incorporated into vegetation and animals, including humans, are probably $\ll 10^{-7}$.

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Like the other actinide radionuclides, environmental plutonium can enter the human body by inhalation, and through food and water. While the food chain is probably the predominant source of the natural ^{239}Pu in the human body, the major route of entry of fallout plutonium into humans and animals has been by inhalation (42).

Taylor (2), using the available published data from measurements in tissues collected at autopsy, calculated median whole-body contents of ^{239,240}Pu in former residents of various countries in the Northern Hemisphere who died between 1959 and 1976; the values ranged from 35 mBq in southern Finland to 179 mBq in Japan with a population-weighted median value of 74 mBq (~30 pg) ^{239}Pu (Fig. 4), a value at least 5 orders of magnitude greater than the calculated <500 ag base load of natural plutonium in the human body (2).

The decreasing levels of ^{239,240}Pu intake since the late 1960s, especially by inhalation, mean that persons born after the cessation of atmospheric weapons testing in about 1970 will have much lower body burdens. Calculations suggest that for persons born in 1970 the fallout ^{239,240}Pu content of the human body would be ~3 mBq (~1 pg or ~5 fmol Pu). Such levels will be very difficult to confirm by direct measurements of autopsy material, even using modern mass spectrometric methods, which offer detection limits of about 0.5 μBq per sample (46).

Large accidental releases of plutonium into the environment can cause significant local or regional increases in population intake. For example, the Chernobyl accident, which released some 61 TBq of ^{239,240}Pu into the northern European environment, may have increased the body content of the people of the Bialystok region of Poland by ~6 mBq (40,46). Compared to the median value of 74 mBq for the amount of plutonium in the bodies of persons who had lived through the whole period of fallout from weapons testing, this Chernobyl-related increase in body content is quite small; however, for the young people of this region who were born after 1970, the Chernobyl-related intake could have more than doubled their body burden (40). The 74-mBq civilization-related load of ^{239,240}Pu in the human body corresponds to the emission of ~6000 α-particles d^{-1}.

5. THE BIOINORGANIC CHEMISTRY OF RADIUM AND RADON

5.1 Radium

In undisturbed uranium and thorium ores, radioactive equilibrium is established between the parent ^{238}U or ^{232}Th and the daughter products in the decay chain. The decay chains pass through ^{226}Ra ($T_{1/2}$ 1600 a) and ^{228}Ra ($T_{1/2}$ 5.7 a), respectively, to stable ^{206}Pb or ^{208}Pb (Fig. 2), at rates corresponding to the amounts of the parent radionuclides in the ore. As long as the ore remains undisturbed, radioactive equilibrium is maintained. Radium, as a member of the alkaline earth group of metals, would be expected to exhibit chemical behavior broadly similar to that

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of calcium. The radium and radon concentrations in the earth's crust are said to average 900 pg kg^{-1} and 400 fg, respectively, concentrations in seawater being 3–4 orders of magnitude lower (6).

Tracy et al. (47) measured the uptake of ^{226}Ra into garden produce grown on soils containing 2.5–830 ng ^{226}Ra kg^{-1} and showed that the CF for soil-to-plant transfer ranged from ~800 to ~1300. Radium is present in all foodstuffs at concentrations ranging from 0.74 to 6.5 pg kg^{-1} (Tables 2 and 3); drinking water concentrations range from ~0.07 to 8 pg kg^{-1}, but drinking water accounts for only ~10% of the daily intake (8). Yu and Mao (27) measured ^{226}Ra and ^{228}Ra concentrations in a range of foodstuffs from Hong Kong and reported concentrations ranging from 0.3 to 39 mBq kg^{-1} for each radionuclide. The daily intake in food and drink was estimated to be ~27 mBq (0.7 pg) $person^{-1}$ for ^{226}Ra and ~70 mBq (~8 fg) $person^{-1}$ for ^{228}Ra. Fisenne et al. (25) measured ^{226}Ra in the diet of New York City dwellers in 1978 and reported concentrations ranging from 2 to 104 mBq kg^{-1}; the daily intake was assessed at 52 mBq (1.4 pg) d^{-1} for ^{226}Ra and 35 mBq (3.9 fg) d^{-1} for ^{228}Ra (Table 4). Estimates of daily dietary intake of ^{226}Ra in other geographical locations range from 0.7 to 3 pg d^{-1} (8). The absorption of radium from the human gastrointestinal tract is assumed to average 20% (9). Radium absorbed from the gastrointestinal tract deposits mainly in the skeleton, where like calcium it is laid down in the hydroxyapatite of the bone mineral.

Comparison of the concentrations of ^{226}Ra measured in human bone samples collected from various countries across the world indicate a range from ~80 to 800 mBq (2–22 pg) kg^{-1} fresh weight (33), with a weighted mean value of 252 mBq (6.8 pg) kg^{-1} fresh weight. Concentrations in subjects from the high radiation background areas in China were ~3 times greater than the maximum observed in other areas. In the Chinese samples the $^{226}Ra/^{228}Ra$ ratios varied between ~1 and 2 (33). On the basis of a $^{226}Ra/^{228}Ra$ ratio of 1 the total mass of ^{228}Ra in the human body would be ~30 fg kg^{-1} fresh weight.

Assuming that 95% of the environmentally derived ^{226}Ra in the body is located in the skeleton, and that worldwide the mean skeletal concentration is 7 pg kg^{-1} fresh weight, with a range of 2–22 pg kg^{-1} fresh weight, the total body mass of radium can be calculated to be ~70 pg, range 18–200 pg (Fig. 4); this is in reasonable agreement with the value of ~30 pg assumed for ICRP Reference Man (8). The calculated average total-body ^{226}Ra content of ~70 pg (2.6 Bq) corresponds to the emission of ~2.2 10^5 α-particles d^{-1}, mostly in the mineral mass of the skeleton.

5.2 Radon

As mentioned above, the ^{222}Rn and ^{220}Rn that are produced by the continuous decay of ^{238}U in the rocks and soil diffuse rapidly into the atmosphere where, together with their short-lived radioactive daughter products, they are inhaled by

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the entire human population (5,49). The levels of radon in the air vary widely according to the geological nature of the ground, being low in areas of basalt and high in areas rich in granite. Radon concentrations within buildings are generally higher than those in the outside air because of the emanation of radon from the wall and floors of the building and of the restricted ventilation in most buildings. Average indoor ^{222}Rn levels in houses vary widely between countries; for example, the level in Australia is 10 Bq m^{-3} (38,48,49) and 20 Bq m^{-3} in the United Kingdom while levels in much of western Europe and parts of the United States may range from ~80 to 180 Bq m^{-3} (38,48). Within most countries there are quite large regional or local variations in indoor radon concentrations; for example, in the United Kingdom the average concentration is ~20 Bq m^{-3}; however, persons residing in areas rich in granite, such as Cornwall, may be exposed to concentrations 5 or more times greater than this. Thoron concentrations are much lower and the radiation doses delivered to the population by ^{220}Rn are less than one-tenth of those that result from ^{222}Rn (48). There is now widespread evidence from experimental and epidemiological studies to show that radon, plus its daughter products, is a human carcinogen (50) and this will be discussed in more detail later.

6. THE BIOINORGANIC CHEMISTRY OF THE FALLOUT RADIONUCLIDES

The two atomic bombs dropped in Japan in 1945, and more especially the atmospheric testing of nuclear weapons between 1945 and the late 1960s, resulted in the release of several other metallic radionuclides into the environment; of these only ^{137}Cs ($T_{1/2}$ 30.2 a) and ^{90}Sr ($T_{1/2}$ 29.1 a), remain of major interest. Like ^{239,240}Pu (q.v.), the fallout ^{90}Sr and ^{137}Cs from nuclear weapon testing, together with much smaller quantities of the shorter-lived ^{134}Cs ($T_{1/2}$ 29.1 a), were deposited widely across the world. In the Northern Hemisphere the total deposition of ^{137}Cs peaked at ~150 PBq (1 PBq = 10^{15} Bq) in about 1963, then declined steadily reaching levels at or below the limits of detection in 1982. A further deposition of ~70 PBq followed the accident at the Chernobyl nuclear power station in the Ukraine in 1986, but deposition dropped to below detectable levels from 1987 onward (51). Deposition in the Southern Hemisphere was about one-sixth of that in the north with the peak activity, ~23 PBq, occurring in 1965; thereafter the activity declined steadily and fell below the limits of detection in 1981. The deposition of ^{90}Sr showed a similar pattern with a peak level of 94 PBq in 1963 in the Northern Hemisphere, and of 15 PBq in 1965 in the Southern Hemisphere. A transient, Chernobyl-related peak of ~2 PBq ^{90}Sr was observed in 1986. A survey of the 1987–88 soil concentrations across Japan revealed median values of 3 Bq (range 0.3–30) Bq ^{90}Sr and 23 (range 0.08–148) Bq ^{137}Cs kg^{-1} in the top 5 cm of soil (52).

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6.1 Cesium

The bioinorganic chemistry of the alkali metal radionuclide ^{137}Cs is broadly similar to that of potassium and the monovalent Cs^+ cation must be regarded as being quite mobile. Cesium is incorporated into almost all foodstuffs, with milk, meat, and fish showing the highest levels. The results of an extensive survey of ^{137}Cs and ^{90}Sr samples in a few types of food across Japan in the years 1987–88 (52) are listed in Table 4. The data show a wide spread of values, much wider than the variations in the calcium and potassium concentrations; this large variation suggests that, except perhaps for fish, there is no simple relationship between the uptake of ^{137}Cs and potassium or ^{90}Sr and calcium. Comparison of the concentrations in the foodstuffs with those in the soil suggest CF values of $\sim 1–5.10^{-3}$ for both radionuclides.

In the human body ^{137}Cs is almost completely absorbed from the gastrointestinal tract (8,9) and becomes more or less uniformly distributed throughout the body tissues, the largest amount being found in the muscle mass. This ^{137}Cs appears to exist in ionic form in the tissues (53); its rate of elimination is relatively slow with biological half-times ranging from 50 to 200 days (9). The rate of elimination from females ($T_{1/2} \sim 80$ days) is shorter than that in males ($T_{1/2} \sim 100$ days). The amount of ^{137}Cs that is found in the human body depends principally on the individual's dietary intake, with persons eating diets rich in meat, fish, or edible fungi showing higher levels than those whose diet is largely vegetarian. For example, Eskimos and residents of Lappland, whose diet is rich in caribou or reindeer meat, show some of the highest levels. Caribou and reindeer feed on lichens, which concentrate large amounts of fallout ^{137}Cs. The levels of ^{137}Cs in humans peaked around 1964 with levels reaching up to $\sim$50 kBq in a 70-kg Eskimo man (54); these peak levels decreased quite rapidly and by the late 1970s levels $\sim$30–110 Bq were being reported (55). The Chernobyl accident increased ^{137}Cs levels in people in some areas of Europe; for example, Pietrzak-Flis and Krajewski (56) estimated that in northeast Poland the dietary intake of ^{137}Cs increased by up to 10-fold following the Chernobyl accident and that human body burdens of ^{137}Cs reached levels of up to 1900 Bq in 1986–87, but declined to $<$450 Bq by 1991–92.

6.2 Strontium

The results of an extensive survey of ^{90}Sr concentrations in a few types of food across Japan in the years 1987–88 (52) are listed in Table 4. The data show a wide spread of values, much wider than the variations in the calcium concentrations; this large variation suggests that, except perhaps for fish, there is no simple relationship between the ^{90}Sr uptake and the calcium concentration. Comparison of the concentrations in the foodstuffs with those in the soil suggest CF values of $\sim 1–5.10^{-3}$ for ^{90}Sr.

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TABLE 4 Concentrations of ^{137}Cs and ^{90}Sr Measured in Soils and Some Foodstuffs in Japan in 1988–89 and Total Daily Intakes of These Radionuclides in the Diet (Ref. 52)

			^{90}Sr (mBq kg^{-1})		^{137}Cs (mBq kg^{-1})	
Material	g Ca kg^{-1}	g K kg^{-1}	Mean ± SD	Median	Mean ± SD	Median
Soil (31)			6.8 ± 7.3[a] (0.3–30.0)[a]	3.0[a]	32.8 ± 37.9[a] (0.85–148)[a]	23.3[a]
Milk (77)[a]	1.11 ± 0.08	1.59 ± 0.11	33 ± 37 (0–277)	26	78 ± 113 (0–777)	48
Rice (39)	0.04 ± 0.01	1.02 ± 0.24	9.6 ± 6.6 0–26	11.1	40 ± 70 0–444	26
Vegetables (74)	0.47 ± 0.28	4.15 ± 2.39	200 ± 274 7–1776	105	42 ± 101 0–814	15
Fish (27)	4.0 ± 4.2 (0.1–13.4) g person^{-1} d^{-1}	3.76 ± 0.73	11 ± 11 0–44 mBq person^{-1} d^{-1}	11	208 ± 70 (78–370) mBq person^{-1} d^{-1}	229
Total diet Japan 1988–89	0.69 ± 0.26	2.1 ± 0.44	116 ± 59 (30–285)	94	229 ± 179 30–285	176
Total diet USA 1980–82			30 ± 8		<50	

[a] Bq kg^{-1}.

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Strontium is an alkaline earth metal and resembles calcium in its general bioinorganic chemistry, except that, unlike calcium, there is no biochemical mechanism that enhances its absorption from the gastrointestinal tract. About 30% of the ^{90}Sr ingested in the diet is assumed to be absorbed from the gastrointestinal tract and of the absorbed radionuclide the largest fraction will deposit in bone (8). The amount of ^{90}Sr reaching human bone is dependent both on the dietary intake and on the physiological activity of the skeleton; thus age at ingestion is an important factor. Papworth and Vennart (57), from a study of orally ingested fallout ^{90}Sr in human bone, showed that skeletal uptake decreased from ~9% at 3 months of age to ~4% at age 5, then began to increase at about age 10 to reach a peak of ~8% at 15–16 years, declining thereafter to 4% at 20 years. Dehos and Schmier compared the age dependence of concentrations of ^{90}Sr in the bones of West German residents in the years 1977, 1980, and 1982 (58). In 1977 the peak activity, ~100–150 mBq ^{90}Sr g^{-1} Ca, was found at age 20–22; by 1980 the peak level was still seen at age ~20 but the concentration had decreased to ~80 mBq ^{90}Sr g^{-1} Ca. For persons aged 30 years or more the concentrations in all three years ranged from ~10 to ~40 mBq ^{90}Sr g^{-1} Ca, which corresponds to a total-body content of ~20–80 Bq ^{90}Sr.

7. HUMAN HEALTH IMPLICATIONS OF ENVIRONMENTAL RADIONUCLIDES

As we have seen in the preceding discussion, the total mass of the element that is associated with the residual primeval radionuclides in the human body is very small, generally less than a few μg, however the radioactivity may vary widely. Table 5 presents a summary of the information in rounded figures; the final column of the table gives, for each of the radionuclides, a rounded value for the number of disintegrations that would be expected to occur each day in the human body. Table 5 indicates that only for the actinide radionuclides and for ^{147}Sm, ^{226}Ra, ^{137}Cs, and ^{90}Sr do the disintegration rates exceed 1 per day. For a total-body content of 1 mg ^{147}Sm the lifetime effective radiation dose is <1 μSv; this is far below the level at which any deleterious effects on human health would be detectable, especially when they must be detected against a 2000-fold higher background irradiation from other natural sources. Radium, thorium, uranium, and plutonium deposit mainly in bone where the α-particle irradiation may cause bone tumors; ^{90}Sr also deposits in bone but the β-particle emission is less effective for inducing bone tumors than α-particles (50). Taylor (60) estimated the risk of bone tumor induction from environmental levels of ^{228,230,232}Th, ^{234,235,238}U, ^{239}Pu, and ^{226}Ra and concluded that these radionuclides might contribute about one-hundredth of the overall spontaneous lifetime risk of developing a bone tumor, ~0.05% in 50 years. Figure 5 presents the risks for each of these radionuclides recalculated using updated risk estimates (59); these revised data suggest that

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TABLE 5 Total-Body Contents of Primeval Radionuclides and Associated Disintegration Rates

	Body content		
Radionuclide	Mass (g)	Radioactivity (Bq)	Disintegrations d^{-1}
^{113}Cd	8E−05	1.6E−08	1.4E−03
^{115}In	1E−07	2.2E−08	1.3E−03
^{138}La	<1E−03	<5E−07	<4E−02
^{144}Nd	<1E−03	<2E−06	<2E−01
^{147}Sm	<1E−03	<2E−02	<2E+03
^{148}Sm	<1E−03	<2E−06	<2E−01
^{152}Gd	<1E−03	<1E−07	<9E−03
^{176}Lu	<1E−03	<2E−06	<2E−01
^{174}Hf	1E−04	9.6E−12	8.3E−07
^{187}Re	5E−11	3.4E−08	2.9E−03
^{190}Pt	5E−09	9.7E−15	8.4E−10
^{232}Th	2E−05	8.1E−02	7.0E+03
^{235}U	9E−05	3.7E−04	3.2E+01
^{238}U	9E−05	1.1E+00	9.5E+04
^{239}Pu	3E−11	7.4E−03	6.4E+02
^{244}Pu	3E−11	<1E−09	<1E−04
^{226}Ra	7E−11	2.5E+00	2.2E+05
^{137}Cs	1E−08	4.5E+02	3.9E+07
^{90}Sr	2E−11	8.0E+01	6.9E+06

their contribution to the total risk might in fact be ~7-fold greater than that previously calculated. However, these radionuclides have been present in human beings ever since the race developed, and they will continue to be present as long as the human race exists; thus any risk they present is an inescapable part of the natural risk of life on planet earth.

The γ-ray emitting ^{137}Cs, because of its more or less uniform distribution through the body, irradiates all the body tissues. In the United Kingdom in the early 1990s the annual dose from fallout ^{137}Cs in the diet was about 0.2 μSv, although in some areas of Europe in the immediate aftermath of the Chernobyl accident the dose rate in 1986–87 may have been as high as 10 μSv (61). A comparison of the late biological effects of intravenously injected ^{137}Cs in beagle dogs showed a close similarity in the pattern and type of tumors induced by external γ-irradiation (62). Using the general ICRP factor for total cancer risk of 0.06 Sv^{-1} (63), the irradiation from the current level of fallout ^{137}Cs poses an additional cancer risk of $\sim 1.10^{-8}$ a^{-1}. However, for the majority of the world's population this is a decreasing risk as the bioavailability of the fallout

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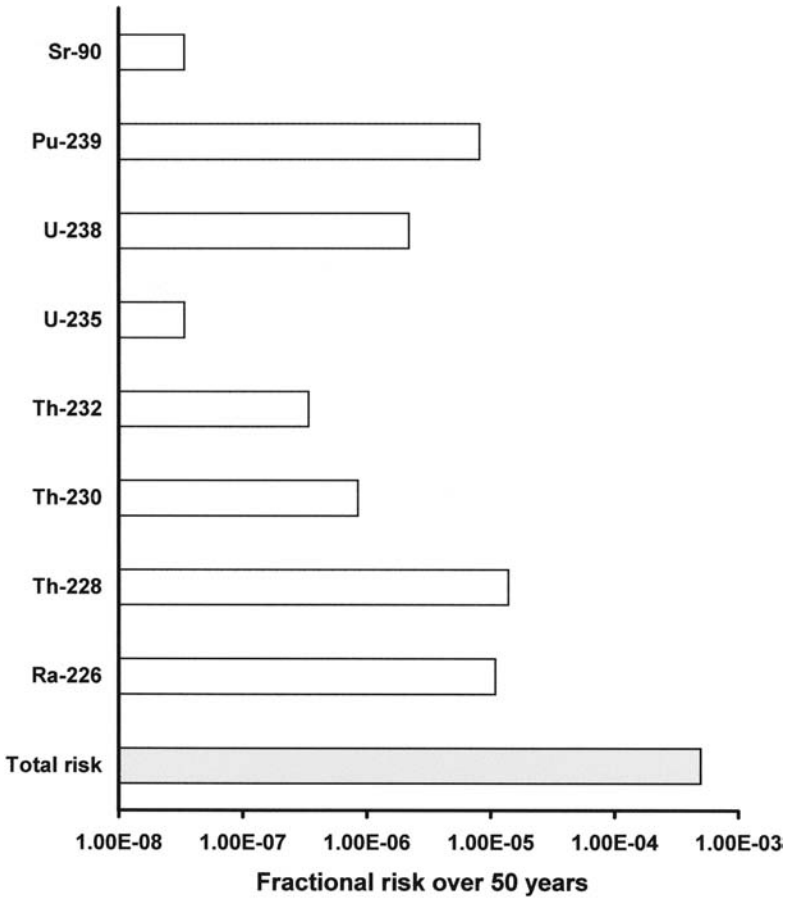

FIGURE 5 The estimated fractional risk of developing a bone tumor from the bone-seeking primeval or fallout radionuclides and their decay product over a 50-year period (60).

^{137}Cs is slowly decreasing and the radioactivity is also being reduced by radioactive decay.

At the average radon concentration in homes in the United Kingdom (20 Bq m^{-3}) radon, in conjunction with smoking, accounts for about 6% of the lifetime risk of developing human lung cancer from all causes (50). A full discussion of this evidence is beyond the scope of this review but the most important conclusions are summarized below. It is interesting to note that what is now recognized to have been radon-induced lung disease was first described by Paracelsus (64)

more than 200 years before the discovery of uranium and some 300 years before the recognition of its radioactivity and of its radioactive daughter products (38).

During the past 30 years, extensive epidemiological studies have been made of the workers in uranium mines around the world. These have demonstrated conclusively that there is a causal link between lung cancer and radon exposure (50). Estimated lifetime risks of developing lung cancer from the inescapable exposure to average indoor radon levels in various parts of the world range from ~1 in 300 in areas of the United Kingdom with an average ^{222}Rn air concentration of 20 Bq m^3 (≡1 mSv a^{-1}) to ~1 in 30 in areas, for example, Finland or Sweden (54), where the ^{222}Rn concentrations are 180 Bq m^3 or more; for smokers these risks are about 3 times greater than those for the general population. The range of doses observed in the United Kingdom is 0.3–100 mSv a^{-1} (65); this range is probably similar to that which would be observed in other countries. The lifetime risks must be considered in relation to the overall lifetime risk of developing lung cancer from all causes, which is about 3–5% for the United Kingdom and United States (50).

In summary it can be said that the levels of primeval and related radionuclides that are presently in the environment pose no significant threat to human health, and appear unlikely to do so in the future. Provided that no one resorts to the use of nuclear weapons, or that there is a resumption of nuclear weapons testing in the atmosphere, the current world levels of ^{90}Sr and ^{137}Cs will decay away over the next century. A matter of some potential concern is the 1200 tonnes or so of unwanted ^{239}Pu that has arisen from the decommissioning of nuclear weapons and from nuclear power production (40). However, the residual primeval radionuclides will remain with us, making a small, but significant contribution to the inescapable natural background irradiation of mankind for many hundreds of millennia into the future.

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5

Metal Carcinogenesis

Michael P. Waalkes
National Institutes of Health, Research Triangle Park, North Carolina

1. INTRODUCTION

Metals are a ubiquitous class of agents both in the natural environment and in the workplace. There are numerous natural and artificial forms of metals. The common occurrence of metals in the human environment is dictated both by their wide natural distribution and by their intensive use in an ever-growing number of industrial processes (1). Clearly, the use of metals has been critical to the progress of human civilization and they are intensely used by modern society. However, metallic agents, once concentrated in the biosphere, generally persist and are not broken down by natural forces, at least not beyond the elemental form. Unlike many products of human enterprise, the use of metal products generally does not consume the innate, natural material. Beyond this, most metals are only sparingly recycled, with a few notable exceptions. Environmental persistence in combination with intensive use by modern society has, over the years, concentrated metals within the human environment. This trend continues generally unabated and provides ample opportunity for human exposure to metals. Thus, human exposure to metals and metal compounds is clearly inevitable. Be-

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yond this, metal exposure, by nature, is always to a multitude of metals and metal compounds and never to a single metallic agent.

The heavy (or transition) metal elements make up a large part of the periodic table and include some of the most toxic agents known, like mercury and cadmium. As a group, heavy metals are an important class of carcinogens. At least three transition metals in one form or another are accepted as human carcinogens by the International Agency for Research on Cancer (1–8). These human carcinogens include cadmium, chromium, and nickel, which have also proven to be carcinogenic in animal models. Several more heavy metals and/or their compounds are suspected to have carcinogenic potential in humans and are active in rodents (1–8). Other known human metallic carcinogens include the metalloid arsenic (4,5) and the alkaline earth metal beryllium (8). There is convincing evidence of beryllium carcinogenesis in animals. The evidence for arsenic carcinogenesis in laboratory animals has been considered limited but recent studies point toward capabilities of inorganic or methylated arsenicals as potential initiators, promoters, or complete carcinogens. In any event, considering that the list of definitive human carcinogens is rather short (5), it is clear that metallic agents, as a class, make up a substantial portion of known human carcinogens. Many more metals are carcinogens in laboratory animals.

Detection of the mechanism or mechanisms of metal carcinogenesis has proven elusive. Many factors are involved in this but, in large part, it is because of the very intricate nature of metal interactions in biological systems (9). This chapter will review the topic of metal carcinogenesis largely following the International Agency for Research on Cancer's (IARC) classification system with special emphasis on known human metallic carcinogens.

2. UNIQUE CHARACTERISTICS OF METALLIC AGENTS AS TOXINS OR CARCINOGENS

Classical theory implicates three more or less overlapping sequential phases of carcinogenesis. These include initiation, promotion, and finally, progression. Initiation involves the alteration of a cell such that it has the ability, under appropriate stimulation, to become a tumor. Promotion involves the stimulation of the initiated cell to accumulate. Progression involves the development of an aggressive, invasive, metastatic malignancy. Generally speaking, the greatest research focus has been directed at elucidating initiating events in carcinogenesis while progression is the least well defined of the phases of carcinogenesis. There is evidence that metallic agents can play roles as initiators, promoters, and progressors.

The metals, as toxicants or carcinogens, are a remarkable group of agents. Although this group can have diverse biological effects, there are several general characteristics that influence toxic outcome. First, all metals have the potential

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to induce adverse reactions in biological systems, even those considered to be essential nutrients. Some essential metals can even be carcinogenic in humans or animals like, for instance, chromium or zinc. However, since many metals are essential in living systems, homeostasis is a key to survival and various biological strategies have developed for the safe transport and storage of metals within the cell. Metal-binding or transport proteins, such as metallothionein (MT; 10) or ferritin (11), are excellent examples of this principle. Thus, metal toxicology has to be considered in the light of systems evolved to intentionally accumulate essential metals. Furthermore, it is thought that the nonessential metals, including many carcinogenic metals, follow the metabolic pathways of similar essential metals (11). Thus, broadly speaking, the carcinogenic metals can be considered mimics of certain essential elements. This mimicry results in the disruption of essential metal function. This is largely due to binding preferences in biomolecules that are similar between the carcinogenic metals and the essential metals they emulate.

Another feature of metal toxicology is the occurrence of acquired tolerance. In this regard, the toxic effects of metals can often be modified by prior, concurrent, or subsequent exposure to the essential metals and clear evidence indicates that the carcinogenic effects of metals are modified by essential elements in chronic rodent studies (12–15). Similarly, essential element deficiency can enhance the carcinogenic potential of metallic carcinogens (16). Such events have frequently been termed ''metal-metal'' interactions and are likely a critical aspect of the mechanisms of metal carcinogenesis (12–15). Thus, although incompletely defined, events in acquired tolerance are likely to be of the utmost importance in assessing the carcinogenic potential to humans. Further, the acute adverse effects of many toxic metals can be mitigated by low, nontoxic doses of the same metal. This acquired self-tolerance to acute toxicity is particularly true for cadmium and has to do with activation of the *MT* gene (10,17). Arsenic will also show acquired self-tolerance (18). How acquired self-tolerance affects carcinogenic outcome is not well defined.

Metal metabolism also has several special features. Biologically speaking, metals, as elements, are indestructable. In essence, they cannot be broken down into less toxic subunits, as is often the case with organic compounds. Thus, enzymatic degradation is not a mechanism available to detoxicate metals (1). Some metallic elements can undergo enzymatic conjugation reactions but how this bears on detoxication is an open question. An example here is the metalloid arsenic, which, with mixed organic and metallic qualities, will undergo enzymatic methylation. However, the role of arsenic methylation in causation or prevention of arsenic carcinogenesis is a matter of some contention. Heavy metal carcinogens generally do not undergo enzymatic conjugation. Conversely, metals typically do not require bioactivation, at least not in the sense that an organic molecule undergoes enzymatic modification resulting in creation of a reactive species. Naturally occurring forms of metals are frequently already reactive species. There

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are adaptive mechanisms that have evolved for metal detoxication however; such as long-term storage in bone or soft tissues. Other detoxication options for metals would include biliary and/or urinary excretion (19–21).

3. METALLIC AGENTS AS CARCINOGENS IN HUMANS

Arsenic was one of the very first agents of any class recognized as a human carcinogen (22). The use of medicinal arsenic compounds was associated with dermal cancers in the classical paper by Hutchinson over 100 years ago (22). Since that time, metallic agents have become an ever-more-important category of human carcinogens. It is fair to say that evidence for the carcinogenic potential of several metals, in both humans and animals, has continued to accumulate over the years. With the addition of two more metals and their compounds in the early 1990s, now at least five metallic elements in one form or another are accepted as human carcinogens (group 1) by the IARC (Table 1; 1–8,23). Specifically, these include arsenic and arsenic compounds, beryllium and beryllium compounds, cadmium and cadmium compounds, hexavalent chromium compounds, and nickel compounds. Considering that the number of agents definitively characterized as human carcinogens of any class is quite small (5), it is quite clear that metal compounds make up a significant portion of this number. Classification in category 1 means that there is clear evidence of human carcinogenicity from various epidemiological studies combined, in almost all cases, with definitive rodent data indicating carcinogenicity. The single exception to this is for arsenic and its compounds, where extensive human evidence of carcinogenic potential supersedes the experimental evidence, which has until recently been considered limited (4,5). More recent animal studies have implicated a carcinogenic potential for inorganic arsenicals (24,25), and shown organoarsenicals to be tumor promoters (26) and, at least in one case, a complete carcinogen (27). However, because of the apparent difficulty in producing tumors in animals with arsenicals there is the legitimate fear that humans may be one of the more sensitive species to arsenic carcinogenesis (28).

Several more metallic agents are classified as suspected or possible human carcinogens (group 2A or 2B; 1–8,23,30). This includes cisplatin, inorganic lead compounds, metallic nickel, iron dextran complexes, methylmercury compounds, cobalt and cobalt compounds, antimony trioxide, and implanted foreign bodies of metallic cobalt, metallic nickel, or certain nickel-chromium-iron alloys (Table 1). This classification is generally based on clear or substantiative experimental data from chronic rodent studies in the absence of sufficient evidence in humans. In the case of cisplatin there are numerous clinical case reports concerning the emergence of secondary malignancies after its use as a cancer chemotherapeutic (5). However, cisplatin is most often used in treatment regimes that include concurrent therapy with multiple agents, some of which are also considered potential

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TABLE 1 Summary of Metallic Agents or Processes Involving Possible Metal Exposure that Have Been Classified as Presenting Human Carcinogenic Risk

Metal or process involving potential metal exposure	Evidence for carcinogenicity in humans	Evidence for carcinogenicity in animals	Overall rating[a]
Arsenic and arsenic compounds	Sufficient	Limited	1
Beryllium and beryllium compounds	Sufficient	Sufficient	1
Cadmium and cadmium compounds	Sufficient	Sufficient	1
Hexavalent chromium compounds	Sufficient	Sufficient	1
Nickel and nickel compounds			
1. Metallic nickel	Insufficient	Sufficient	2B
2. Nickel compounds	Sufficient	Sufficient	1
Underground hematite mining with exposure to radon	Sufficient		1
Iron and steel founding	Sufficient		1
Cisplatin	Insufficient	Sufficient	2A
Cobalt and cobalt compounds	Insufficient	Sufficient	2B
Iron dextran	Insufficient	Sufficient	2B
Inorganic lead compounds	Insufficient	Sufficient	2B
Methylmercury compounds	Inadequate	Adequate	2B
Implanted foreign bodies of metallic cobalt, metallic nickel, or certain Ni/Cr/Fe alloys	Inadequate	Adequate	2B
Welding fumes and/or gases	Limited	Inadequate	2B

[a] Rated by IARC convention as follows: 1, carcinogenic to humans; 2A, probably carcinogenic to humans; 2B, possibly carcinogenic to humans. See ref. 5 for details.
Source: From IARC (1–8,29,30).

carcinogens (5). For the other metallic agents human evidence is lacking or ambiguous. It should be kept in mind that most metallic agents have not been tested adequately for carcinogenic potential in animals.

The status of lead compounds, which are last analyzed by IARC in 1987 (5), deserves some additional comment. Evidence is mounting that occupational exposure to inorganic lead compounds is, in fact, a causative factor in human

carcinogenesis (31–35). Several recent epidemiological studies, including one meta-analysis, have pointed toward the kidney and lung as target sites of lead in humans (31–35). There are also hints of gastrointestinal and nervous system tumors (33,34,36). Clearly, with these new data it is time for a reanalysis of the potential carcinogenicity of occupational exposure to lead and lead compounds.

Several occupations involving potential exposure to metallic agents, or involving participation in processes with potential metal exposures, are also considered to be a human carcinogenic hazard. Processes potentially involving metallic agent exposures that show clear evidence of an association with human cancer include iron and steel founding and underground hematite mining with coexposure to radon (5). Clearly the iron exposure from hematite mining would probably be secondary in effect to the radon exposure. Iron and steel founding would include exposure to various mixtures of potentially carcinogenic metallic fumes as well as exposure to other carcinogens, such as polyaromatic hydrocarbons, silica, and formaldehyde (5). Welding fumes or gases are rated as possible human carcinogens largely on the basis of limited human data and on the knowledge that these gases and fumes often contain carcinogenic metals (5,6). For instance, welding fumes and gases often contain nickel and chromium, both established human carcinogens (6). Aluminum production is definitively associated with human cancer but there are quite likely exposures to nonmetallic agents that account for this finding (5), although the role of metallic agents has not been definitively excluded.

What probably should be considered as a special case of metal carcinogenesis is the occurrence of local tumors arising at the site of a corroded implanted metallic prosthetic device (37,38). There are at least a dozen clinical case reports of tumors arising at the site of a metallic orthopedic device (37–39). Although this probably represents an underreporting of implant-associated tumors, considering the number of implantations of such devices yearly, cancer at the site of a metallic implant is a very rare event indeed (37). The tumors induced are most frequently sarcomas of one type or another (37,39) and in many cases the tumors at the site of metallic implants are linked with a deterioration or corrosion of the implant, presumably creating local release of the metal components of the device and a high local concentration. The devices associated with these tumors frequently contain chromium and occasionally nickel, both known human carcinogens, and postoperative hypernickelemia and nickeluresis have been reported (40). Additionally, indwelling metal fragments, such as those sustained from bullet or shrapnel wounds, may occasionally give rise to local cancers in humans after a long residence time (41,42). These results in humans and supportive animal data led the IARC to classify certain implanted foreign metallic bodies as possible human carcinogens (30). It should be kept in mind that the formation of tumors at the site of indwelling metal fragments or metallic orthopedic devices

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probably has an element of foreign-body-type carcinogenic response as a mechanistic component (43).

Many exposure situations are associated with metal carcinogenesis in humans (1–8,23,45–50). Most of these involve occupations in metal refining, smelting, fabrication, or other metal processing. Electroplating with metals is a potential occupational exposure situation while soldering can lead to significant exposures of lead or cadmium. Welding is another important example of industrial metal exposure, although the precise metal fumes or gases depend on the metals being welded. Iatrogenic metal carcinogenesis can occur, and medicinal metal preparations, like arsenic and cisplatin, are likely human carcinogens (5,22) but the risk/benefit analysis still favors their chemotherapeutic use. Exposures occurring during chemical production using metals, such as for certain chromium pigments or nickel-containing catalysts, have been associated with human cancer (6). Individuals involved in certain mining processes and in iron or steel founding may be at greater risk for metal-induced carcinogenesis, although exposures to nonmetallic carcinogens are likely important (5). It is quite possible that many other occupational activities or metals could be added in the future. There are also some cases in which environmental exposures to metals have been linked

TABLE 2 Definitive Target Sites of Metals Accepted as Carcinogens in Humans[a]

Target site or tissue	Metallic agent, metallic compound or process involving potential metal exposure
Lung	Arsenic and arsenic compounds
	Beryllium and beryllium compounds
	Cadmium and cadmium compounds
	Hexavalent chromium compounds
	Nickel compounds
	Underground hematite mining with exposure to radon
	Iron and steel founding
Sinonasal cavity	Nickel compounds
	Hexavalent chromium compounds
Urinary bladder	Arsenic and arsenic compounds
Kidney	Arsenic and arsenic compounds
Liver	Arsenic and arsenic compounds[b]
Skin	Arsenic and arsenic compounds

[a] Includes only those agents rated as carcinogenic to humans (category 1; see Table 1) and including recent data from the NRC (49) for arsenic that show three or more separate studies with the same site.
[b] Specifically hemangiosarcoma.

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to human cancer but these are generally limited with most inorganics (5,8). Environmental arsenic exposure, however, clearly results in development of human cancers (49). Arsenic exposure occurs to the greatest extent from contaminated drinking water and secondarily from contaminated foods (49). Tobacco smoke is thought to be an important nonoccupational source of metal exposure, including exposure to cadmium and nickel.

With regard to target sites, the earliest report of a metallic agent as a carcinogen was that of skin tumors in humans undergoing oral therapy with medicinal arsenical preparations for various diseases, including, in fact, cancers (22). Inhalation exposure to arsenic has also led to formation of dermal carcinomas in humans (Table 2). The skin as a target site for metals in humans has, however, proven unique to arsenic and arsenic compounds (1–8). In fact, for the accepted human metallic carcinogens, the respiratory system is the single most frequent target site in humans and inhalation exposure to compounds of arsenic, beryllium, cadmium, chromium, and nickel is associated with pulmonary carcinomas (1–8). Sinonasal cavity tumors are also associated with exposure to hexavalent chromium and nickel compounds (6). Metal-induced respiratory tumors have occurred exclusively from inhalation exposure (5,51). The preponderance of the lung as a target site in metal carcinogenesis is probably due to this being the point of first contact during occupational exposures. Exposure to arsenic has also been

TABLE 3 Possible Target Sites of Metals Accepted as Carcinogens in Humans[a]

Possible target sites	Metallic agent or compound
Liver	Hexavalent chromium[b]
	Cadmium and cadmium compounds
Esophagus	Hexavalent chromium[c]
Prostate	Cadmium and cadmium compounds
	Arsenic and arsenic compounds
Gastrointestinal	Arsenic and arsenic compounds
	Cadmium and cadmium compounds
Hematolymphatic	Arsenic and arsenic compounds
Kidney	Cadmium and cadmium compounds
Nasal	Arsenic and arsenic compounds

[a] Includes only those agents rated as carcinogenic to humans (category 1; see Table 1). Includes target sites evidenced in one or two epidemiological studies as having a significant ($p \leq 0.05$) association.
[b] Occurred only in males. Exposure to nickel also occurred in this cohort.
[c] Some exposure to benzo[*a*]pyrene probably also occurred in this cohort.

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repeatedly associated with hepatic angiosarcomas in humans (5,49,52,53) and because of the rare nature of this tumor it seems a safe conclusion that arsenic is an etiological factor. Arsenic exposure is also associated with various other tumors in humans including tumors of the urinary bladder and kidney. There is also growing evidence for respiratory and renal cancer following exposure to lead compounds (31–35) but these compounds are not considered human carcinogens at this point. There are also several other possible target sites of the metals accepted as carcinogens in humans (Table 3).

Of the processes accepted as carcinogenic to humans involving potential exposure to metals, mining of certain iron ores is associated with pulmonary tumors, but only with concurrent exposure to radon (Table 2; 5,54). Similarly, occupation in iron and steel founding industries contributes to lung cancer incidence but this is likely due, at least in part, to nonmetallic carcinogens, including exposure to polycyclic aromatic hydrocarbons (5). Based on several rodent studies, it has been suggested that iron particles may act as a carrier for organic carcinogens in humans thus increasing the residence time in the lung (5).

4. METALLIC AGENTS AS CARCINOGENS IN ANIMALS

Of those metallic agents or metallic compounds analyzed by the IARC to date, 10 are considered to have shown sufficient or adequate evidence of carcinogenicity in animals (Table 1). These include beryllium and beryllium compounds, cadmium and cadmium compounds, cisplatin, cobalt and cobalt compounds, hexavalent chromium compounds, inorganic lead compounds, iron dextran, nickel compounds, metallic nickel, and methylmercury compounds (1–8). The data for arsenic are considered limited but the most recent evaluation is now over 10 years old and additional data have shown methylated arsenicals as multiple-site tumor promoters after organic carcinogens (26). One study has also shown dimethylarsinic acid as a complete carcinogen for the ray urinary bladder (27). Some evidence indicates that inorganic arsenicals can act as cocarcinogens in mouse skin (24) or can induce premalignant lesions of the uterus, testes, and liver in mice (25). A reevaluation of arsenic and arsenic compounds is probably in order.

Not all metals have been analyzed by the IARC and many other metallic agents and/or their compounds have shown some evidence of carcinogenicity in animals (Table 4). These include aluminum, trivalent chromium, copper, manganese, platinum, titanium, and zinc, all of which have produced tumors in one or more studies (44–47,55–57). However, some of these metals show carcinogenicity only when given by rather unusual routes. For example, salts of both copper and zinc, when injected directly into the testes of rodents or fowl, induce the formation of malignant testicular teratomas (44–47). Although teratoma formation can be modified by endocrine status, such as breeding cycle in birds, it is

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TABLE 4 Selected Target Sites of Metal Carcinogenesis in Experimental Animals[a]

Target site	Tumor	Metal or metal compounds	Exposure route[b]
Adrenal	Carcinoma/adenoma	Cadmium	sc
		Nickel	inh
Bone	Osteosarcoma	Beryllium	iv, ios
Brain	Glioma	Lead	po
Hematopoietic	Lymphoma/leukemia	Cadmium	sc, po
		Lead	ip
Injection site	Sarcoma	Cadmium, cobalt, chromium, iron carbohydrates, or nickel	sc or im
		Colloidal silver, gadolinium, tin, titanium, or yttrium	im
		Aluminum dextran, cisplatin, or manganese	sc
Kidney	Carcinoma/adenoma	Lead	po, trpl
		Nickel	ir, trpl
		Methylmercury	po
Liver	Adenoma	Cadmium	sc
Lungs	Carcinoma	Beryllium, cadmium, chromium, or nickel	inh
Pituitary	Carcinoma	Nickel	trpl
	Adenoma	Cadmium	sc
Prostate	Carcinoma/adenoma	Cadmium	sc, im, po, ipro
Skin	Carcinoma	Cisplatin	trpl
	Papilloma	Arsenic	po
Testes	Leydigoma	Cadmium	sc, im, po
Testes	Teratoma	Cadmium, zinc, copper	ites
Urinary bladder	Carcinoma	Arsenic (dimethylarsinic acid)	po

[a] In addition to the data cited in the text includes data from refs. 137–140.

[b] Defined as: im, intramuscular; inh, inhalation; ios, intraosseous; ip, intraperitoneal; ipro, intraprostatic; ir, intrarenal; ites, intratesticular; iv, intravenous; po, oral; sc, subcutaneous; trpl, transplacental. Intratracheal instillation studies confirmatory of inhalation studies are not included. Only selected initiation/promotion studies are included.

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still difficult to assess the carcinogenicity of these metals. If they are only carcinogenic by such obscure routes, human risk would be minimal.

Experimental animal systems have to a large extent reproduced the metal-induced tumors seen in humans (Table 4). Inhalation or intratracheal exposure in rodents to compounds of beryllium, cadmium, chromium, and nickel has resulted in pulmonary carcinomas (1–8). This is clearly supportive of the human epidemiological evidence for the role of these metals in the etiology of pulmonary carcinogenesis. The one major exception to this is the case of arsenic where evidence of pulmonary carcinogenicity in rodents is presently considered as limited.

Accumulating evidence indicates that parenteral or oral exposure to cadmium can result in prostatic proliferative lesions and prostate tumors in rats (58–65). The finding of cadmium-induced prostate cancer in rats supports a possible role of cadmium in the etiology of this important human malignancy. Cadmium will also frequently induce tumors of the testes.

Other sites of metal carcinogenesis in rodents include injection site sarcomas seen with metals such as cadmium, cobalt, chromium, manganese, nickel, and titanium (1–8,44–47,55–57). These sarcomas arise at subcutaneous or intramuscular injection sites. Sarcomas have arisen with some frequency in dogs around implanted metallic intramedullary bone splints that have undergone corrosion in situ (37,39), and this is probably an example of locally occurring (''injection site-like'') metal implant tumors in nonrodent species. Again the occurrence of local sarcomas arising at the site of implanted metallic prosthetic devices, particularly those having undergone corrosion, could be considered as a special case of metal carcinogenesis in humans that is similar to the injection site sarcoma formation in rodents.

The rodent testis has been shown to be a target of metallic agents. Intratesticular injection of copper, zinc, and cadmium will produce testicular teratomas, while intratesticular nickel injections produce local sarcomas (6,44–47). The relevance of tumors formed by direct injections of metals into tissues is questionable particularly if the tumor is not duplicated by other routes of exposure. Systemic exposure to cadmium can result in Leydigomas (8), although cadmium-induced disruption of hormone homeostasis resulting in excess mitogenic stimulation of the testicular interstitium appears critical in their formation (66). Cadmium treatment has also been associated with rare testicular tumors in rodents but not in sufficient numbers to allow causal linkage (15,67).

The kidney has been a target site of several metals in chronic rodent experiments. Chronic oral lead exposure has been linked to tumors of the rodent kidney (4,68). Lead will induce both brain tumors and renal tumors, which is consistent with emerging human data. With renal tumors, chronic nephropathy due to lead and the resulting proliferative repair was suspected as the causative factor in carcinogenesis (69). However, lead exposure can induce renal tumors in the ab-

sence of chronic nephropathy (70), indicating that overt nonmalignant renal disease is not obligatory. Methyl mercury and nickel will also produce renal tumors in rodents. Direct injection of nickel compounds into the kidney results in sarcomas while parenteral exposure to nickel followed by a renal tumor promoter will also result in renal cortical adenomas and carcinomas (6,44–47,71). Transplacental exposure to nickel followed by postweaning promotion by barbital will also induce renal tumors, as well as malignant pituitary tumors (72). Transplacental exposure to cisplatin induces preneoplastic foci within the renal cortical epithelium (73).

Intravenous injection of beryllium compounds in laboratory animals can produce osteosarcomas (8). Osteosarcomas are likewise formed by implantation or direct injection of beryllium into the bone (8). The bone is an unusual target site for metallic agents although several metals will concentrate in bone.

Transplacental exposure to cisplatin followed by postnatal exposure to TPA administered topically induces squamous cell papillomas (73). Sequential exposure to TPA and then arsenic will also produce skin tumors in mice (24).

Dimethylarsinic acid (DMA), the methylated form of arsenic, is a complete carcinogen for the rat bladder (27). DMA is also a promoter of carcinogenesis in the bladder, lung, kidney, and thyroid in rats when given after exposure to organic carcinogens (26). Arsenic exposure will also induce preneoplastic lesions of the uterus, testes, and liver in mice, which may be consistent with an estrogen-like mechanism (25).

A recent study has shown that preconception exposure of male mice to trivalent chromium will cause tumors of the adrenal and thyroid in the offspring that they sire (74). This is the first study to show activity for trivalent chromium as a carcinogen in rodents or humans. The possible role of chromium, and other metals, in preconception carcinogenesis deserves further study.

Another recent study (75) showed that repeated exposures to cadmium resulted in the more rapid onset and increased malignancy of subcutaneous injection site tumors in rats. The aggressive nature of the tumors formed by repeated exposure to cadmium was reflected in a higher rate of invasion into the subdermal muscle layers and bone as well as greater tendency of the tumors to metastasize to the lungs. This is strong evidence that cadmium may act as a ''progressor'' in the sense that the malignant progression of tumors formed by cadmium is enhanced by repeated exposures to the metal. Although cadmium is certainly an effective single-dose carcinogen in rodents (8), it is obvious that human populations, during an average lifetime, would experience repeated exposures to cadmium. Additionally, it should be pointed out that, in general, tumor metastasis is more often the cause of cancer death than the primary tumor. The observation that repeated exposures to cadmium has an impact on tumor progression could have important implications in exposed humans. Industrial cadmium exposures are undoubtedly multiple events over many years. Furthermore, it is estimated

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that cigarette smoking will double the total lifetime body burden of cadmium, because of tobacco's cadmium content (76). So in this case there would be repeated exposure to cadmium along with all the other organic carcinogens contained in tobacco smoke and a role of ''progressor'' for Cd could be quite important.

Overall, in animals metallic carcinogens seem to show multiple target sites and the target sites are common to two or more metallic agents. The exceptions to the latter are the bone (beryllium), brain (lead), and prostate (cadmium) where only a single metallic agents is active as an animal carcinogen.

For chemical compounds accepted as human carcinogens, commonality of target sites between humans and rodents is a very frequent phenomenon (77), and is likely of mechanistic significance. This is certainly the case with the metallic carcinogens. The pulmonary system is the primary example of a common target site between the human and rodents. All known human metallic carcinogens, with the exception of arsenic, are effective pulmonary carcinogens in humans and rodents following inhalation. This includes beryllium, cadmium, hexavalent chromium, and nickel. The example of arsenic notwithstanding, this is a remarkable correlation. Other sites that have at least some concordance between animals and humans include the prostate (cadmium), brain (lead), kidney (lead and cadmium), urinary bladder (arsenic), and skin (arsenic).

5. DETERMINING FACTORS FOR TARGET SITES IN METALLIC AGENT CARCINOGENESIS

Several factors likely contribute to determining the target site of metallic carcinogens. One is that metals as toxins or carcinogens tend to be highly tissue specific. This can involve both toxicokinetic and toxicodynamic elements. Another determinant can be tissue proximity to locally high concentrations of the carcinogenic metal.

Specific disposition of metallic carcinogenic in a tissue is likely an important determinant of target site, although little direct evidence for this is available from human studies. The accumulation of lead or methyl mercury in the rodent kidney (69,78) is a case where specific disposition of the carcinogenic metallic compound could be related to tumor formation (5,8,69). Cadmium also concentrates in the rodent and human prostate (62,79), consistent with tumor formation at this site. Beryllium accumulates in the bone, which is in accord with beryllium-induced osteosarcomas in animals (8). Overall, however, specific disposition does not appear to be a primary factor in dictating target site in metal carcinogenesis. For instance, several of the target sites of cadmium carcinogenesis in rodents, including the testes, accumulate very little of the metal.

Specific sensitivity factors likely help dictate target tissues in metal carcinogenesis. Absence or suppression of normal defense mechanisms against the par-

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ticular metal may contribute to an uncommon sensitivity. For instance, MT, a metal-binding protein, is clearly a key element in endogenous or acquired cellular tolerance to cadmium toxicity and possibly carcinogenicity. There is evidence that the *MT* gene is not basally active or only poorly responsive to stimulation in certain target tissues of cadmium carcinogenesis in rodents (61,80). Furthermore the *MT* gene is more active and responsive to stimuli in the lungs of rodent species resistant to the pulmonary carcinogenicity of cadmium (81,82). This tissue-specific expression of MT may well contribute to sensitivity to cadmium carcinogenesis. In fact, activation of the *MT* gene inhibits cadmium-induced genotoxicity in cultured cells (83,84). Tolerance systems also exist for several other metals, including nickel and arsenic (18).

Tissue proximity to locally high concentrations of metal is also a determining factor in metal carcinogenesis. For instance, the inhalation of a metal particle or corrosion of a metallic implant would result in the direct exposure of adjacent cell populations to a high local concentration as the solid metal deteriorates from physical or biochemical processes and is, in all likelihood, an important contributing factor to development of malignancies. Within the lung after inhalation of metal-containing particulates, very high concentrations could thus occur locally. First the metal particle probably would not be entirely inert in physiological solutions and surface loss of metal would be expected. Additionally, as the body recognizes the particle as foreign and directs efforts as its dissolution, inflammatory responses in the lung may help release further metal. In animal models, the proximity to locally high concentrations of carcinogenic metal clearly occurs at repository-type injection sites of metallic agents, At such sites, biological reactions to an irritating deposit would also help dissolve and release metal locally. The sarcomas that can arise in humans or dogs around implanted metallic devices that have undergone corrosion are an example of tumors occurring in juxtaposition to a source of high local metal concentrations. Tumors can form at the site of injection of metal that is complexed with other molecules resulting in prolongation of the local residence time of the metal. Tumors arising at the site of repository iron injections, such as iron-dextran (5), or cadmium-protein complexes (9) are two examples.

6. DUALITIES IN METAL CARCINOGENESIS

6.1 Essentiality and Carcinogenicity

An important concept in metal carcinogenesis is that some metallic agents are both carcinogenic and essential. Chromium is the best example of this duality (6). Likewise, the essential elements zinc and copper can induce malignancies, at least under unusual circumstances of exposure, such as direct injection into

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the testes (44,46). Similarly, iron, injected in combination with carbohydrates, will also produce injection site tumors (5,46). Thus, biological systems, which have mechanisms by which essential metals can be safely stored and utilized, do not have an unlimited capacity and, as these systems become overwhelmed, even essential metals can take on toxic characteristics, including carcinogenicity. The carcinogenicity of certain essential metals is a major challenge in risk assessment and nutrition, particularly if metal-induced cancer is assumed to be a nonthreshold event. The nonessential metals that mimic essential metals may behave in a similar fashion. Here the storage and defense mechanisms normally in place for the essential metal, which are used by the toxic mimic metal, may be overwhelmed by excess exposure with nonessential elements.

6.2 Carcinogenic and Anticarcinogenic

Metallic agents can be both carcinogenic and anticarcinogenic (46,85–88). This can be based on dose or time sequence but in certain cases can also be a specific tissue response. An excellent example of this duality is cisplatin, a widely used cancer chemotherapeutic. The efficacy of certain platinum compounds as chemotherapeutics comes from their ability to disrupt DNA, which, with sufficient time, probably results in tumor formation (5). There is currently great excitement for the revival of arsenic as a cancer chemotherapeutic and it appears able to specifically induce apoptosis in malignant lymphocytes (89). It is thought that arsenic has specific activity against acute promyelocytic leukemia and shows promise against other malignancies. Any use of arsenic must be tempered with the knowledge that it is in fact a potent, multisite human carcinogen (90), but when faced with a deadly disease drastic cures may well be in order. Other evidence also indicates that cadmium, a potent rodent and human carcinogen, can be specifically destructive to liver and lung tumor cells and reduce tumor formation in mice (85–88). Here it appears that cadmium causes specific necrosis within liver tumor cells preserving the normal surrounding cells (88). The sensitivity of liver tumor cells appears to be dictated by poor expression of MT (88). Cadmium can also perturb the growth and limit the metastases of human tumor xenografts in mice (91).

So the ability of metallic agents to act as both carcinogens and anticarcinogens is not uncommon. In this regard, the carcinogenic metals are frequently very effective systemic or site-specific toxins in addition to their carcinogenic effects. Thus toxicity may abolish a carcinogenic response at higher doses. The cancer-cell-specific apoptosis induced by arsenic (89), and tumor-cell-specific necrosis induced by cadmium (88), are probably examples of this principle. A reduced neoplastic response can result from what appears to be a dose-related spectrum of toxic effects ranging from cytotoxicity and carcinogenicity to cytolethality in

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specific target cell populations. The wide variability of cell sensitivity to the toxic effects of the individual metallic agents can have a direct bearing on the eventual carcinogenicity of a given dose in a given tissue.

7. METAL-METAL INTERACTIONS IN METAL CARCINOGENESIS

Treatment with essential metals will often modify metal carcinogenesis, and these effects have been termed ''metal-metal'' interactions. This should not be taken to mean that metal-metal complexes are formed, but rather that the essential metals antagonize the toxic manifestations of the carcinogenic metals. These studies have been valuable in defining potential mechanisms in metallic agent carcinogenesis and clearly are very important in defining carcinogenic potency and efficacy of a given metal. Several studies have shown the inhibition of tumor formation by one metallic agent by exposure to another (12,14,15,92). For instance, in the early 1960s it was found that zinc markedly reduces the cadmium carcinogenesis at the subcutaneous injection site and in the testes (93). Later it was shown that cadmium-induced injection site sarcomas can be prevented by magnesium treatments (94). Inhalation of zinc markedly reduces the pulmonary carcinogenicity of cadmium (95). The antagonism by zinc of cadmium carcinogenicity may lie in the ability of zinc to induce the production of large amounts of MT, a protein that avidly binds cadmium (10,17). Magnesium and manganese will also prevent nickel-induced injection site sarcomas in rats (96,97).

Metal-metal interactions are not always inhibitory and enhanced response after exposure to another metal can also occur. For instance, calcium treatment enhances lead-induced renal tumors in rats (68) likely by exacerbating nephrotoxicity. Zinc increases the incidence of cadmium-induced prostatic tumors in rats (59) probably by reducing cadmium toxicity in the testes. Dietary deficiencies of essential elements can also alter response to carcinogenic metals, as with zinc deficiency, which can increase the incidence of cadmium-induced injection site sarcomas (16). Metal-metal interactions do not appear to modify the target site but, rather, typically alter tumor incidence or progression within a single site.

8. HYPOTHETICAL MECHANISMS IN METAL CARCINOGENESIS

There are several difficulties in attempting to define mechanism in metal carcinogenesis. First, metals can have a vast array of biological effects, which can depend largely on the dose. All too often in vitro studies attempting to define mechanism use levels of metal that would be incompatible with in vivo survival. The results of such studies have to be interpreted very carefully and may often be more

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misleading than helpful. Furthermore, many metals, by nature, display the tendency to form ionic bonds, which are more readily disrupted than covalent bonds. This creates a distinct handicap for defining the actual location of a metal within a cell with any degree of certainty after the cell has been disrupted. Cell disruption undoubtedly releases many loosely bound metals, which creates the distinct possibility for artifactual distribution. Take, for instance, metal bound to DNA, which represents a large variety of biological ligands for metals and could associate with metals after cell disruption. Additionally, defining the location of metals in intact or fixed cells is essentially impossible if the metal is not very highly concentrated, such as within a lead inclusion body or phagocytized metal particles, and the use of immunohistochemical or other localization techniques has not been very fruitful. So meaningful studies that define the point of action within the cell are unavailable. Add to this the major difficulties in defining the mechanism of any carcinogen, which include the lengthy time period between initiation and final tumor formation, and defining mechanisms in metal carcinogenesis presents formidable obstacles.

In fact, a mechanism has not been clearly defined for any metallic carcinogen. For a proposed mechanism in metal carcinogenesis to be plausible, it should at least fulfill certain minimal criteria (15). First, the proposed mechanistic event or events should occur within the target tissues of the particular metallic carcinogen and should occur to a lesser extent in nontarget tissues. Descriptions of molecular events that occur in nontarget tissues or cells could be more misleading than elucidating. Additionally, the noncarcinogenic metals should be utilized as negative controls, and should be inactive or only sparingly active in stimulating the molecular events proposed as mechanistic. Furthermore, the specific antagonisms by noncarcinogenic or essential metals, where known from chronic carcinogenicity testing, should occur and should modify molecular events accordingly. There are several examples of biological antagonism of metal carcinogenesis in chronic animal testing, including the observations that zinc antagonizes cadmium carcinogenesis or that magnesium reduces nickel, lead, or cadmium carcinogenesis (see above). Whatever the proposed molecular mechanisms are, they should incorporate and account for these metal-metal interactions.

Carcinogenic agents are often classified as either genotoxic or nongenotoxic (epigenetic). Genotoxic carcinogens would be expected to act by causation of DNA damage with subsequent mutations resulting in the modification of gene expression and subsequently leading to tumor formation. This would not necessarily require direct-acting agents. For instance, redox-active metals can produce radicals that in turn could attack DNA. Nongenotoxic, epigenetic mechanisms would apply where an agent effects tumor formation through some other event than DNA damage and subsequent mutation. The distinction between these subsets of carcinogenic mechanism can, at times, be nebulous and a carcinogen

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should not be seen as less important because it has an epigenetic mechanism. In any event, metallic agents potentially can be directly or indirectly genotoxic or could function through a variety of nongenotoxic mechanisms.

Direct genotoxic effects of metals would include direct DNA strand breaks (single or double stranded), binding to DNA with resultant conformational changes and/or cross-linking between the DNA strands or DNA and proteins (55,98–100). Metal damage of DNA or binding to DNA could potentially modify conformation and consequently modify gene expression or produce mutations (55,98–102). For instance, nickel binding to DNA produces conformational changes that can result in altered DNA methylation in specific regions, which, in turn, results in inactivation of an important senescence gene (101,103). For chromium, the cellular reduction of hexavalent chromium creates free radicals that may attach DNA, as well as chromium reductant species that can directly bind DNA or cause strand damage (104–107). Reduced accuracy of DNA replication and/or decreased DNA repair and synthesis are also distinct possibilities with DNA-bound metals (55,98,99,102,108,109). These events would be expected to result in mutation for initiation.

With regard to indirect DNA damage, several metals can cause the generation of DNA-damaging radicals. These, in turn, can cause various DNA lesions, including single- and double-strand breaks and DNA-protein cross-links (104, 110–116), and thereby induce mutations (117,118). Metals that could generate radical species include nickel, chromium, copper, and iron. Some evidence indicates that metals can replace zinc in DNA-binding zinc finger proteins (119), which are typically transcription factors that show a high site-specific DNA binding (120). This could potentially place a redox active metal in close proximity to DNA, possibly facilitating radical attack (119). Other indirect genotoxic mechanisms could include inhibition of DNA repair by carcinogenic metals, which can happen at very low levels (109,121–123) and would be expected to increase mutation rates. Also, alterations in the activity of DNA replication enzymes (DNA polymerases) can reduce accuracy of replication and presumably cause mutations (55,98,99,102).

Altered gene expression, including either overexpression or underexpression, if protracted could result in transformations without an obligatory mutational event. For instance, alterations in cellular signal transduction pathways by metal interactions may also stimulate expression of cellular proliferation genes, including oncogenes (124,125). Likewise, changes in DNA methylation status can allow for aberrant expression of genes important in the control of proliferation. For instance, with arsenic exposure chronic consumption of cellular methyl groups appears to result in DNA hypomethylation and aberrant gene expression (126), at least in some cells. In addition, the binding of carcinogenic metals to zinc finger-loop protein transcription factors that regulate gene expression could clearly alter protein conformation and allow disruptions in the control of gene

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expression (119,120). It is thought that metal substitution of cadmium, nickel, and cobalt for the zinc within finger-loop proteins could be an indirect mechanism for gene activation or inactivation associated with carcinogenesis (120) and clearly such metal replacement can reduce the DNA-binding affinity of finger-loop proteins (127). There is also evidence that cadmium can replace zinc in p53 protein, a metal-binding transcription factor that helps control DNA repair, and this substitution inactivates p53-mediated responses to DNA damage, presumably allowing damage to go unrepaired (128). Likewise cadmium can effectively block apoptosis induced by DNA-damaging agents (129) apparently through inhibition of enzymes critical to cellular dedication to apoptosis (130). This perturbed apoptosis could allow the survival of cells with damaged DNA that would otherwise be eliminated (129,130). On the other hand, chromium effectively induces cellular apoptosis (107,131–133) and the dual potentials of chromium in carcinogenesis and apoptosis may coexist in response to chromium-induced DNA damage. It is thought that a small population of cells with chromium-induced DNA damage may escape from cell cycle checkpoints, thus becoming transformed as the first step to malignancy (132,133). Thus stimulation of the rate apoptosis, as with chromium, may actually reduce the probability of a damaged cell within a given population from undergoing apoptotic cell death and may confer a selective growth advantage on that cell and its clonal descendants, thereby acting as a prelude to development of neoplasia (134).

It is quite evident that the definition of mechanism in metal carcinogenesis will require much further work. It should be recognized that, in all likelihood, not all metals will have the same mechanism and that the same metal may have different mechanisms in different tissues.

9. PROBLEMS IN ASSESSING CARCINOGENIC RISK FROM METALS

There are important issues confronting the assessment of human carcinogenic risk associated with exposure to metals, but these are not necessarily unique to metallic agent carcinogens. A major issue is that frequently there are only small populations that have been exposed to well-defined levels of a given metal in the absence of exposure to other metals. Exposure populations can also otherwise be limited and, with occupational exposure to metals, females have been infrequently available for analysis. This creates a whole subset of our population in which the potential sensitivity to metal carcinogenesis is essentially undefined. Additionally, human metal exposures are invariably of a complex nature, including erratic rates of exposure and/or differing periods of exposure. The nature of metal exposure often changes with time and the advent of new technologies and different industrial applications. Concurrent exposure to multiple species of the same metal is quite likely, and although metal speciation is an important consider-

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ation for defining carcinogenic potential, it is difficult, if not impossible, to define precisely in human exposure situations. Human exposures are also almost invariably to multiple toxic agents. The toxicological implications of multiple exposures, of which one or more is a metal, on carcinogenic outcome are largely unknown. Individuals are never exposed to only a single metallic element over a lifetime and the interactions of two or more metallic agents greatly complicate assessment of carcinogenic risk.

Another major problem in defining carcinogenic risk of metals is the general lack of adequate data concerning dose effect. Data are often from high-level exposures in humans or single high-dose studies in animals. Though excessive exposures have occurred, these are now rare and chronic low-level metal exposure now is more likely to occur both in the workplace and through the environment (135). It is certainly conceivable that large doses of metals would exceed the available endogenous detoxication pathways and result in pathophysiological changes that would not occur during low-level exposure. As with any carcinogen, the lack of adequate dose-response data makes extrapolation to effects at chronic low-level exposure difficult and available data generally do not allow definitive elimination of threshold effects. The essentiality of some metals also is a complication in assessing risk and one can be placed in the unenviable position of proposing recommendations for daily intake of a known human carcinogen.

Metals can also show acquired self-tolerance and cross-tolerance. For instance, in rodents cadmium pretreatment will induce tolerance to further cadmium exposure while preexposure to zinc mitigates cadmium toxicity (15). Likewise, cells made tolerant to arsenic also show cross-tolerance to nickel (18). Even the sequence of exposure can have an impact on metal toxicity, as with arsenic and cadmium (136). Thus single-dose carcinogenesis studies potentially do not involve the physiological adaptive mechanisms that would be functioning in cases of repeated or continuous exposures, making interpretation of risk difficult.

10. CONCLUSION

In summary, metallic agents are a very important class of carcinogens. Owing to the nature of modern life, we are all constantly exposed to carcinogenic metals through the environment. This makes defining mechanism a very important task. However, metals are probably the least studied of any major class of human carcinogens and, on this basis alone, deserve additional effort. There are several inherent difficulties in the study of metal toxicology and it is clear that we know very little about many aspects of the molecular mechanisms of metal carcinogenesis. It is, however, likely that mechanisms will prove to be specific to the individual metal and potentially to the particular target tissue as well.

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Arsenic in the Environment: A Global Perspective

Prosun Bhattacharya and Gunnar Jacks
Royal Institute of Technology, Stockholm, Sweden

Seth H. Frisbie
Better Life Laboratories, Inc., Plainfield, Vermont

Euan Smith and Ravendra Naidu
CSIRO Land and Water, Glen Osmond, South Australia, Australia

Bibudhendra Sarkar
The Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada

1. INTRODUCTION

Arsenic (As) is widely distributed in the environment and is known to be highly toxic to humans. Both natural and anthropogenic activities result in the significant input of As to the environment. Natural processes like erosion and weathering of crustal rocks lead to the breakdown and translocation of arsenic from the primary sulfide minerals, and the background concentrations of arsenic in soils are strongly related to the nature of parent rocks. An extensive range of anthropogenic sources may enhance concentration of As in the environment. Some of

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these activities include industrial processes that contribute to both atmospheric and terrestrial depositions, such as mining and metallurgy, wood preservation, urban and industrial wastes, and applications of sewage sludge and fertilizer (1–3). Among the two modes of As input, the environment is mostly threatened by anthropogenic activities. The fate of As accumulated in the surface environment depends essentially on its retention and mobility in the host medium, soil and groundwater, and is most vulnerable for biota.

Arsenic is known to be essential for life in small amounts (4), but sufficiently high exposures to inorganic As in natural environments, such as water, sediment, and soil, have proved to be toxic for plants, animals, and humans. Arsenic exposure caused by groundwater used for drinking in different parts of the world (5,6) has emerged as an issue of great concern. However, As ingestion might also occur through consumption of foods and locally from air. High levels of As exposure are commonly observed among the persons residing around mining areas and smelters, and those working in the wood preservation and pesticide industries using copper-chrome-arsenate (CCA) chemicals and other arsenical preparates, primarily through the inhalation of As-rich aerosols. A limited amount of this As intake is, however, metabolized by the liver to the less toxic methylated forms and excreted through urine. Studies in Denmark, the United Kingdom, and Germany have shown that the average estimate of As intake through food of plant origin is 10–20 μg As/day (7). These values are equivalent to only 10–12% of the estimated dietary intakes of As in these three countries. Bioaccumulation of As in crops grown in areas with elevated atmospheric deposition, contaminated lands, and areas irrigated with contaminated groundwater has raised concern about As ingestion through diet (8–10).

Geochemical behavior of As is very similar to that of phosphorus, which is an important nutrient. Wide distribution of As in natural environments, the geochemical characteristics of As, and an increased dependence on groundwater for drinking have resulted in severe As toxicity for several millions of people worldwide. This chapter explores the environmental behavior of As, with special reference to the abundance and distribution of As in the lithosphere, sediments, soil environment, and groundwater, various pathways of As emission to the environment, methods for As determination in drinking water, and some techniques for remediation of As-contaminated soil and groundwater systems.

2. OCCURRENCE, DISTRIBUTION, AND SOURCES OF ARSENIC EMISSION

2.1 Occurrence and Distribution

Arsenic is a natural constituent of the earth's crust and ranks twentieth in abundance in relation to the other elements. The average As content in continental

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crust varies between 1 and 2 mg As/kg (11,12). Arsenic is widely distributed in a variety of minerals, but commonly occurs as arsenides of iron, copper, lead, silver, and gold, or as sulfides (13–17). Realgar (As_4S_4) and orpiment (As_2S_3) are the two common As sulfides where As occurs in reduced form while As occurs in oxidized form in the mineral arsenolite (As_2O_3). Loellingite ($FeAs_2$), safforlite (CoAs), niccolite (NiAs), rammelsbergite ($NiAs_2$), arsenopyrite (FeAsS), cobaltite (CoAsS), enargite (Cu_3AsS_4), gerdsorfite (NiAsS), glaucodot [(Co,Fe)AsS], and elemental As are other naturally occurring As-bearing minerals (18).

2.2 Sources of Arsenic Emission

From its origin in the earth's crust, As can enter the environment through natural and anthropogenic processes. Two principal pathways of As emission in the environment, are (a) natural processes and (b) industrial activities. Arsenic is released in the natural environment through natural processes such as weathering and volcanic eruptions and may be transported over long distances as suspended particulates through water or air. Industrial activity is, however, the more important source of As emission and accounts for widespread As contamination (3,4). In the following section, we discuss these two principal modes of As emissions and their comparison among these two sources.

2.2.1 Natural Sources

Mean global atmospheric emission of As from natural sources is about 12.2 gigagram (19). These sources include windblown dust from weathered continental crust, forest fires, volcanoes, sea spray, hot springs, and geysers (20,21). Emissions of As from volcanic eruptions vary considerably, as high as 8.9 gigagrams/year from Mount Saint Helens in the United States to about 0.04 gigagram/year from Poas in Costa Rica (20). Arsenic emission through volcanic eruptions is mostly in the form of dust—ca. 0.3 gigagram/year compared to nearly 0.01 gigagram/year as volatile forms (22).

Typical contents of As in different crustal materials are presented in Table 1. Local concentration of As occurs in the hydrothermal ore deposits such as in the arsenopyrite, orpiment, realgar, and other base metal sulfides (13). In sedimentary environments, As occurs as sorbed oxyanions in oxidized sediments. The concentrations of As vary between 0.6 and 120 mg/kg in sand and sandstones and as high as 490 mg/kg in shales and clay formations (11). Arsenic is incorporated in diagenetic pyrite (FeS_2), formed widely in sediments rich in organic matter, especially black shales, coal, peat deposits, and phosphorites (21,23,24). Coals from different geological basins contain 0.5–80 mg As/kg and the average As concentration for world coal is reported to be 10 mg/kg (25,26). High-As-bearing coals have been reported from the former Czechoslovakia (maximum

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TABLE 1 Abundance of Arsenic (mg As/kg) in Crustal Materials (11,28)

Rock type	Range
Igneous rocks	
Ultrabasics	0.3–16
Basalts	0.06–113
Andesites	0.5–5.8
Granites/silicic volcanics	0.2–13.8
Sedimentary rocks	
Shales and clays	0.3–490
Phosphorites	0.4–188
Sandstones	0.6–120
Limestones	0.1–20
Coals	0.5–80

1500 mg As/kg) and Guizhou province of China (as high as 35 g/kg) (27–29). Peats may also contain significant quantities of As; for example, Finnish peat bogs contain 16–340 mg As/kg on a dry weight basis (23).

Arsenic concentration in seawater is reported to be around 2.6 μg/L (30), while rainwater derived from uncontaminated mass of oceanic air contains an average 19 ng As/L (31). In natural lakes, levels of As range from 0.2 to 56 μg/L (32), but a level as high as 15 mg/L has been reported in Mono Lake, in California (33). River water contains low As, but a significant partitioning is observed among the As concentrations in the suspended particulates and the aqueous phase (34). High levels of As are noted in both dissolved and particulate phases in rivers influenced by contamination from anthropogenic sources in Europe and North America (35–37). Low As concentrations are, however, reported from pristine river-estuarine systems of Krka, Yugoslavia (37) and Lena, Russia (38). Among the major rivers in the United States, the Columbia River in Oregon has an average As concentration of 1.6 μg/L (34). In Yellowstone National Park, the Madison River contains 250–370 μg/L of dissolved As (39). Concentrations of dissolved As are, however, lower and vary between 16 and 176 μg/L upstream and 25 and 50 μg/L downstream of the park. Among the major rivers of Bangladesh, dissolved As concentrations vary between 0.7 and 1.1 μg/L in the Padma River, while in the Meghna River, the concentrations vary between 0.6 and 1.9 μg/L (Bhattacharya, 2001, unpublished data). Low levels of As (0.6 μg/L) are noted upstream of the river at Bhairab Ghat, Ashuganj, but the concentrations are higher (1.9 μg/L) downstream of the river near Laxmipur. In China, dissolved As concentrations in the Huanghe River are found to increase from 1.4–1.5

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μg/L in upstream water to 2.3–2.4 μg/L in the water in the middle and lower reaches of the river (40).

The cycling of As is caused by the interactions of natural water with bedrock, sediments, and soils as well as the influence of local atmospheric deposition. Weathering and leaching of geological formations and mine wastes result in elevated concentrations of As in natural waters in several areas. Mobility of As is constrained in the surface water because of the prevalence of oxic conditions. On the other hand, reducing conditions offered by the aquifers lead to the mobilization of As, thereby increasing the risk of groundwater contamination. Natural occurrence of As is widely reported in groundwater in several parts of the world, and the concentrations vary significantly depending on the redox characteristics of the groundwater and the lithological characteristics of the bedrock (41,42).

2.2.2 Anthropogenic Sources

The major producers of As_2O_3 (''white arsenic'') are the United States, Sweden, France, the former USSR, Mexico, and southwest Africa. The uses of As compounds are summarized in Table 2. Arsenic compounds such as monosodium methylarsonate ($NaCH_3HAsO_3$), disodium methylarsonate ($Na_2CH_3AsO_3$), and diethylarsenic acid [$(CH_3)_2AsO(OH)$] are widely used as agricultural insecticides, larvicides, and herbicides. Sodium arsenite (NaH_2AsO_4) is used for aquatic weed control and for sheep and cattle dips. Arsenic acid (H_3AsO_4) is used to defoliate cotton bolls prior to harvesting and as a wood preservative. As_2O_3 is used to decolorize glass and in the manufacture of pharmaceuticals. Elemental As is mainly used in Pb, Cu, Sb, Sn, Al, and Ga alloys (18,43).

Mining, smelting, and ore beneficiation, pesticides, fertilizers, and chemical industries, thermal power plants using coal, wood preservation industries using CCA, and incinerations of preserved wood wastes contribute to significant influx of As to the environment (3,44). Global emissions of As in the atmosphere have been estimated to be 0.019 gigagram (0.012–0.026 gigagram), but in soil and

TABLE 2 Commercial Uses of Arsenic Compounds in the United States (18)

Use	As (metric tons)	Percentage
Pesticides	26,000	65
Wood preservatives	7,200	18
Glass	3,800	10
Alloys and electronics	1,100	3
Miscellaneous	1,500	4

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aquatic environment, the estimated figures are 0.082 and 0.042 gigagram, respectively (45). However, there has been a substantial decrease in the atmospheric emission of As in Europe, from circa 0.005 gigagram in 1986 to 0.00031 gigagram in 1995 (46,47).

Mining and Ore Beneficiation. Elevated concentrations of As, as well as other metals such as cadmium, copper, iron, lead, nickel, and zinc, are commonly encountered in the acid mine effluents. The principal source of As in mine tailings is the oxidation of arsenopyrite (FeAsS) following the reaction:

$$\mathrm{FeAsS\ (s) + 13Fe^{3+} + 8H_2O \Leftrightarrow 14Fe^{2+} + SO_4^{2-} + 13H^+ + H_3AsO_4\ (aq)}$$

Arsenopyrite can be oxidized by both O_2 and Fe^{III}, but the rate of oxidation by Fe^{III} is faster than for pyrite (48). The rate of this reaction was reported as 1.7 $\mu mol/m^2/s$, a reaction faster than a similar oxidation reaction for pyrite. Under extremely acidic environment, with a pH of about 1.5 and an aqueous As concentration at >10 mmol/L, As precipitates as scorodite ($FeAsO_4 \cdot 2H_2O$) (49). Under acidic conditions (pH < 3), As^V may substitute SO_4 in the structure of jarosite [$KFe_3(SO_4)_2(OH)_6$] in different mine wastes (50). Adsorption of As on $Fe(OH)_3$ surfaces was found to be the principal sink for As in studies of acid mine drainage (51). However, the adsorption of As by $Fe(OH)_3$ may be only transient as changes in redox conditions (Eh) and pH may result in dissolution of $Fe(OH)_3$ with consequent mobilization of As. Effluents and water in tailings ponds are often treated with lime to increase pH levels to stabilize the dissolved As and other metals as precipitates.

Agriculture. Over hundreds of years, inorganic arsenicals (arsenic trioxide, arsenic acid, arsenates of calcium, copper, lead, and sodium, and arsenites of sodium and potassium) have been widely used in pigments, pesticides, insecticides, herbicides, and fungicides (52–57). At present, As is no longer used in agriculture in the West, but persistence of the residues of the inorganic arsenicals in soils is an issue of environmental concern (58–61). Studies by Kenyon et al. (62) and Aten et al. (63) have indicated elevated concentrations of As in vegetables grown in soils contaminated by lead arsenate used as an insecticide in apple orchards. The recalcitrant nature of arsenical herbicides has, however, been observed in agricultural soils particularly around old orchards (64). Biomethylation of As (65,66) is a mechanism through which a significant quantity of methylarsines may be released into the atmosphere following the application of As compounds to the soil. A relatively faster production of dimethyl- and trimethylarsines has been reported from grasslands treated with methylarsenic compounds while grass treated with sodium arsenite indicated slow release of methylarsene into the atmosphere.

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TABLE 3 Common Water-Soluble Arsenic-Based Chemicals Used for Wood Preservation (3)

Preservative	Year of use	Composition	Percent	Percent metal in pure form
Boliden S25	1951–1954	Zn(II) oxide (ZnO)	11.6	9.3 Zn
		Copper(II) oxide (CuO)	3.9	3.1 Cu
		Chromium trioxide (CrO_3)	23.0	12.0 Cr
		Diarsenic pentoxide (As_2O_5)	36.0	23.5 As
		Water (H_2O)	25.5	
K33, CCA type B	1952–1990	Copper(II) oxide (CuO)	14.8	11.8 Cu
		Chromium trioxide (CrO_3)	26.6	13.8 Cr
		Diarsenic pentoxide (As_2O_5)	34.0	22.2 As
		Water (H_2O)	24.6	
Celcure/C33 (or equivalents)	1983–1990	Copper(II) sulfate ($CuSO_4 \cdot 5H_2O$)	23.2	8.2 Cu
		Copper(II) oxide (CuO)	2.8	
		Chromium trioxide (CrO_3)	40.0	14.0 Cr
		Diarsenic pentoxide (As_2O_5)	22.7	14.8 As
		Water (H_2O)	11.3	

Wood Preservation. The use of CCA and other As-based chemicals in wood preservation industries has caused widespread contamination of soils and aquatic environments (3,67–73). CCA had attained wide-scale industrial application as a wood preservative owing to biocidic characteristics of Cu^{II} and As^{V}. The preservative chemical used for pressure impregnation comprises a water-based mixture of dichromic acid ($H_2Cr_2O_7$), arsenic acid (H_3AsO_4), and Cu^{II} as divalent cation at variable proportions (Table 3) (3). Chromium is used to bind As and Cu into the cellular structure of the wood. Fixation of CCA is dependent on the transformation of Cr^{VI} to Cr^{III}, a reaction that is dependent on the temperature and water content of the wood. Cr^{III} forms insoluble complexes with both As and Cu (74). Further stabilization of these complexes takes place after complete fixation of the As and Cu in the wood tissues and minimizes the risk of leaching of the CCA components from the processed wood. Among the active ingredients of CCA wood preservatives, As is most mobile and toxic to a broad range of organisms, including human beings.

Studies around an abandoned wood preservation site at Konsterud, Kristinehamns Community in Central Sweden (70,71) revealed soil As concentrations between 10 and 1067 mg/kg, and the order of abundance for metal contaminants was found to be As $>$ Zn $>$ Cu $\geq$ Cr. Sediments in a drain adjacent to the

cemented impregnation platform contained an average 632 mg As/kg. Arsenic concentrations in the reference soils (119 mg/kg) were lower than in the contaminated area, but exceeded the level of As in average glacial till (75). Analyses of water in a stream found As concentration of 238 μg/L (70). Groundwater contamination must therefore be considered as an imminent risk close to wood preservation sites, and especially at older sites where precautions against spills and material handling were not taken adequately.

Coal Combustion and Incineration of Preserved Wood Products. Combustion of high-As-bearing coals is known to be a principal pathway of As emission in the Guizhou province of southwestern China (28,29). Open coal-burning stoves used for drying chili peppers have been the principal cause of chronic As poisoning in a population of nearly 3000. Fresh chili peppers have less than 1 mg/kg As, while chili peppers dried over high-As coal fires were reported to contain more than 500 mg/kg As (28). Consumption of other tainted foods, ingestion of kitchen dust containing as high as 3000 mg/kg As, and inhalation of indoor air polluted by As from coal combustion are the other causes of chronic As poisoning.

A possible pathway for exposure through air particulates is the incidental use of preserved wood in open fires, indoors or outdoors. Incineration of CCA-impregnated wood from a sawmill was found to be a source of As contamination to the environment (76). The content of As in air particulates from open fires was found to exceed the German air quality standards by 100-fold (77). The ashes, spread on lawns or vegetable cultivations, pose further risk to human health. In addition, tobacco smoke is another source of As emission in the indoor environment. It is interesting to note that mainstream cigarette smoke contains 40–120 ng As per cigarette (78).

Comparison of the Contributions of Arsenic from Natural and Anthropogenic Sources. An overview of the sources of natural and anthropogenic emission and the biogeochemical cycle of As is presented in Figure 1. Natural emission of As in the atmosphere is estimated to be around 2.8 gigagrams/year as dust and 21 gigagrams/year as volatile phases. Among the natural sources, wind-blown dust from crustal weathering, forest fires, vegetation emissions, volcanoes, and sea spray are significant (20,79,80). Anthropogenic emissions of As account for as high as 78 gigagrams/year and are thus significantly higher compared to the natural inputs (79). The concentration of As can therefore be appreciably high in the areas affected by anthropogenic activities. A considerable amount of As is released by the combustion of fossil fuels, especially coal, from wood preservation industries as well as the use of the preserved wood products. Mining and smelting of ore minerals including sulfides of copper, lead, and zinc, as well as gold processing, have contributed to significant environmental As emissions in the past, but changes in smelting processes during the last decade have signifi-

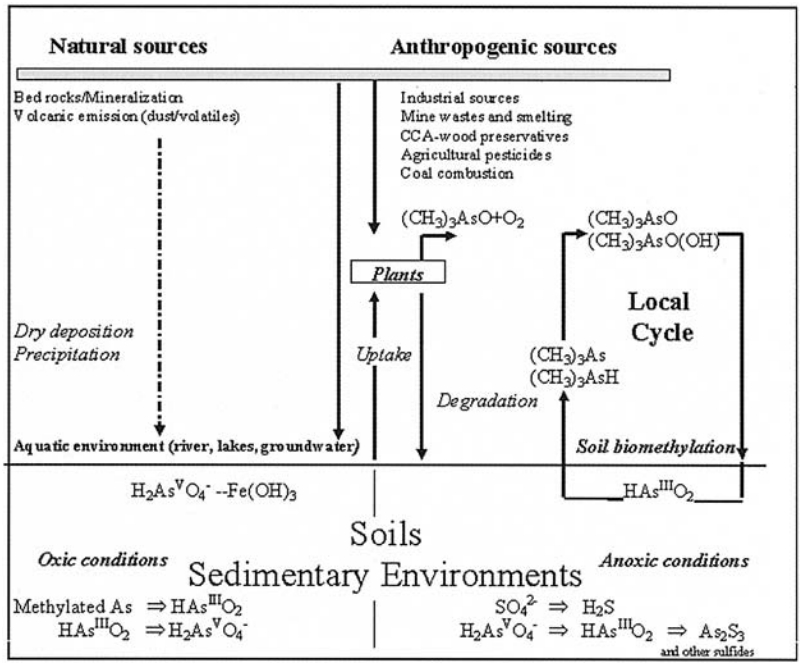

FIGURE 1 Natural and anthropogenic sources and biogeochemical cycling of As in sedimentary environment. (Modified from ref. 3.)

cantly reduced the emission of As from these sources. However, according to an estimate made by the USEPA, nearly 6,000,000 people living within 12 miles of these copper, zinc, and lead smelters may be exposed to 10 times the average atmospheric levels of As in the United States (78). In another study it has been shown that nearly 40,000 people were at risk of exposure to As levels exceeding the national atmospheric levels by 100 times in the vicinity of some copper smelters (43). Significant bioaccumulation of As occurs in crops grown in contaminated soils around lead smelters (81).

3. GEOCHEMISTRY OF ARSENIC IN SOILS AND NATURAL WATER

3.1 Chemistry of Arsenic in Soil

The natural content of As in soils varies considerably (17) but is mostly in a range below 10 mg/kg (82–85). The background concentration of As in soils is

governed by the lithology of the parent rocks. Arsenic concentrations in Swedish tills (<0.06 mm) range between <5 and 175 mg/kg, with a median value of 8 mg/kg (O. Selenius, personal communication, 2000). Availability and dispersal of As in the soil environment are influenced by several factors (16,71,86). Climatic and geomorphic characteristics in an area, such as rainfall, surface runoff, rate of infiltration, and the groundwater level and its fluctuations, affect the mobility and distribution of As (87). The speciation and mobility of As in soils are also governed by the soil physical characteristics, such as grain size and mineralogy, and chemical characteristics like redox potential (Eh) and pH conditions of the soils (88). Sorption characteristics of As in soils and bioavailability are also governed by the composition of clay minerals (89–92).

3.1.1 Weathering of Primary Sulfide Minerals

Geochemical cycling of As is triggered by chemical weathering. Arsenic is released in the soil environment owing to weathering of the arsenopyrite (FeAsS) or other primary sulfide minerals. Important factors controlling the weathering reactions are: (a) the presence of water and its composition, (b) pH, (c) temperature, (4) reactivity of the species with CO_2/H_2O, (5) hydrolysis, (6) solubility, and (7) redox characteristics of the species. The release of As from FeAsS involves both hydrolysis and oxidation. Weathering of arsenopyrite in the presence of dioxygen (O_2) and water involves oxidation of S^{2-} to SO_4^{2-} and As^{III} to As^{V}, both taking place through the reduction of O_2 (93). The complete reaction could be represented as:

$$4FeAsS + 13O_2 + 6H_2O \Leftrightarrow 4SO_4^{2-} + 4AsO_4^{3-} + 4Fe^{2+} + 12H^+$$

The half-redox reactions are written as:

$$O_2 + 4H^+ + 4e^- \Rightarrow 2H_2O \qquad E^O = 1.23\ V$$
$$S^{2-} + 4H_2O - 8e^- \Rightarrow SO_4^{2-} + 8H^+ \qquad -E^O = -0.76\ V$$
$$AsO_2^- + 2H_2O - 2e^- \Rightarrow AsO_4^{3-} + 4H^+ \qquad -E^O = -0.56\ V$$

Once released from the mineral, As can be mobilized by different physical as well as chemical processes (94).

3.1.2 Speciation and Solubility of Arsenic in Soil and Water

Arsenic in the soil environment normally occurs in the +III and +V oxidation states (16). In soils and natural waters, As typically occurs as weak triprotic oxyacids. In reducing environment, arsenous acid dominates in the form of $H_3As^{III}O_3^0$ at a wide range of pH values while the protonated $H_2As^{III}O_3^-$ forms only at pH > 9.0. At higher pH and in an oxidized environment, As^V is present as $H_2AsO_4^-$ (pH < 7.0) or as $HAsO_4^{2-}$ (pH > 7.0) (88,95–98). Arsenic acid is

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a moderately strong oxidizing agent and is readily reduced to arsenous acid (98), according to the equation:

$$H_3As^{V}O_4 + 4H^+ + 2e^- \Leftrightarrow H_3As^{III}O_3 + 2H_2O \qquad E^0 = 0.559 \text{ V}$$

The typical dissociation diagrams for arsenic and arsenous acids are presented in Figure 2. In the natural environment, speciation of As changes qualitatively according to the thermodynamic predictions (86). In the As-H_2O-O_2 system, stable inorganic As species are $H_3As^{III}O_3$, $H_2As^{V}O_4^-$, $HAs^{V}O_4^{2-}$, or As(s). However, in the presence of dissolved S in the system, a range of As sulfides (AsS_2^-, As_2S_3, and $HAsS_2$) are stable (34). The E_H-pH diagram for 10^{-5} M aqueous As in the presence of dissolved O_2 and S is given in Figure 3.

As^{III} is more toxic and more mobile in soils than As^{V} (34,99–102). Arsenic is readily mobile as methylated species, such as monomethylarsonic acid

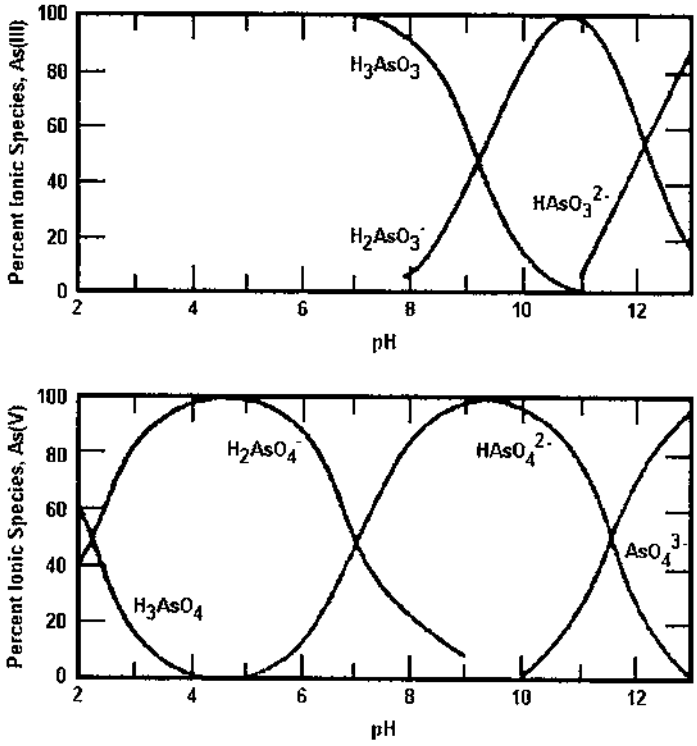

FIGURE 2 Acidic dissociation diagram for $H_3As^{III}O_3$ and $H_3As^{V}O_4$. (Adapted from ref. 271.)

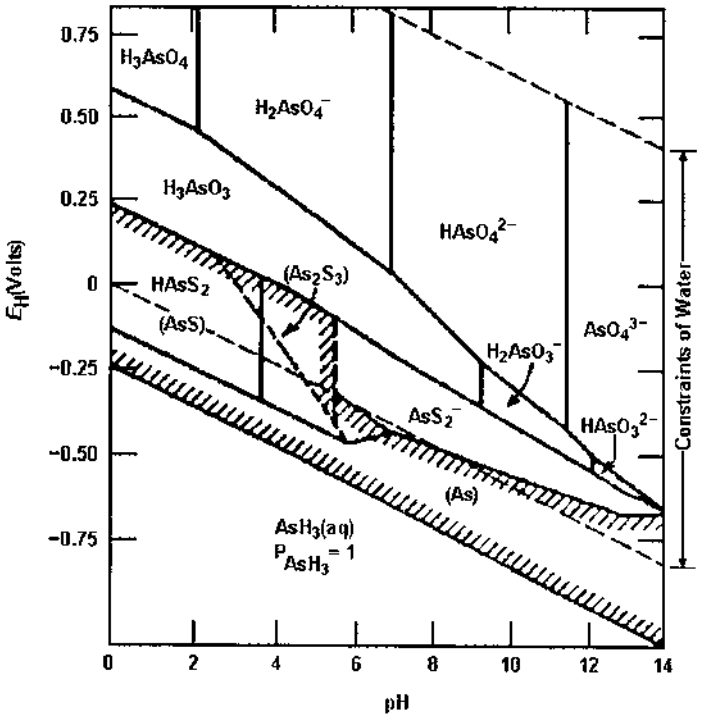

FIGURE 3 E_H-pH diagram for As at 25°C and 1 atmosphere with total As 10^{-5} M and total S 10^{-5} M. Solid As compounds are enclosed with parentheses in the cross-hatched area. (Adapted from ref. 34.)

[MMAA, $CH_3AsO(OH)_2$] and dimethylarsinic acid [DMAA, $(CH_3)_2AsO(OH)$] by reaction of $H_3AsO_3^0$ with methylcobalamin in the presence of anaerobic bacteria (103). However, these volatile forms are not stable under oxidizing conditions and get back to the soil environment in inorganic forms (16,104).

Ferric hydroxide generally plays a much more important role in controlling the concentration of As in soils as well as in aqueous media. The precipitation of ferric hydroxide can be expressed by the reaction:

$$Fe^{III} + 3H_2O \Leftrightarrow Fe(OH)_3 + 3H^+$$

This reaction has critical importance for retention and mobilization of As in soils. Both As^V and As^{III} are adsorbed on $Fe(OH)_3$, but affinity for adsorption is higher for As^V as compared to As^{III}. The adsorption optimum for As^{III} is around pH 7.0, while As^V adsorbs optimally at pH 4.0 (105). The absolute magnitude

of adsorption is higher for arsenate, As^V, over the pH range <9.0 for arsenate (106). Arsenic is readily desorbed from $Fe(OH)_3$ with an increase in pH and also due to competing anions like PO_4, MoO_4, and SO_4 for the adsorption sites (107). The geochemical behavior of As^V and P is strikingly similar and both form complexes with Fe, Al, and, under specific circumstances, even with Mn (86, 100,108,109). Short-range-order secondary aluminosilicates, imogolite and allophane [termed collectively imogolite-type materials (ITM)], and ferrihydrite are the commonly occurring minerals in spodic horizons in Swedish podsols (110). These minerals are characterized by large surface area and high positive surface charge under acidic pH and effectively adsorb the bulk of As^V (71,110,111). Both As^V and As^{III} behave as chelates and precipitate with many metallic cations (112). $Ca_3(AsO_4)_2$ is the most stable As^V species in well-oxidized alkaline environments. Under reducing conditions and high concentrations of Mn in the soils the solubility of As is controlled by $Mn_3(AsO_4)_2$ (88).

In oxidized soils (E_H = 0.2–0.5 V), As^V is immobile and coprecipitated with $Fe(OH)_3$, a mineral phase that dissolves under moderate to low reducing conditions (E_H = 0–0.1 V) and controls the solubility of As^V (113). On aging, amorphous $Fe(OH)_3$ gets transformed goethite (FeOOH) and releases part of the adsorbed As owing to a decrease in reactive surface area (114). Complexation of As by the dissolved organic matter and humic acids in soil environments prevents sorption and coprecipitation of As with solid phases leading to an increased mobility of As in soil and water (61,115).

In the basic and acidic effluents from waste dumps from the gold-processing units in Canada (116), dissolved As represented 1% and 13% of the total As, respectively. At pH 9.5, the stream contained 910 mg/L particulate As and 10.1 mg/L soluble As, while the other waste stream at pH 3.1 contained 880 mg/L particulate As and 132 mg/L soluble As.

Despite the high affinity of soil for As, the kinetics of As retention by soils to levels below toxicological concern may be very slow. Lead arsenate and copper acetate-arsenate, once commonly used insecticides, may require decades to be converted to nonphytotoxic forms in soils (95). An encapsulation of As by other precipitates is assumed to be the mechanism of detoxification in these soils.

3.2 Arsenic in Groundwater

3.2.1 Aqueous Speciation, Mobility, and Global Occurrence

The origin and mobility of As in the groundwater environment has received significant attention in recent years. Water is the major pathway for the influx of As in the environment, although atmospheric inputs contribute significantly to the As concentrations in natural aquatic ecosystems. Elevated concentrations of As in natural waters are known to have resulted from weathering and leaching

of As-rich geological formations, drainage from mine tailings and wastes, and thermal springs and geysers in several parts of the world. However, As is mobilized in groundwater through complex geochemical processes in natural environments (6,16). Anoxic conditions in the subsurface environments enhance As mobility, which renders groundwater more vulnerable for As contamination as compared to surface water. Groundwater contaminated with As used for drinking is thus an issue of major concern owing to severe toxic effects on human health.

In groundwater, inorganic As commonly exists as As^{V} (arsenate) and As^{III} (arsenite), the latter being considered to be more mobile and toxic for living organisms (117). In aqueous environments prokaryotes and eukaryotes reductively biomethylate inorganic As to DMAA and MMAA (118), but the toxicity of these methylated forms is less. Biomethylation is a more subtle, but persistent process, which may affect mobility and transport of As in groundwaters. Biomethylation involves degradation of organic matter and conversion of As^{V} to the more soluble As^{III} species and mobilizes As from the aquifers into groundwater. Although little is known about formation of methylated arsenicals in groundwater, it is important to emphasize the need to understand the biogeochemical interactions in the aquifers as methylation increases solubility of As species and thereby affects the dispersion of As in the environment (119). Anoxic conditions in aquifers may enhance As methylation through degradation of organic matter by bacteria (H. Hasegawa, personal communication, 2000). Interestingly, methylated As species also sorb onto Fe oxides with an affinity in the order of $As^{V} >$ DMAA $> As^{III} >$ MMAA (120) and may therefore affect the distribution and mobilization of As.

On a global scale, As is widely reported in groundwater from several countries. Natural occurrences of As are known in groundwaters of the United States (summarized in ref. 121), Canada (122), Argentina (123,124), Mexico (125–128), Chile (129,130), Ghana (131), Hungary (132), the United Kingdom (133,134), Finland (135), Taiwan (136,137), China (138–140), Japan (141), southern Thailand (142), West Bengal, India (6,143–147), and lately from Bangladesh (148–154). A similar problem of As contamination in groundwater may also emerge in the Mekong Delta (155,156). Some of the salient aspects of the distribution, concentration, and possible mechanisms for the release of As in groundwater in a few of these affected countries are summarized in Table 4.

3.2.2 Drinking Water Criteria for Arsenic

Arsenic in drinking water affects human health and is considered one of the most significant environmental causes of cancer in the world (157). Keeping in view the toxic effects of inorganic As on humans and other living organisms, it is necessary to understand the level of As in drinking water, and its chemical speciation, to establish regulatory standards (121). The FAO health limit for As in groundwater was 50 μg/L, but in view of recent incidences of As poisoning in

the Indian subcontinent, a decrease in the groundwater As concentration to 5–10 μg/L is being considered by a number of regulatory bodies throughout the world. The provisional WHO guideline value adopted for As in drinking water is 10 μg/L, which is based on a 6×10^{-4} excess skin cancer risk, which is 60 times higher than the factor that is typically used to protect public health. WHO states that the health-based drinking water guideline for As should be 0.17 μg/L. However, the detection limit for most laboratories is 10 μg/L, which is why the less protective guideline was adopted (158–160).

The U.S. Environmental Protection Agency (USEPA) drinking water standard for As, 50 μg/L, was set by the EPA in 1975, based on a Public Health Service standard originally established in 1942 (161). On the basis of the investigations initiated by National Academy of Sciences, it was concluded that the previous standard did not eliminate the risks of long-term exposure from low As concentrations in drinking water causing skin, bladder, lung, and prostate cancer. There are several noncancer effects of As ingestion at low levels, including cardiovascular disease, diabetes, and anemia, as well as reproductive and developmental, immunological, and neurological disorders. To achieve the EPA's goal of protecting public health, recommendations were made to lower the safe drinking water limit to 5 μg/L, which is higher than the technically feasible level of 3 μg/L (162). Recently the USEPA has established a health based *nonenforceable* maximum contaminant level goal (MCLG) for zero As and an *enforceable* maximum contaminant level (MCL) of 10 μg As/L in drinking water (163), which would apply to both nontransient, noncommunity water systems and community water systems as against the previous MCL of 50 μg As/L set by the USEPA in 1975. However, the current drinking water guideline for As, 10 μg/L, adopted by WHO and the USEPA is higher than the Canadian and Australian maximum permissible concentrations of 5 and 7 μg As/L, respectively.

3.2.3 Determination of Arsenic in Natural Water

Sampling Considerations. Well-defined sampling protocol is essential for the determination of As in water samples. Arsenic occurs predominantly as As^{III} and As^{V} in natural waters along with dissolved Fe at varying concentrations. It is necessary to prevent postsampling oxidation of Fe^{II} to Fe^{III} and consequent precipitation in the form of $Fe(OH)_3$, which is an efficient scavenger for a series of contaminant species including As. Thus, sampling must be carried out very cautiously in the field for the determination of As (164). Water samples need to be filtered through an 0.45-μm membrane online filter and then acidified with suprapure HCl/HNO_3 to pH below 2.0, and headspace in the sampling bottle should be avoided. In cases where colloidal iron is suspected in the water sample, it is recommended that several replicates be taken.

Oxidation of As^{III} to As^{V} is considered a relatively slow process (165). Speciation of As^{III} and As^{V} in groundwater can be carried out in the field using

TABLE 4 Comparison of Arsenic Occurrences in Groundwater from Selected Parts of the World

Country/region	Area affected	Depth of well	Arsenic conc. (μg/L)	Mechanism of contamination	Ref.
Bangladesh, BDP (52 districts)	118,012 km^2	8–260 m	<2–>900	Reduction of Fe oxyhydroxides/ sulfide oxidation(?) in alluvial sediments?	148,151,179
West Bengal, India, BDP (8 districts)	34,000 km^2	14–132 m	<1–1300	Reduction of Fe oxyhydroxides/ sulfide oxidation (?) in alluvial sediments	6,151,169
China, Xinjiang Inner Mongolia (HAB)	4800 km^2	Shallow/deep	<50–1860	Reducing environment in alluvial sediments	138,139,140,201
Taiwan	—	Deep	Up to 1820	Oxidation of pyrite in mine tailings	136,137
Thailand (10 districts)	10 districts	Shallow	120–6700	Oxidation of mine wastes and tailings	142

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Ghana	1600 km^2	70–100 m	2–175	Oxidation of arsenopyrite in mine tailings	131,203
Argentina (Chaco-Pampean Plains)	10 million km^2	Shallow aquifers	100–4800	Volcanic ash with 90% rhyolitic glass	123,124
Chile	—	Shallow and deep wells	100–1000	Volcanic ash	129,130,134,197
Mexico, Zimapán, Lagunera	—	Shallow and deep wells	300–1100	Oxidation of sulfide from mine wastes	126,127,204,205
Hungary (Great Hungarian Plain)	4263 km^2	80–560 m	25–>50	Complexation of arsenic with humic substances	132
USA	Large areas	53–56 m	100–>500	Desorption of arsenic from Fe oxyhydroxides/ sulfide oxidation	121,213
Canada (Nova Scotia)	—	8–53 m	18–146	Oxidation of sulfides	122
United Kingdom (Cornwall)	—	Shallow wells	>10	Oxidation of sulfides from mine wastes	133,211

Source: Adapted from ref. 156.

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Ficklin's method (166). Columns with dimensions of 100 × 70 mm are packed with a slurry of 2.3 g anion resin. The resin is chloride based, and is converted to acetate forms before use in the field. The columns need to be capped tightly to prevent drying. At the sampling sites, groundwater is filtered through a 0.45-μm online filter and acidified with 0.5 ml suprapure concentrated HCl; 5 ml of the acidified groundwater is then passed through the ion exchange column followed by 15 ml of 0.12 M HCl, added in three more portions. Four 5-ml fractions are collected, and the first two fractions contain As^{III}, while the last two contain As^{V} (166). Arsenic can thereafter be analyzed in the four separate fractions. This method has been successfully used by von Brömssen (151) and Hermansson (152) for As speciation in the field. The method is however, complicated to use in the field, especially because of the time required for pretreatment of the resin and the runs for separation.

However, it is possible to separate As^{III} from As^{V} immediately in the field using specially devised Disposible Cartridges, special online filters (167). These Disposible Cartridges are packed with adsorbents that selectively adsorb As^{V} while As^{III} in water passes through the filter. Thus, As^{III} can be separated from water at pH between 4 and 9 by simply attaching the cartridge to an online 0.45-μm filter fitted to a syringe in the field. The filtrate collected in a separate bottle needs to be acidified for measurement of As (regardless of later oxidation) as As^{III}.

Laboratory Measurements. Silver diethyldithiocarbamate (SDDC) method. The SDDC method can be accurate, precise, and sensitive; however, this analytical method requires highly skilled and intensive labor, demands a well-ventilated work area for safe operation, generates significant volumes of toxic wastes, and is subject to matrix interferences. This method can be used to measure arsenite ($H_3As^{III}O_3$, $H_2As^{III}O_3^-$, $HAs^{III}O_3^{2-}$, and $As^{III}O_3^{3-}$), arsenate ($H_3As^{V}O_4$, $H_2As^{V}O_4^-$, $HAs^{V}O_4^{2-}$, and $As^{V}O_4^{3-}$), and total inorganic As (arsenite plus arsenate) in aqueous samples. The analyte is selectively reduced to arsine (AsH_3). The arsine is distilled from the sample matrix through aqueous lead acetate [$Pb(CH_3COO)_2$] supported on glass wool to remove hydrogen sulfide (H_2S); the arsine is collected in a stabilized organic solvent, where the arsine is reacted with silver diethyldithiocarbamate [$AgSCSN(C_2H_5)_2$] to produce a red derivative that is determined spectrophotometrically at 520 nm (168).

Total inorganic As is determined in the absence of methylarsenic compounds after reduction to arsine by aqueous sodium borohydride ($NaBH_4$) at pH 1. Methylated arsenicals, if present, are reduced to methyl arsines at pH 1, which form colored interferences. As^{III} is determined after selective reduction to arsine by aqueous sodium borohydride at pH 6. As^{V}, MMMA, and DMMA are not reduced under these conditions. As^{V} is determined in a separate run after the removal of As^{III} from the sample as arsine (168).

Hydride generation–atomic absorption spectroscopy (HG-AAS) method. HG-AAS is the preferred method of the American Public Health Association (APHA), the American Water Works Association (AWWA), and the Water Environment Federation (WEF) for determining As in water. This method can be used to measure total As (inorganic plus organic) in aqueous samples. Inorganic and organic forms are oxidized to As^{V} by acidic digestion. This As^{V} is quantitatively reduced to As^{III} with sodium iodide (NaI). This As^{III} is further reduced to arsine (AsH_3) with sodium borohydride ($NaBH_4$), directed into an argon/hydrogen flame, and quantified by atomic absorption spectroscopy. Interferences are minimized because As is removed from the sample matrix prior to detection (168).

4. CASE STUDIES ON ARSENIC CONTAMINATION IN GROUNDWATER

Chronic exposure of As due to drinking of contaminated groundwater is a global catastrophe affecting several millions of people particularly in the developing world. Chronic As poisoning has been reported from Argentina, Bangladesh, Chile, China, Ghana, Hungary, India, Mexico, Taiwan, Thailand, the United Kingdom, and the United States (134,156), where groundwater has been used primarily for drinking. Similar incidences of chronic poisoning and cancer have been found globally among the population exposed to groundwater with As concentrations even below the former drinking water standard of 50 μg/L. The situation in the Bengal Delta Plain (BDP) in Bangladesh and in West Bengal, India, one of the densely populated regions of the world, is still critical where several millions are suffering from chronic As-related health effects (6,145,148,169) due to wide-scale dependence on groundwater for drinking. The occurrence, origin, and mobility of As in groundwater of sedimentary aquifers is primarily influenced by the local geology, hydrogeology, and geochemistry of the sediments as well as several other anthropogenic factors such as the land use pattern (6). The following section deals with the salient aspects of groundwater As occurrences in different parts of the world.

4.1 Argentina

A population of nearly 1,200,000 in rural Argentina depend on groundwater with As concentrations exceeding 10 μg/L and the local Argentinian permissible limit of 50 μg/L. The most affected areas are extended parts of the Pampean plain, some parts of the Chaco plain, and some small areas of the Andean range where drinking-water wells contain 50–2000 μg As/L (123,124,170,171). ''Bell Ville Disease,'' a local term describing the As-induced skin cancer, and other cancers of the kidney and liver are associated with As exposure (172) through groundwater.

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The sedimentary aquifers in the region comprise Tertiary aeolian loess-type sediments in the Pampean plain and predominantly fluvial sediments of Tertiary and Quaternary age in the Chaco region. Drinking water for the rural population is supplied from the shallow aquifers and contains around 200 μg As/L (nearly 30% as As^{III}) besides high concentrations of fluoride (2.1 mg/L) (124). Only some larger towns and the cities use deeper aquifers, which locally also contain As (viz. in Santa Fe province) or they import water from other sites.

4.2 Bengal Delta Plain (Bangladesh and West Bengal, India)

The natural incidence of high-As groundwater in the vast tract of alluvial aquifers within the BDP in Bangladesh and West Bengal, eastern India, has caused a health crisis for a population of over 75 million in the region. Nearly 50 million in Bangladesh are drinking well water with As levels above the acceptable limits. Manifestations of chronic As-related diseases such as *arsenical dermatosis*, *hyperkeratosis*, and *hyperpigmentation* and cancers of the skin have been identified by several epidemiological studies (145,173). Large-scale exploitation of groundwater resources to meet the rising demand of safe water for drinking and agriculture has resulted in this largest As calamity in the world. In addition, As exposure from the diet (9,10,149,153) and the synergetic effects of As and other toxic metals in groundwater and air and their impact on human health also need to be studied in detail.

Manifestations of As toxicity were first identified in West Bengal in 1978, but chronic As poisoning from groundwater was not discovered before 1982–83 (174,175). Natural As occurrences are now encountered in groundwater in eight districts of West Bengal, which covers an area of 37,493 km^2 in the Indian part of the BDP (6,176). Nearly 200,000 people were diagnosed with arsenicosis in West Bengal (177,178); 38.3% of the analyzed groundwaters from West Bengal (176) indicated As levels below 10 μg/L, 44.3% samples indicated As levels above the Bureau of Indian Standards (BIS) drinking water limits, while 55.6% samples had As concentrations below the BIS limit (50 μg/L).

Arsenic was first identified in Bangladesh's well water by the Department of Public Health Engineering in 1993 (134). Of 64 districts in Bangladesh, in 60 districts covering approximately 118,000 km^2 (nearly 80% of the country), groundwaters in a majority of wells have As concentrations exceeding the WHO limit [10 μg/L (158)] and 30% of the groundwater contains As at levels >50 μg/L, the Bangladesh drinking water standard (179). Arsenic concentrations exceeding 1000 μg/L and as high as 14 mg/L in shallow tube wells are reported from 17 districts in Bangladesh (180). According to the national data set, based on the DPHE/UNICEF field kit results, the central and southeast regions in the BDP are most affected. The most systematic laboratory study was conducted by

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DPHE/BGS (181), and the most severely As-affected regions coincided with the area demarcated by the field kit survey. Notably, high-As groundwaters occur in the Chandpur, Comilla, Noakhali, Munshiganj, Brahman Baria, Faridpur, Madaripur, Gopalganj, Shariatpur, and Satkhira districts. In addition, high As levels are also found in isolated ''hot spots'' at the southwestern, northwestern, northeastern, and northcentral regions of the country (Fig. 4). Interestingly, groundwater in the Hill Districts is mostly free from high-As concentrations for yet unknown reasons (181).

The Pleistocene aquifers in the upland Barind and Madhupur tracts are considered to be free from As (134,182). The arseniferous aquifers located in the Holocene BDP lowlands are predominantly confined to depths of 20–80 m (6,41,156). Widespread mobilization of As from the BDP aquifers cannot be attributed to any anthropogenic activities in the region, and evidence indicates a predominantly geogenic source and mode for release of As into the groundwater (6,9,147,150,183,184). However, there exist many uncertainties in understanding

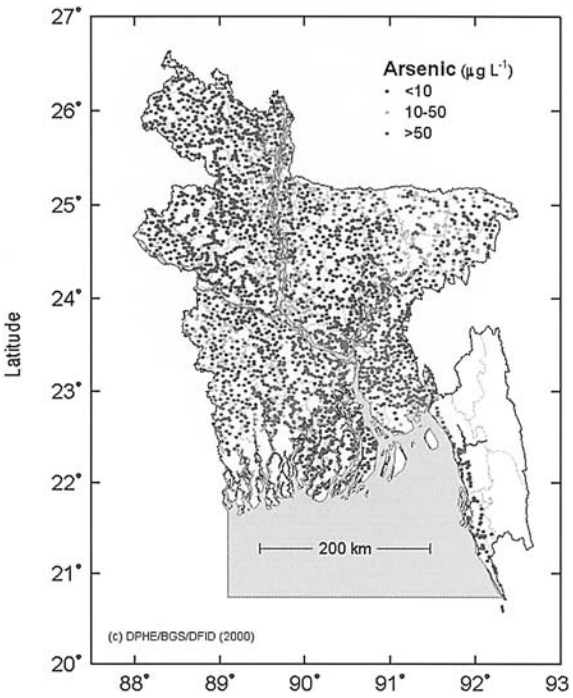

FIGURE 4 Distribution of As in groundwater from BDP aquifers in Bangladesh (From Refs. 181,182).

the sources and mechanisms for As release in groundwater. Several isolated high-As geological domains in the Himalayas and adjoining highlands might have been the provenance of As in the sedimentary aquifers (134,184,185). Two conflicting hypotheses have been widely suggested to explain the mechanisms of As mobilization in the sedimentary aquifers of the BDP. The first hypothesis suggests that As is released by the oxidation of pyrite (FeS_2) or arsenopyrite (FeAsS) following lowering of the water table during groundwater pumping (186). The second hypothesis that is widely accepted suggests that As is released due to desorption from or reductive dissolution of Fe oxyhydroxides in a reducing aquifer environment (6,41,131,147,149–154,187).

The distribution of As in groundwater from shallow and deep aquifers was also mapped under the USAID program (149,153) (Fig. 5a,b). Distribution of As (41,153,184,187,188) in deep BDP groundwater in Bangladesh and West Bengal (Fig. 6) indicate that As levels are typically above the drinking water limits up to a depth of <150 m. The deep aquifers (>150 m) in general produce groundwater with As concentrations below the WHO limit of 10 μg/L (41).

Groundwater pH is predominantly near neutral to slightly alkaline (pH 6.5–7.6). The E_H values vary between +0.594 and −0.444 V, which suggests a mildly

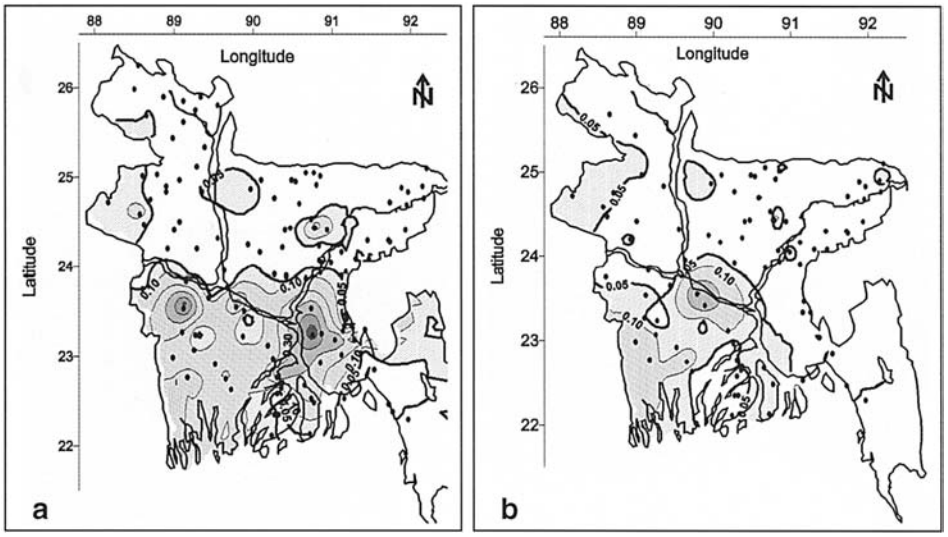

FIGURE 5 Map showing the distribution of As in groundwater (in mg/L) from tubewells in Bangladesh (149,153). (a) Wells less than 30.5 m (100 feet) below ground surface (bgs); (b) wells greater than 30.5 m (100 feet) bgs. (•) Sampling locations.

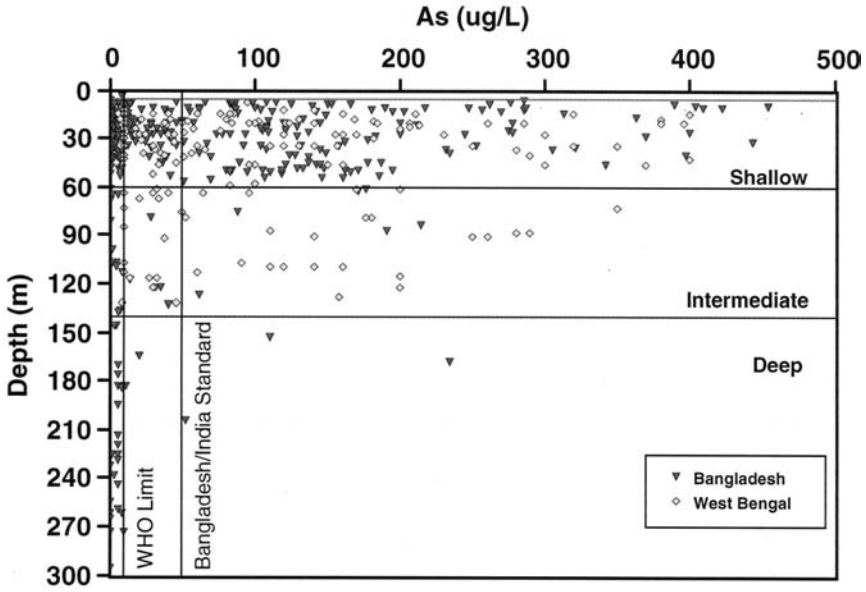

FIGURE 6 Distribution of arsenic in deep groundwater of the aquifers in the BDP in Bangladesh and West Bengal. (Data from refs. 41,151,188.)

oxidizing to moderate/strong reducing groundwater environment in the BDP. The water types are generally Ca-HCO_3 or Ca-Mg-HCO_3, although Ca-Na-HCO_3 type and Na-Cl type water are also encountered in selected patches (183,184,189). Bicarbonate (320–600 mg/L) dominates as the major anion in groundwater and shows an apparent depth and lithological control (41,119,187). Sulfate ($\leq$3 mg/L) and nitrate ($\leq$0.22 mg/L) concentrations are generally low, and concentrations of phosphate (0.05–8.75 mg/L) are high in the BDP groundwaters. Distribution of total Fe (Fe_{tot}) varies considerably (0.4–15.7 mg/L) along with total As (As_{tot}; 2.5–846 μg/L) in groundwater. As^{III} is the prevalent aqueous species and accounts for about 67–99% of the total As in well water. The concentration of dissolved organic carbon (DOC) in the groundwaters ranged from 1.2 to as high as 14.2 mg/L.

To understand the hydrogeochemical controls on As contamination in groundwater, we need to address some of the key elemental relationships. In the investigated groundwaters from Bangladesh, definite positive correlation was noted between Fe_{tot}-HCO_3 ($r^2 = 0.57$, Fig. 7a), Fe_{tot}-PO_4 ($r^2 = 0.50$, Fig. 7b), and Fe_{tot}-As_{tot} ($r^2 = 0.42$, Fig. 7d). A positive correlation was also indicated for the distribution of HCO_3 and DOC ($r^2 = 0.38$, Fig. 7c). It is interesting to note that

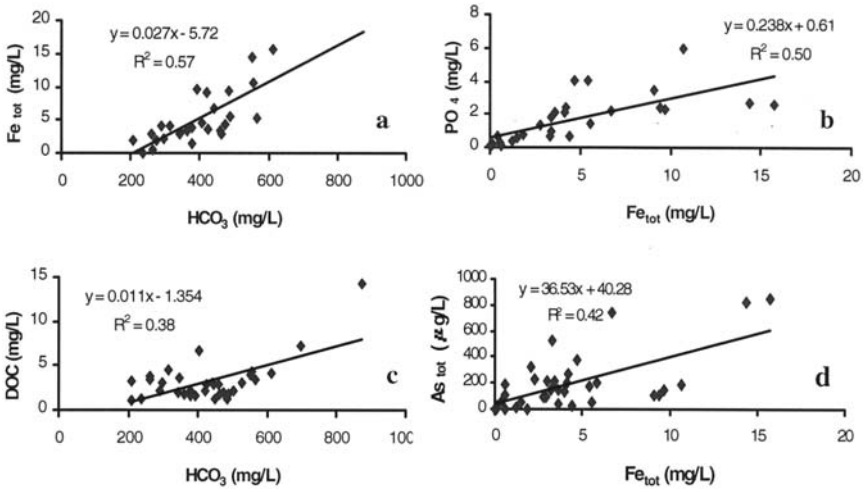

FIGURE 7 Salient chemical characteristics of groundwater from BDP aquifers in Bangladesh ($n = 36$), showing the relationship between: (a) Fe_{tot} and HCO_3; (b) PO_4 and Fe_{tot}; (c) DOC and HCO_3; and (d) Fe_{tot} and As_{tot}.

studies by Bhattacharya et al. (187) indicated specific trends in the relationships between HCO_3 and DOC in the groundwater from wells at specific depths. At shallow depths (7.9–28.5 m) the correlation was low ($r^2 = 0.35$), while water samples representing the group of deeper wells (67.1–255.3 m) indicated strong positive correlation ($r^2 = 0.77$). Distinct negative correlation was, however, observed between HCO_3 and DOC ($r^2 = 0.42$) in water samples from wells at depths of 29–62.5 m, which suggests anaerobic degradation of DOC. It is also appropriate to mention that the concentration of ammonium is high in BDP groundwaters (up to 10 mg/L), which could come from dissimilatory nitrate reduction or from in situ degradation of organic matter. In view of the low nitrate levels even in near-surface environments, the latter alternative seems more likely. High DOC levels are consistent with the dominance of As^{III} in groundwater, which suggests reduction of organic matter by microorganisms and conversion of As^{V} to As^{III} in the sedimentary aquifers. The source of DOC in BDP groundwater is not known and is a subject of further investigation. However, the pool of organic matter in the BDP aquifer sediments (119,187) may act as a source for the DOC in the groundwater. Low sulfate concentrations in BDP groundwater (151,152,187) can be attributed to sulfate reduction but not sufficient enough to cause precipitation of sulfides on a regional scale. However, framboidal pyrites have been identified in the S-rich clayey sediments in some parts of the aquifer segments, viz. at Tungipara (154). Correlation between concentrations of HCO_3

with Fe_{tot}, As_{tot}, and DOC indicates that several terminal electron-accepting processes (TEAP) are active in the BDP aquifers, which drives the reductive dissolution of Fe oxyhydroxides in the aquifers. Reductive dissolution of Fe^{III} in sediments mobilizes Fe^{II} and As. Some of the key redox reactions in the BDP aquifers controlling the groundwater chemistry are:

$CH_2O + O_2 \Rightarrow CO_2 + H_2O$	(Organic matter oxidation by O_2)
$CO_2 + H_2O \Rightarrow H_2CO_3 \Rightarrow H^+ + HCO_3^-$	(Dissociation and hydrolysis)
$5CH_2O + 4NO_3^- \Rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O$	(Denitrification)
$2CH_2O + SO_4^{2-} \Rightarrow 2HCO_3^- + H_2S$	(Sulfate reduction)
$4Fe^{III}OOH + CH_2O + 7H_2CO_3 \Rightarrow 4Fe^{II} + 8HCO_3^- + 6H_2O$	(Reductive dissolution of Fe oxides)

The mobilization of As in groundwater is caused by desorption of As oxyanions (147,190) or by reductive dissolution of the Fe (oxy)hydroxide, leading to the release of both Fe and As in aqueous solution. Oxyanionic As species are commonly adsorbed on the reactive surfaces of the Fe and Mn (oxy)hydroxide in the sediments, which are characterized by pH-dependent surface charge. At a lower pH, they attain net positive charge leading to significant adsorption of As^V species, but with an increased alkalinity, the oxide surfaces attain the point of zero charge (PZC) and releases the As oxyanions through desorption. Although the source of As in the alluvial sediments is geogenic, further research is in progress to understand the complex (bio)geochemical interactions in the BDP aquifers, and the effects of land use pattern.

The redox status in the aquifers is influenced by the practice of wetland cultivation in the BDP leading to the mobilization of As (6). Reducing conditions in soils flooded during paddy cultivation leads to the production of methane (191,192). Rice cultivation produces 3–4000 kg of straw per crop and a root biomass equivalent to 400 kg/ha C (193), which is a very good substrate for methane fermentation under anaerobic conditions (194). Consequently, Fe^{III} reduction observed in the soil zone is commonly reflected by increased concentrations of Fe^{II} in groundwater at intermediate depths of 45–60 m in BDP aquifers (195). Interestingly, methane emission is recorded at several well sites in the Bangladesh part of the BDP (196). In situ methane production may also lead to methylation of As through the anaerobic degradation of organic matter and transform As^V to the more soluble As^{III} species and methylated arsenicals thereby affecting the overall mobility and transport of As in groundwater (119).

To supply safe drinking water, major strategies should include identification of the wells yielding water with As concentrations at levels <50 μg/L, the national drinking water standard in India and Bangladesh. Screening of tubewells, appears to be a promising short-term measure for the supply of drinking water at safe As levels (144,153). Deeper tubewells drilled in several parts of West Bengal and Bangladesh provide As free water to the rural and semiurban popula-

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tion (144,153). However, the relatively low apparent success rate coupled with the potential of a prolonged effort at a high cost suggest that drilling deeper wells should be a minor component of an overall strategy used to provide safe drinking water (147,153). Possibilities to remove As from groundwater should include techniques that are low cost, effective, and socially acceptable by the rural population in the affected households.

4.3 Chile

Approximately 400,000 residents of northern Chile drink water from public supplies that are diverted from rivers in the Andes Mountains to the arid regions. However, many of these rivers have high levels of natural As that often ends up in northern Chile's drinking water (129,130,134,197). High As in drinking water has been associated with increased mortality from bladder, lung, kidney, and skin cancers (198). Epidemiological studies in Chile indicated that exposure to As concentrations through drinking water containing <50 μg/L had had severe toxic effects on human health (199).

The public water system of Antofagasta (the largest city in this region) had approximately 800–1000 μg/L of As. A population of nearly 200,000 is served by a full-scale conventional treatment plant for As removal since 1970. The concentration of As in drinking water was lowered to 40 μg/L (197,200), but this experience suggests that for source water with high As concentrations and a greater proportion of As^{III}, stringent standards for As (<20 μg/L) could not be met by conventional coagulation. Moreover, the cost of As removal also increased drastically to reach an effective As removal below 20–30 μg/L (200).

4.4 China

Large areas in the Xinjiang and Inner Mongolia provinces of China have drinking water wells where high As concentrations (50–1860 μg/L) are reported (138–140,201). The source of As in both provinces is geogenic. In the Kuitun area of Xinjiang province of China, endemic arsenicosis, fluorosis, and combined As and fluoride poisoning was encountered where nearly 102 drinking water wells had levels of >100 μg/L As and >1 mg/L fluoride (140). A concentration of chronic As poisoning was discovered during a national water quality survey in the Huhhot Alluvial Basin (HAB) of Inner Mongolia in 1984. HAB is an alluvial and lacustrine basin surrounded by the mountains toward the east, south, and north and open to the west. The basin has an aerial extent of about 4800 km^2 at an average elevation of 1050 m above the mean sea level. The northern part of the HAB is a depression (lowland) in the front of alluvial and lacustrine fans. The middle part of the HAB is the alluvial and lacustrine plain of the Daheihe River. The southwestern part of the HAB comprises the alluvial and flood plain of the Yellow

River. The groundwater occurs in the Q_4 sediments (139) and is characterized by a high concentration of As derived from the adjoining highlands. The HAB is predominantly an agricultural area with wheat, rice, millet, corn, green beets, potatoes, and sunflowers as the primary cultivated crops. There are no sources of anthropogenic emissions of As from industries and mines into the atmosphere, water, or soil and no arsenical pesticides have been used. Analyses of the surface soils, air, fish, and crops, however, do not show levels of As above the regulatory limits. Approximately 5.3% of the people from this basin had visible hyperkeratosis with hyperpigmentation or hypopigmentation (139).

The concentration of As in groundwater from the HAB exceeds the provisional limits of the WHO for safe drinking water by a factor of more than 5–100 as well as the Chinese national drinking water standard by factors >1–20. Typical As concentrations in groundwater from several shallow aquifers were in the range of ≤1800 μg/L, while in deep aquifers concentrations of As are in the order of ≤360 μg/L. Arsenic concentrations in the water supply wells were greater than the national standard of 50 μg/L in 63 of the 305 samples (20.7%). In deep wells, levels of As were greater than the national standard in 18 of 33 investigated wells (54.6%). The As concentrations in surface waters ranged up to 20 μg/L, were not elevated above the limits of the national drinking water standards, but exceeded the provisional WHO limits. As^{III} was found to be predominating (52–75%) in both shallow and deep wells in the region. These waters were also high in fluoride and low in dissolved oxygen, sulfate, nitrate, selenium, iron, and manganese. The aquifers rich in organic matter seem to provide a reducing environment that facilitates mobilization of As. This organic matter stimulated microbial respiration causing depletion of dissolved O_2 and anoxic environment leading to high concentrations of dissolved As^{III} (139).

4.5 Ghana

Approximately 1600 km^2 of Obuasi has rivers that are contaminated with up to 7900 μg/L of As. The well water in this region has up to 175 μg/L of As. Oxidation of naturally occurring arsenopyrite (FeAsS) in the areas of gold mining is the most likely source of As contamination. In addition to producing dissolved As, the oxidized arsenopyrite apparently reprecipitates to form scorodite ($FeAsO_4 \cdot 2H_2O$), arsenolite (As_2O_3), and arsenates (131,202,203).

4.6 Hungary

In the southern part of the Great Hungarian Plain (GHP), an area of 4263 km^2 with a population of nearly 456,500 in five towns and 54 villages, groundwater from the Pleistocene aquifers containing As at levels about the national permissible limit of 50 μg/L is used for public drinking water supplies. The aquifers comprise sediments deposited by the river Danube, while in the eastern part of

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the plains the sediments were brought by the Maros and Körös Rivers. The groundwaters were predominantly Ca-Mg-HCO_3 and Na-HCO_3 type. Typical As concentrations in the region vary between 25 and 150 μg/L, and it was concluded that the distribution of As in the GHP groundwaters was controlled by the humic substances (132).

4.7 Mexico

The Lagunera region of north central Mexico has widespread chronic As poisoning. This poisoning results from drinking As-affected well water. The source of As is assumed to be geological deposits. Symptoms include changes in skin pigmentation, keratosis, blackfoot disease, gastrointestinal problems, and skin cancer (204).

Mining has been an important economic activity in the Zimapán Valley in Mexico and several towns were developed around these mines. Oxidation of arsenopyrite and solubilization of scorodite in the mine wastes, generated during centuries of silver, zinc, and lead mining (126,127), leach As into the aquifers and cause natural As contamination in the drinking water wells of the region. Groundwater is the only drinking water source for the community of nearly 10,000 inhabitants in Zimapán. The highest levels of As found in groundwater range up to 1100 μg/L. The shallow wells are contaminated from the mine tailings and fumes emanated by the smelters and have As concentration up to 530 μg/L (205). Chronic As poisoning, which includes skin cancer, and kidney and liver diseases are common among the residents in the Zimapán area.

4.8 Taiwan

In Taiwan, a population of approximately 20,000 were exposed to As due to drinking groundwater from artesian wells containing up to 1820 μg/L As. Nearly 1141 cases of chronic As poisoning were diagnosed in 1975. The term ''blackfoot disease'' was coined to describe arsenicosis of the lower legs (137,206). In addition, As exposure is also associated with skin, bladder, kidney, ureter, urethral, liver, and lung cancers. However, recent improvements to the drinking water supply have reduced As exposure, which in turn has reduced the incidence of cancer (207–209).

4.9 Thailand

Eight villages from the Ron Philbun District have wells that are polluted with up to 6700 μg/L of As. A total of 824 cases of ''Kai Dam,'' a local term for chronic As poisoning, were reported in 1997 (142). The source of this pollution is oxidation of naturally occurring arsenopyrite (FeAsS) during mining and smelting operations (210).

4.10 United Kingdom

The old Cornwall and Devon mining and smelting regions currently have agricultural soils and household dusts with up to 1000 mg/kg or more of As (211). Treated surface water is currently used for drinking owing to extensive groundwater contamination. The concentrations of As in untreated and treated surface waters are 10–50 μg/L and typically less than 10 μg/L, respectively (133,134).

4.11 United States

Nearly 10% of groundwater resources in the United States indicate As concentrations exceeding the drinking water guideline of 10 μg/L (190). In general, highest As concentrations are encountered in the western part and large areas of the midwest and northeast, exceeding the national and WHO drinking water guideline value of 10 μg/L. However, in the southeastern part of the country, As con-

FIGURE 8 Arsenic concentrations in groundwater of the United States. (Adapted from refs. 212,213.)

centrations are low (212,213). The most prevalent mechanisms of widespread concentrations of As are desorption and reductive dissolution of iron oxides and oxidation of sulfide minerals, in addition to upflow of geothermal water and evaporative concentrations (190,214,215). A national-scale assessment of As-affected water wells based on the analysis of 18,850 wells from 595 counties during the past two decades done by the U.S. Geological Survey (USGS) is summarized in Figures 8 and 9.

The estimated percentage of public water-supply systems in the United States with various limits of As concentrations in groundwater is presented in Figure 10. Nearly 13.6% of these systems use groundwater with As concentrations above 5 μg/L, compared to about 7.6% that exceed 10 μg/L (212,213). The counties with As concentrations exceeding various potential new United States drinking water standards in 10% of more of their wells are shown in Figure 9. Approximately 24% of these counties had As concentrations exceeding 10 μg/L in at least 10% of their wells (212,213).

An independent survey of Alaska, Arizona, California, Idaho, Indiana, Nevada, Oregon, and Washington evaluated the effect of geological environment

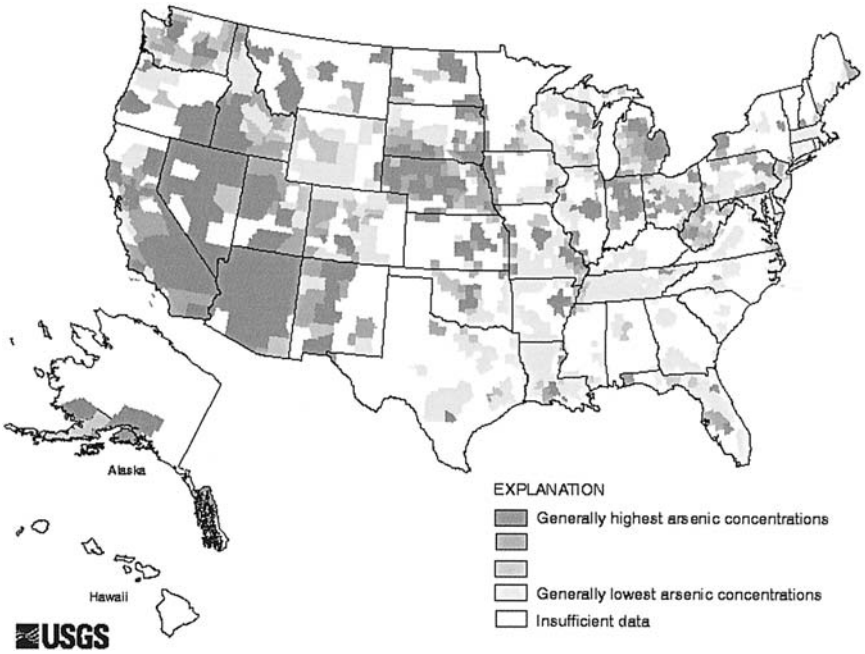

FIGURE 9 Counties with As concentrations exceeding the new MCL (10 μg/L) by 10% or more in groundwater. (Adapted from refs. 212,213.)

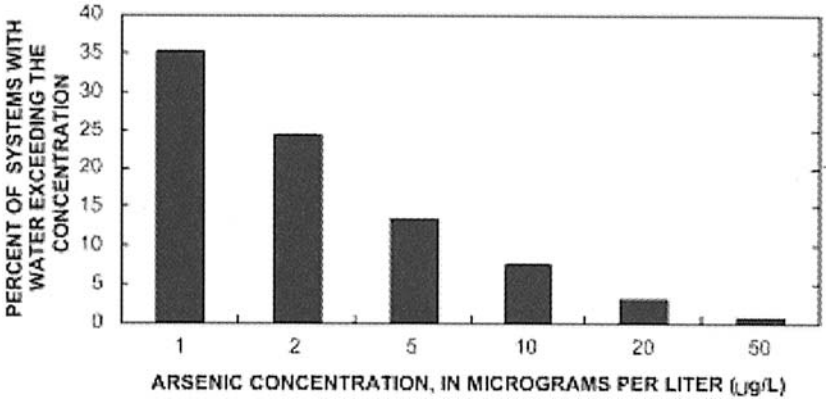

Figure 10 Distribution of As concentrations in the wells of public water-supply systems that serve between 1000 and 10,000 people. (Adapted from ref. 212.)

on groundwater As concentration. Aquifers made from basins that were filled with alluvial (windblown) or lacustrine (lake) deposits had As concentrations that ranged from 50 to 2750 µg/L. Aquifers in volcanic terrain, adjacent to geothermal systems, and in uranium and gold mining areas had As concentrations that ranged from 170 to 3400, 800 to 15,000, and 130 to 48,000 µg/L, respectively (216). In addition, five public drinking water supply systems in Nevada had As concentration above 50 µg/L standard (217).

5. MANAGEMENT OF ARSENIC-CONTAMINATED SOILS

The natural background concentration of As in soils is an important factor in assessing the environmental quality and strategies for subsequent remediation. Remediation of As in contaminated soil systems is much more complicated and often involves designing economically feasible and effective techniques that are site-specific. Both in situ and ex situ remediation technologies have been developed, although neither of these technologies have gained popularity because of the inconsistencies in results as well as involvement of high costs in the remediation process. In situ processes reflect all technologies directed to an unexcavated soil that remains relatively undisturbed. Ex situ processes treat soils that are disturbed either on- or off-site. In addition to these remediation technologies, As-contaminated soils may be managed through chemical fixation techniques that reduce the bioavailability of As. The chemical fixation technique is a particularly relevant management method for diffuse contamination where phytoavailability or mobility of As is being controlled.

5.1 Chemical Fixation

Among the in situ remediation technologies, chemical fixation technique is often used to reduce the mobility of contaminants. Such a process either minimizes the potential for groundwater contamination by reducing contaminant leaching or environmental and human heath risks through reduced contaminant availability. Chemical fixation involves addition of additives to the soil that immobilize hazardous elements. Principles of the process of the leaching of toxic metals in soils and the process of chemical fixation of these metals in soils as applicable to the cleanup of hazardous wastes have been discussed by Connor (218). He reports that chemical fixation techniques stabilize contaminants in soil by converting these into a less mobile chemical form and/or by binding them within an insoluble matrix offering low leaching characteristics. Chemical fixation processes have been applied both in situ and ex situ, the latter being both on- and off-site. Such treatments often involve application of oxyhydroxide minerals that enhance the As-binding capacity of soils. For instance, the potential for using Fe (hydr)oxide in acidic fly ash as a possible process of controlling As sorption was investigated by van der Hoek and Comans (219). Using controlled leaching experiments they studied the sorption characteristics of As and Se on crystalline and amorphous Fe (hydr)oxide. They found that virtually all As and Se at the fly ash surface was associated with amorphous iron (hydr)oxides in the fly ash matrix. Using isotopic exchange experiments they concluded that at pH <10 the oxyanions were partly coprecipitated with secondarily formed amorphous iron (hydr)oxide, a process that reduced their availability.

Specific adsorption of As^{V} by Fe-oxyhydroxide surfaces was discussed by Hingston et al. (220) and studies in later years demonstrate that under field conditions the chemistry of As is largely controlled by both poorly ordered (oxalate extractable) and crystalline free (citrate-dithionite extractable) Fe-oxide minerals (71,110,147,221,222). The effect of Fe-oxyhydroxide on As sorption was further confirmed in our laboratory by investigating the kinetics of As adsorption by soils. It was found that addition of 20% goethite to soil increased As adsorption by 450% (165–750 mg/kg) after 25 min in an Alfisol soil (7, 4, and 87% clay, silt, and sand, respectively) collected from northern New South Wales, Australia (Fig. 11). According to Sadiq (223), chemisorption of As oxyanions on soil colloid surfaces, especially those of Fe-oxide/hydroxides and carbonates, is a common mechanism for As solid-phase formation in soils. Such processes reduce the bioavailability and leachability of As.

A fixation process in which As^{V}-contaminated solids are treated by the 1:1 addition of ferrous sulfate ($Fe^{II}SO_4 \cdot 4H_2O$) solution to produce ferric arsenate $Fe^{III}AsO_4$ (equation 1 below) was described by Sims et al. (224). Although the exact mechanism for the oxidation of Fe^{II} to Fe^{III} is not described, it is apparent

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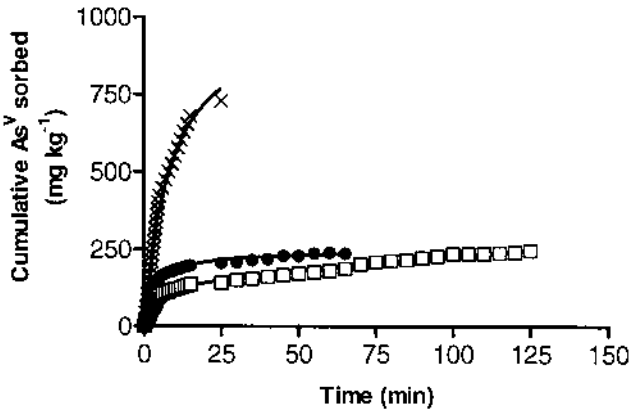

FIGURE 11 Effect of goethite on cumulative As adsorbed by an oxisol from Northern New South Wales, Australia (Smith and Naidu, unpublished data).

from the discussions that O_2 availability is one of the key factors controlling the process (equation 2).

$$Fe^{II}(H_2O)_6^{2+} \Rightarrow Fe^{III}(H_2O)_6^{3+} + e^- \quad (1)$$

$$Fe^{III} + As^{V}O_4^{3-} \Leftrightarrow Fe^{III}As^{V}O_4\ (s) \quad (2)$$

However, these investigators assume that $FeAsO_4$ is an insoluble phase. Artiola et al. (225) investigated the effectiveness of hematite (Fe_2O_3), siderite ($FeCO_3$), ferrous sulfate ($FeSO_4$), and gypsum ($CaSO_4 \cdot 2H_2O$) in reducing leachable As in soils as evidenced by the toxicity characteristic leaching procedure (TCLP) test. They concluded that ferrous sulfate was the only additive that effectively fixed As. They proposed that the prerequisite to the fixation process was oxidation of Fe^{II} to Fe^{III} by atmospheric O_2 and the pH of the soil that was to be kept above 5. This was followed by reaction of Fe^{III} with the As^V to form $FeAs^VO_4$ (equation 2) in the soil. Chemical bonding of As^V to Fe^{III} was claimed to be the factor stabilizing As^V rather than a simple ion exchange process at surface sites of Fe oxides. It has also been shown that the mole ratio between the amount of Fe added to fix As is important in controlling the stability of the Fe-As compound. Krause and Ettel (226) reported that at Fe/As ratios >8, solubility of As was less than 1 mg As/L, while at Fe/As ratios <4, As solubility was increased to more than 7 mg As/L. This suggests that Fe arsenates may be suitable for the disposal of As only if a sufficient amount of Fe is added.

Other chemical additives that enhance fixation through cementation process using lime have also been investigated. For instance, Sandesara (227) proposed

addition of H_2SO_4, $Ca(OH)_2$, and $FeSO_4$ to the slurry of an As-bearing soil to precipitate $FeAsO_4$ in a cement binder. Using a modification of this process, Voigt et al. (228) investigated the potential to fix As-contaminated soils in the field. Their method consisted of mixing soil with a slurry of $FeSO_4$ and water to initiate a reaction between Fe and As. After 20 min, the soil-SO_4 slurry was mixed with portlandite [$Ca(OH)_2$]. Using this technique they were able to successfully treat soils contaminated with 0.1–0.2 weight percent As so that these soils were suitable for waste disposal as determined by the TCLP test. Detailed investigation of the chemistry of the fixation process by Voigt et al. (228) found no direct evidence for the formation of $FeAsO_4$ phases in the fixed soils as theorized by Connor (218). Using sequential extraction they demonstrated that the fixation process involving $FeSO_4$ and portlandite decreased the exchangeable As observed in the untreated bulk soil. The fixation occurs through reaction with $FeSO_4$ and cementation by phases such as portlandite, ettringite, and Ca silicate hydrate. The cement and portlandite combined with the soil to form a barrier preventing mobilization of the As but also increased the pH of the soil to alkaline conditions. Other mineral phases, such as hoernesite, were observed to form in the soils and this was attributed to the presence of large amounts of Mg recorded in these soils. It has also been postulated that Fe/As compounds are not stable over a wide range of pH conditions, owing to the dissolution of Fe/As precipitates at low pH, as well as the transformation of amorphous ferric oxyhydroxide to goethite over time, which may influence the stability of the Fe/As structure. As discussed above, Krause and Ettel (226) reported that ferric arsenates were stable over a wide range of pH and the stability increased with increasing Fe/As ratios. Furthermore, they (226) found that after aging the Fe/As precipitates for nearly 1350 days, the 1:1 precipitates were unstable, and As solubility increased from 160 to 299 mg As/L after 698 days. In contrast, As solubility was consistently $<$0.2 mg As/L in Fe/As precipitates with ratios $>$4:1 after 1355 days of contact.

5.2 Electroremediation

Electrokinetic remediation of polluted sites is the physical removal of contaminants through the application of a low direct current (DC). Removal of contaminants may occur through the following processes; electromigration, electroosmosis, or electrophoresis (229–231). Electromigration of charged-bearing particles (e.g., clays) and dissolved ions occurs when a low-level DC is applied between two electrodes. Positively charged ions and particles are attracted toward the cathode and negatively charged particles and ions are attracted toward the anode. Electroosmotic-induced movement of ions occurs because of a drag interaction between the bulk of the liquid in a soil pore and thin layer of charged fluid next to the pore wall that, like the electromigration movement of a single ion, is moved under the action of the electric field in the direction of the bulk

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flow. The liquid velocity from electroosmosis is proportional to the zeta-potential of the surface and to the applied electric field strength (229). The movement of charged particles due to electrophoresis plays only a limited role in compact soil systems since the solid phase is restrained from movement (229,233).

Currently much of the research on electroremediation is being undertaken at the bench scale. However, electroremediation technology has been applied to remediate contaminated soils in both Europe and the United States. Electroremediation technology was used to remove As from a heavy clay soil at the site of a former timber treatment plant in the Netherlands (234). At the end of the remediation process three-quarters of the contaminated site had As concentrations less than the reference objective of 30 mg As/kg. The electroremediation process therefore provides an alternative treatment for the removal of strongly sorbed elements such as As.

5.3 Bioremediation

Bioremediation technology is based on the remediation of contaminated sites using either microorganisms or plants to detoxify the site largely by transforming or degrading the pollutants. Bioremediation is a well-established technology for the removal of organic soil contaminants. The use of microorganisms to transform inorganic contaminants is, however, still at the laboratory stage. Unlike the organic contaminants, which can break down into smaller units and can be lost from the system, the actual degradation of metals does not occur, although the oxidation and subsequent removal of the contaminant metals as precipitates have been recorded (232). Bioremediation processes generally operate well under conditions that enhance the activities of either the native microorganisms or the introduced species, particularly for those elements that transform or degrade under conditions suitable for the optimum performance of the microorganisms. Bioremediation processes are based on four different criteria, and involve: (a) stimulation of the activity of indigenous microorganisms, (b) inoculation of microorganisms with specific biotransforming ability, (c) application of immobilized enzymes, and (d) use of plants to remove or transform pollutants. Stimulation processes enhance the activities of indigenous microorganisms by addition of nutrients, regulation of redox conditions, pH change, or removal of other limiting conditions.

At the bench scale, Cr, Hg, and As have shown potential for bioremediation in soils. While the detoxification of Cr has involved aerobic reduction of the Cr^{VI} to Cr^{III} (235,236) in the soils, remediation of Hg (237) and As has involved transformation of the contaminants to volatile phases. Bioremediation of As-contaminated soils is based on the ability of microbes, mold, or fungi to transform As into either the less toxic forms or a form that allows volatilization of As. The key mechanisms of As removal include biooxidation and biomethylation.

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Biooxidation processes involve development of bioremediation techniques that detoxify As-contaminated soils and water using microbes that oxidize toxic As^{III} to the less toxic As^{V} species. Arsenic oxidizing microbes were first identified in cattle-dipping fluids in South Africa (238). A bacteria, provisionally referred to as *Bacillus arsenoxydans*, was able to grow in culture media containing up to 1% As_2O_3 as As^{III}, which brought about its oxidation to As^{V}. In his studies on spontaneous oxidation of As^{III} in cattle-dipping fluids from Queensland, Turner (239) isolated 15 strains of heterotrophic bacteria belonging to the genus *Pseudomonas* that tolerated up to 0.1 M As^{III} and also oxidized As^{III} to As^{V}. Later studies by Abdrashitova et al. (240) indicated that *Pseudomonas putida* was resistant to As and effectively oxidized As^{III} to As^{V}.

Biotransformation (oxidation) of As has widely been used in the purification/treatment of As-contaminated water. As discussed above, oxidized As is removed from the water sample using chemical precipitation technique. Studies by Osborne and Ehrlich (241) have indicated that isolated microorganisms *Alcaligenes faecalis* and other *Alcaligenes* spp. oxidized As^{III} to As^{V} in culture experiments under aerobic conditions. These investigators found that the use of respiratory inhibitors prevented further oxidation of As^{III}, indicating that oxygen served as the terminal electron acceptor. A variety of microorganisms have also been found to reduce As^{V} to As^{III} and As^{V} may even be an electron acceptor in anaerobic respiration (242). While such oxidizing bacteria play an important role in the remediation of contaminated water and waste materials, oxidation of As^{III} in contaminated soils does not reduce the total As concentration. Therefore, the use of such As-oxidizing microbes does not enable the cleanup of contaminated soils.

The most common bioremediation process for As involves biomethylation. The methylation of As^{III} and As^{V} has been observed in studies with fungi, bacteria, and algae in defined growth media amended with As. This process has been observed under both aerobic and anaerobic conditions. The earlier works of Challenger et al. (66) demonstrated that two fungi, *Scopulariopsis brevicaulis* and *Aspergillus niger*, were able to produce trimethylarsine from various As compounds. Quoting the work of Challenger (65), Frankenberger and Losi (243) discussed the importance of fungal metabolism of As where a number of poisoning incidents in Germany and England were caused by trimethylarsine gas. Since these studies several species of fungi that are able to volatilize As have been identified, which include *Penicillium* sp., *Candida humicola*, and *Gliocladium roseum*, capable of converting methylarsonic and dimethylarsinic acids to trimethylarsine (244). Effects of phosphate and other anions on trimethylarsine formation by *C. humicola* were investigated in subsequent studies by Cox and Alexander (245), and it was found that the transformation from inorganic As to methylarsonic acid was inhibited by the presence of phosphate although it enhanced its formation from dimethylarsinic acid. Recent studies by Andrews et al.

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(246) have reported that filamentous fungi, e.g., *S. brevicaulis*, can cause biomethylation of As.

More recent studies by Huysmans and Frankenberger (247) demonstrated the potential for the biomethylation of As compounds using the fungus *Penicillium* sp. that was isolated from agricultural evaporation pond water. These investigators used a minimal medium in which 100 mg As/L methylarsonic acid served as the As source, with pH 5–6 and an incubation temperature of 20°C. They also found that the amino acids phenylalanine, isoleucine and glutamine promoted trimethylarsine production with an enhancement ranging from 10.2- to 11.6-fold over the control without amino acid. In contrast, addition of carbohydrates and sugar acids to the minimal medium suppressed trimethylarsine production. However, no reason for the suppressed biomethylation of As was given by these investigators. Reviewing the literature on bioremediation of heavy metals with fungi, Frankenberger and Losi (243) concluded that soil fungi may play an important role in the transformation and mobility of As chemicals used in agriculture. Numerous investigators have reported the natural biomethylation of As in soils. For instance, soils amended with inorganic and methylated As herbicides produce dimethylarsine and trimethylarsine (248–250). These studies indicate that the organisms responsible for the volatilization of As come from a number of species that have the capacity to produce alkylarsines. Despite these studies, limited effort has been directed toward optimizing conditions for the remediation of As-contaminated soils using fungi. In addition, there is little information in the literature on the nature of fungi present in As-contaminated soils.

Although the biomethylation process has been successfully used to remediate As-contaminated water, success with the remediation of soils has been limited and there is little information on the reasons for the lack of success with the biomethylation process involving soils. Since microorganisms can only utilize soluble species, adsorption processes can also indirectly influence rates of species transformations by limiting the solubilities of reactant species available to organisms. Studies on the influence of adsorption on the rates of microbial degradation of As species in sediments indicated that rates of microbial As degradation were influenced by the As adsorption characteristics in the soils (251).

Another bioremediation technique involves bioleaching of As in combination with indigenous microbes. This was recently demonstrated by Bachofen et al. (252), who used bioleaching in combination with the indigenous microbial population to remediate As-contaminated soils. In this study, they bioleached contaminated soils with a liquid containing a dilute nutrient solution (glucose 1 g/L, NH_4HCO_3 0.34 g/L) at a rate of 60 ml/h. They found that *Methanobacterium thermoautotrophicum* bacteria were resistant to As^V concentrations of up to 350 mol As/L and had no effect on growth or methanogenesis. Continuous cultures fed with As^V transformed As depending on the phosphate concentration to volatile As species with a yield of 25%. Leaching of the contaminated soil in percola-

tion columns with indigenous microbes solubilized and slightly volatilized As present in the soil columns. Although the actual mechanism of microbial action was not discussed by the investigators, evidence in the literature suggests that microorganisms can solubilize As from insoluble minerals under oxidizing environment (253). In their leaching studies Bachofen et al. (252) found that an undefined bacterial population solubilized nearly 50% of insoluble As_2S_3 within 2 months. Such microbes could be used to bioleach the As from contaminated soils, although more work needs to be done to investigate the mode of action of the microbes.

5.4 Phytoremediation

Phytoremediation technology uses plants to absorb contaminants from the soil and translocate them to the shoots. Contaminants may then be removed by harvesting the aboveground tissue for subsequent volume reduction (ashing) and storage. Combination of this technique with electrochemical technology could enable extraction and isolation of pure metals. A small number of plant species were identified capable of growing on metal-rich soils; these included Ni, Cr, Cd, and Zn accumulator plants (254). Following this study there have been numerous investigations using metal accumulator plants for potential remediation of contaminated soils. The potential for phytoremediation of Cd- and Zn-contaminated soils using *Thlapsi caerulescens* was investigated by Baker et al. (255), and it was observed that *T. caerulescens* grown in soil plots contaminated with 444 mg Zn/kg and 13.6 mg Cd/kg accumulated these metals 10 times greater than those in the soil. Based on these concentrations, they concluded that 13 croppings of *T. caerulescens* would reduce the concentration of these metals to the 300 mg Zn/kg limit set by the Commission of the European Communities. However, in contrast to investigations on the phytoremediation of Cd and Zn, no work has been conducted on phytoremediation of As-contaminated soils. One reason for the lack of investigations on the phytoremediation of As-contaminated soils may be the similarity in As and P chemistry. In soils with adequate P supply, preferred uptake of nutrient P may reduce the potential for plants to hyperaccumulate As (256). There appears to be a higher affinity for P than As with a discrimination ratio of 4 : 1. In a study of the potential for removal of As from solution by water hyacinth [*Eichhornia crassipes (Mart) Solms*], it was found that high concentrations of P inhibited As uptake by the plant (257).

In contrast to soil remediation, phytoremediation of contaminated water has been investigated by numerous researchers. Arsenic accumulation by *Rhaphydophyceae chattonella* Antiqua was studied in seawater containing an As concentration of up to 50 mg As/L by Yamaoka et al. (258). They found that the plant species grew well at 50 mg As/L and survived even at 200 mg As/L concentration. Arsenic concentration was unaffected by P and 52% of the As accumu-

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lated in the living cell was found in the intracellular fraction, 27% in the lipid fraction, and 21% in the cell wall. Water hyacinth (*Eichrhornia crassipes*) culture was used in a study to improve the quality of stabilization pond effluent that contained As among other metals by Dinges (259). He found that plant tissues contained large amounts of Cl, K, Mg, P, Cr, and As. Based on these results, he recommended that hyacinth treatment systems, in conjunction with stabilization ponds, should be quite economical to construct and operate in warm areas of the United States and in similar regions throughout the world.

Although a number of As-accumulating plants have been identified, there is no report in the literature on the application of such plants for phytoremediation of contaminated soils. A list of 72 plant species tolerant for As on the arseniferous mine wastes in Rhodesia was compiled by Wild (260). Of these, five species were introduced exotics, 13 were exotic or African weed species, and 50 were indigenous species. These species were found to grow on soils with As concentrations ranging from 5000 to 30,000 mg As/kg. The ability of these plants to accumulate As may be enhanced by selective breeding, plant genetic engineering, and appropriate agronomic practices. For successful phytoremediation of metal-polluted soils, a strategy should be considered that combines rapid screening of plant species possessing the ability to tolerate and accumulate heavy metals with agronomic practices that enhance shoot biomass production and/or increase metal bioavailability in the rhizosphere (261).

5.5 Soil Washing

Soil washing, one of the potential methods for chemical remediation, is a process where metals in the contaminated soils are mobilized through acidification. The effect of pH on metal solubility has been demonstrated by several workers (70,262–264). Solubility and subsequent removal of heavy metals from contaminated soils during chemical remediation involve geochemical processes such as desorption from the surface-reactive soil components, dissolution of the unstable mineral phases (i.e., carbonates and other metal oxides), and formation of soluble metal complexes. The remediation process involves excavation of the contaminated soil mass and separation of fine-grained fractions of the soils (71). These fine-grained fractions contain clay minerals, amorphous oxides of Fe, Al, and Mn characterized by a pH-dependent surface charge, and poorly crystalline secondary surface-reactive aluminosilicate phases like imogolite (ITM) in the spodic horizons of the podzolic soils (110). Such surface-reactive soil components effectively adsorb the bulk of anionic metal contaminants such as $As^{V}O_4^{-}$, $H_3As^{III}O_3^{0}$, $Cr^{VI}_2O_7^{2-}$, $Cr^{VI}O_4^{2-}$, and several other metal cations depending on the soil pH.

Fine-grained fractions of As-contaminated soils from the site of a former wood preservation industry (3,70,71) were used for the remediation experiments, with oxalate as the extraction medium. Oxalate was chosen as a suitable extrac-

tant because it forms a complex with positively charged metal cations and is a powerful dissolving agent for Fe and Al oxides and hydroxides (265,266). Moreover, oxalate medium for chemical remediation is based on the fact that it is biodegradable in natural soil environment (267).

Bench scale remediation experiments were carried out on the As-contaminated soil samples from the wood impregnation site at Konsterud, in Central Sweden, using oxalic acid and acid ammonium oxalate solutions (3,70,71,268). The soils (<0.125 mm) were sequentially extracted with 0.4, 0.5, and 0.7 M oxalic acid and acid ammonium oxalate solution at pH 1.4, 2.5, and 4.0. The soil-extractant mixture was shaken rigorously for 5 min and filtered. The soil residue from the first leaching step was further extracted twice and the filtrates were analyzed for metal content. Since As was found to be the principal contaminant in the wood preservation site, major emphasis was focused on the removal of As during remediation. The amount of remaining As in the two contaminated soil samples, shown in Figure 12, reveals that nearly 93–99% As could be extracted from the contaminated soil by optimizing the concentration and pH of the oxalate media (Table 5). However, nearly 58–82% Cu, 38–98% Cr, and 56–70% Zn were also removed from these soils in the process (268). The bulk of As in the soils was oxalate-extractable and the solubility was maximum at pH 0.9–1.4 and associated with the oxalate-extractable fractions of Fe, Al, and Si

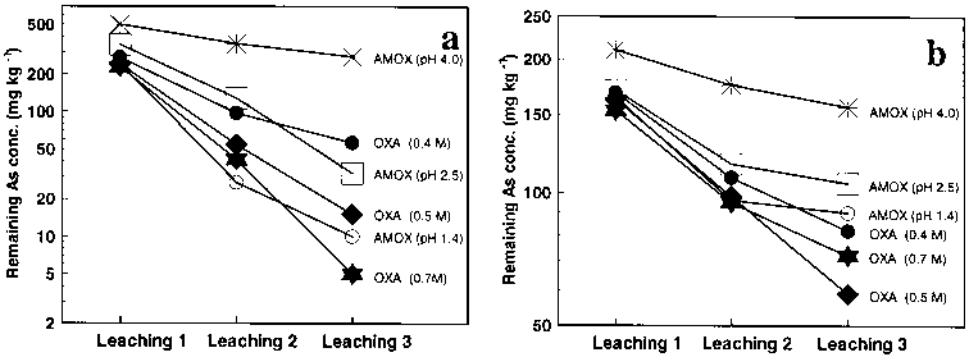

FIGURE 12 Experimental results for the removal of As from contaminated soils at a wood preservation site in Konsterud, Central Sweden (268). Shown are the remaining metal concentrations in the fine-grained (<0.125 mm) fractions in two soils: (a) Kc-32 (pH = 5.6; As_{tot} = 912 mg/kg) and (b) Kc-45 (pH = 4.9; As_{tot} = 261 mg/kg) following three-step sequential leaching of the soil sample. Note logarithmic scale in the y-axis. OXA, oxalic acid; AMOX, ammonium oxalate.

TABLE 5 Results from the Bench Scale Experiments for Chemical Remediation of Arsenic in CCA-Contaminated Soil from Konsterud, Central Sweden by Soil Washing

Extractant	Soil sample	pH	Initial As (mg kg^{-1})	As extracted (mg kg^{-1})				Remaining As (mg kg^{-1})	As removal %
				Leaching 1	Leaching 2	Leaching 3	Total		
Oxalic Acid									
0.4M	Kc-32	0.9	912	640	175	41	855	57	93.8
0.5M	(pH 5.6)	0.9		671	187	39	897	15	98.4
0.7M		0.9		684	187	36	907	5	99.4
0.4M	Kc-45	0.9	261	93	60	26	179	82	68.7
0.5M	(pH 4.9)	0.9		99	64	39	202	59	77.6
0.7M		0.9		108	58	23	189	72	72.6
Ammonium oxalate									
0.2M	Kc-32	1.4	912	645	213	17	875	37	95.9
0.2M	(pH 5.6)	2.5		567	217	96	880	32	96.5
0.2M		4.0		413	148	75	635	277	69.6
0.2M	Kc-45	1.4	261	97	68	6	171	90	65.3
0.2M	(pH 4.9)	2.5		91	54	11	156	105	60.1
0.2M		4.0		51	35	19	105	156	40.4

Source: Data from ref. 268.

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in the contaminated soils (3,71). Similar efficacy of As (and Cu) removal (about 96–98%) was achieved by oxalate extraction of the contaminated soil materials from another wood preservation site in Ljungby Community, South Sweden (3).

6. MANAGEMENT AND REMOVAL OF ARSENIC FROM CONTAMINATED WATER

The removal of As from water is an important worldwide issue. Several millions of people are drinking water with elevated levels of As compared to the drinking water standards. Elevated As concentrations in groundwater within the developing countries with poor infrastructure demand technologies that are effective and affordable for provision of a safe drinking water supply to the affected population. Conversely, many people in the developed world are also drinking water with unsafe levels of As. Large number of treatment technologies are available to remove As from water, ranging from sophisticated technology such as ion exchange and reverse osmosis to the much simpler, and often highly effective, coagulation-flocculation techniques.

The majority of the contaminated water remediation techniques are based on mechanisms that involve an initial oxidation of As^{III} to As^{V} and subsequent precipitation using chemicals. Successes in the water treatment for As in the past have generally relied on the relatively poor solubility of arsenate (As^{V}). If As^{III} is present in the influent, then an oxidant such as chlorine (Cl_2), potassium permanganate ($KMnO_4$), or oxygen (O_2) is typically used to oxidize As^{III} to As^{V} prior to As removal. Coagulation, absorption to activated alumina, ion exchange with strong-base anion-exchange resins, and reverse osmosis are conventional technologies that have been used to treat As-contaminated water (97). The use of new adsorbents, in situ passive reactive barriers, bioremediation with chemical precipitation, and aquifer oxygenation are some of the emerging technologies for the in situ removal of As from groundwater. In addition, many low-cost technologies for As removal in the developing world are being researched keeping in view the sustainability and people's participation in the treatment systems (269). A review of each of these technologies is discussed in the following sections. The discussion of conventional coagulation is stressed since its principles are readily applied to these other technologies.

6.1 Conventional Technologies for Treating Arsenic in Water

6.1.1 Coagulation

Conventional coagulation involves the formation of large, nondispersed particles from a colloid, such as hydrated $Fe_2(SO_4)_3$, and a solute, such as $H_2As^{V}O_4^{-}$. An

analysis of three studies on conventional coagulation treatment for As removal is discussed in the following sections.

In the first study, the effects of coagulant (ferric sulfate, alum, and lime), chlorination, and pH on As removal were explored (97). The highest removal rates were observed when ferric sulfate was mixed with chlorinated water at pH 8 or less. Conventional coagulation of 50 μg/L of As^V with 30 mg/L of ferric sulfate [$Fe_2(SO_4)_3$] at pH 8 or below removed greater than 95% As while leaving less than 1 μg/L of dissolved As after treatment. Similarly, conventional coagulation of 50 μg/L of As^V with 30 mg/L of alum [$AlK(SO_4)_2 \cdot 12H_2O$] at pH 7 or below removed more than 90% As, leaving less than 10 μg/L of dissolved As after treatment (Fig. 13). Conventional coagulation of 50 μg/L of As^V with 40 mg/L of ferric sulfate [$Fe_2(SO_4)_3$] at pH 7.5 removed 99.9% As while leaving less than 1 μg/L of dissolved As after treatment (Table 6).

The data suggested that ferric sulfate was more efficient at As removal than alum, and that alum was more efficient at As removal than lime. Chlorination enhanced As removal at each reported combination of coagulant and pH. The enhanced removal of As in chlorinated water was likely due to the oxidation of As^{III} to As^V. The efficiency of As^V removal by the ferric and aluminum salts was greatly reduced at higher pH values.

The second study explored the effects of coagulant (ferric chloride, alum, and lime) and chlorination on As removal (104). The results supported those from the first study: The ferric salt was the most efficient coagulant, and chlorination enhanced As removal. In the second study, the highest removal rates were observed when ferric chloride was mixed with chlorinated water at an unspecified pH.

Conventional coagulation of 800 μg/L of As in 30 mg/L Cl_2 with 50 mg/L

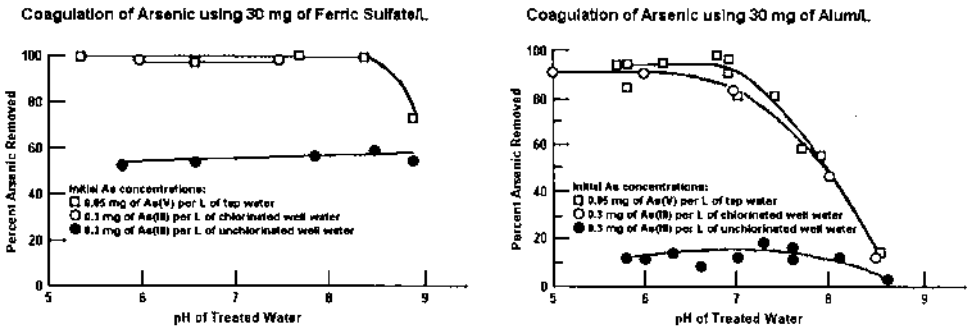

FIGURE 13 Effects of pH and chlorination on arsenic removal by ferric sulfate and alum (291).

TABLE 6 Removal of As^V by Adsorption with Ferric Sulfate (97)

Initial pH	Dose of $Fe_2(SO_4)_3 \cdot H_2O$ (mg/L)	Percent as removal	
		Coagulation and sedimentation	Coagulation, sedimentation, and filtration
5.0	10	66	96.5
	20	87.7	96.9
	30	90.5	96.4
	40	91	95.2
	50	96.8	99
6.0	10	66.2	97.1
	20	81.8	97.5
	30	91.3	98.3
	40	95	99.6
	50	94	98.4
7.5	10	74.6	94
	20	78.5	97
	30	94.5	96.5
	40	91.5	99.9
	50	96.5	97.5
8.0	10	63	88.6
	20	65	89.5
	30	93	96.5
	40	93	96.3
	50	95.4	96.5

of ferric chloride ($FeCl_3$) removed approximately 100% As while leaving less than 10 μg/L of As after treatment (see Table 7). Based on this experiment, a pilot plant was constructed in which a combination of conventional schemes was tried. The most effective treatment, with 100% removal of As, involved oxidation with 20 mg/L Cl_2, coagulation with 50 mg/L $FeCl_3$, sedimentation, and sand filtration. The sand filter eventually had to be washed with NaOH to remove the coagulated As. The permanent water treatment plant utilized an aerator, a mixer, a sedimentation tank, two slow sand filters, a storage tank, an elevation tank, and a sand-washing basin.

The third study investigated the effects of pH resulting from the addition of ''lime'' (the author did not specify whether quicklime (CaO) or hydrated lime [$Ca(OH)_2$] was used) and chlorination on As removal (104). The highest removal rates were observed in chlorinated waters at pH values between 10.9 and 11.5

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TABLE 7 Removal of 0.8 mg of As/L in Raw Water by Oxidation and Coagulation (104)

Specification	Concentration (mg/L)				
Cl_2 added	5	10	20	30	40
$FeCl_3$ added	50	50	50	50	50
Free residual Cl_2	0	0.3	1.0	2.0	3.0
Fe in supernatant	0.5	0.2	0.2	0.2	0.2
As in supernatant	0.12	0.08	0.01	Trace	Trace
As removed (%)	82.5	90.0	98.7	~100	~100

(Fig. 14). An estimate of the concentration of As remaining after lime softening could not be inferred from the provided information.

The removal of As by conventional coagulation using ferric chloride, ferric sulfate, and alum in large-scale treatment of As-contaminated water removed between 70 and 95% of As from contaminated water, but the process produces

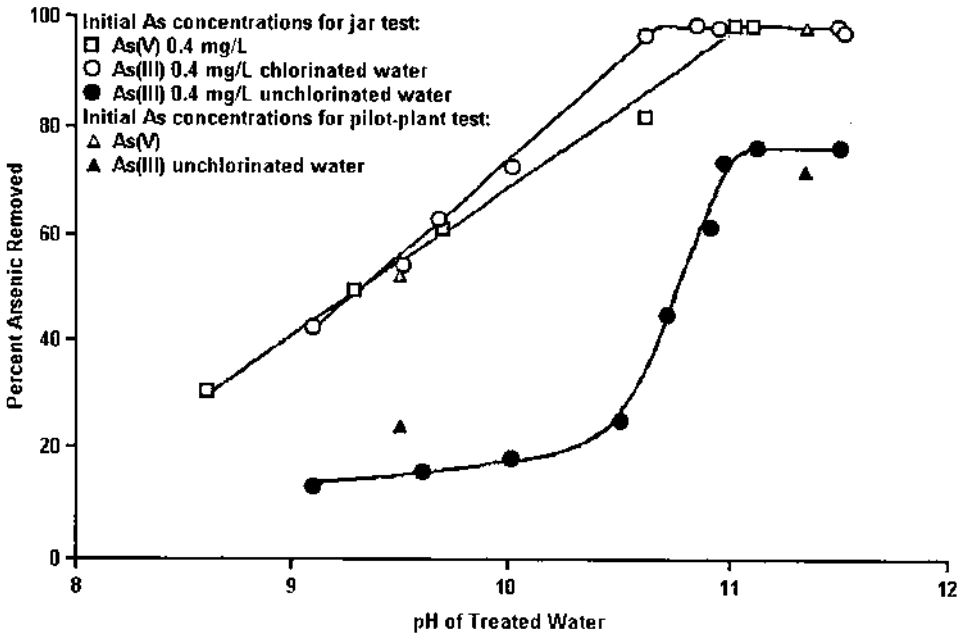

FIGURE 14 Effect of pH on arsenic removal by lime softening (291).

amorphous sludge materials that are typically disposed of in landfills. Disposal of As-containing wastes in landfills has the potential to be a source of contamination if these sludges are not stable. Stabilization of the amorphous waste material may be achieved by solidification of the material into cement. Similarly, heating of the waste material at 60–70°C was reported to increase the stability of the Fe hydroxide at high pH, through the transformation of amorphous Fe into the more stable crystalline Fe forms (270).

6.1.2 Sorption to Activated Alumina

Activated alumina (Al_2O_3) strongly sorbs arsenate (As^V). Arsenic-saturated activated alumina can be regenerated by anion exchange with OH^-. In detail, an activated alumina process involves removal, backwash, regeneration, neutralization, and steps of rinsing.

A variety of parameters should be considered when designing an activated alumina process. For example, the equilibrium capacities of activated alumina for As^V were maximized at pH values less than 7, while As^{III} was best removed at pH values less than 9 (271); however, activated alumina and As slowly reach equilibrium. Therefore, an optimum pH for column conditions should be determined. Pilot studies would have to be conducted to develop design and operating criterion such as optimum pH, operating capacity, removal flow rate, and regeneration and neutralization steps (97).

6.1.3 Ion Exchange with Strong-Base Anion-Exchange Resins

Limited testing has been done with strong-base anion-exchange resins. The initial cost of the resin will probably be higher than that of activated alumina, but the lower cost of regeneration with sodium chloride (NaCl) may make strong-base anion-exchange resins more cost effective than activated alumina (97).

6.1.4 Reverse Osmosis

Reverse osmosis (RO) requires external pressure to reverse natural osmotic flow. As pressure is applied to the saline solution, water flows from a more concentrated saline solution through the semipermeable membrane. RO membrane has a thin microporous surface that rejects impurities, but allows water to pass through. The membrane rejects bacteria, pyrogens, and 85–95% of inorganic solids, especially the polyvalent ions such as As oxyanions, which are rejected more efficiently than the monovalent ions. The effectivity of the RO process depends on the chemistry of the inlet water. Efficacy of RO was observed for a wide range of pH (3–11) and hence RO was applicable for treating groundwater contaminated with As.

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6.2 Emerging Technologies for Treating Arsenic in Water

6.2.1 Fe Oxide as an Absorbent

The strong affinity for As by Fe oxide surfaces has also been widely used in the water purification processes. New adsorbents developed by Hlavay and Polyak (272) for the removal of As^{III} and As^{V} ions from synthetic and deep-well waters using Al_2O_3 and/or TiO_2 coated with freshly precipitated $Fe^{III}(OH)_3$ removed both As^{III} and As^{V} ions by chemical reactions on the surface of the $Fe^{III}(OH)_3$. Similar to the reaction between the $H_2PO_4^-$ and $Fe(OH)_3$ precipitates (273), the neutral functional group of {═FeOH} reacts with $H_2AsO_3^-$ ions, and surface compounds of {═$FeAsO_3H_2$}, {═$FeAsO_3H^-$}, and {═$FeAsO^=$} can be formed (274). Many water treatment plants through out the world have used chemical fixation processes similar to this. However, such processes may prove expensive in developing countries where the effectiveness of the process is dependent on the local availability of the necessary materials.

6.2.2 In Situ Remediation Using Passive Reactive Barriers

An alternative method for the removal of As from water, notably groundwater, is the use of Fe oxide containing materials as passive reactive barriers. This type of treatment offers a low-cost alternative for the removal of As from pollution plumes. Arsenic concentration was reduced from between 1 and 3 mg As/L to $<$0.2 mg As/L over a 92-day period in laboratory column experiments using a reactive barrier containing spodic B horizon material (275). Laboratory studies at the CSIRO laboratories in Adelaide, Australia indicated that a significant amount of As may be sorbed by high-Fe-and-Al-containing materials obtained relatively cheaply. In a short column study it was observed that pyritic and oxidic materials sorbed between 2500 and 5000 mg/kg of As^{V}, respectively (Fig. 15), indicating that these cheap and easily obtained materials may also be suitable as alternative barrier wall materials. However, there still remains some doubt as to the stability of these natural materials over time as well as their efficiency to sorb As^{III} from aqueous solutions. Although there has been some concern about the long-term stability of sorbed As over time, it has been reported that as long as a high Fe-As ratio is maintained, ferric arsenates may be extremely insoluble and useful for the safe disposal of As.

A common problem that has been reported extensively in the literature is the inefficiency of As^{III} removal by sorbents compared to As^{V}. The most common treatment to enhance As removal is the inclusion of a preoxidation step, through the use of an oxidizing reagent such as Cl_2 or hydrogen peroxide. However, this will increase the cost and complexity of the water treatment. Several researchers have reported that As^{III} may be removed far more effectively than As^{V} over a much wider pH range. Matis et al. (276) reported that As^{III} was removed far more successfully than As^{V} over a wide pH range between 2 and 10 in goethite

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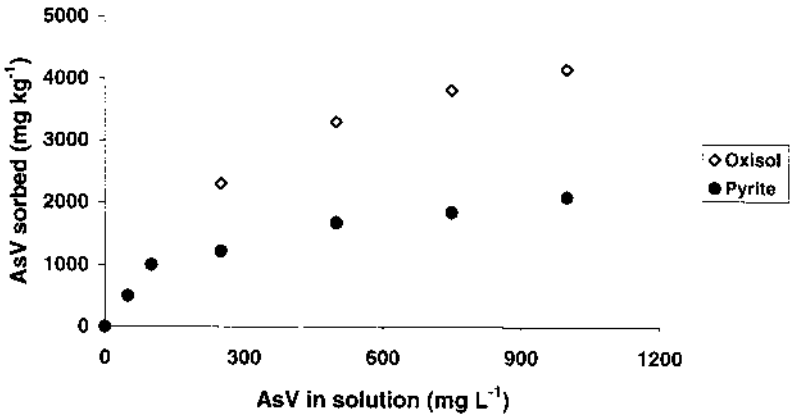

FIGURE 15 Column studies of As^{V} sorbed by Fe- and Al-containing materials (Smith and Naidu, unpublished data).

concentrations of 0.5 and 1 g/L in solution. These experiments highlight the potentially useful and inexpensive treatment materials that may be used for the removal of As^{III} from solutions. However, further studies are required before the use of such naturally occurring materials as used by Lindberg et al. (275) can be considered for the removal of As^{III} from aqueous solutions.

6.2.3 Bioremediation with Chemical Precipitation

Another technique reported recently combines bioremediation and chemical precipitation processes to remove As. The potential for a biological treatment process to remove elemental As and arsenide under reducing conditions and as precipitates of iron hydroxides under oxic conditions were investigated (117,277,278). These investigators subjected As-contaminated groundwater to aerobic, aerobic-anaerobic bioreactor systems and through a cell containing $FeCl_3$. It was found that the concentration of As in groundwater remained unchanged when the bioreactor was operated only under aerobic conditions. However, under combined aerobic and anaerobic operating conditions, As concentrations in the groundwater were reduced by more than 70% in the first anaerobic cell and by another 50% in the second anaerobic cell. The addition of $FeCl_3$ to the second aerobic cell was found to increase the total removal of As up to 99.7%. The enhanced removal of As was attributed to precipitation of the most oxidized form of As (As^{V}) with Fe^{III}. The lowest effluent concentration of As achieved by this process was 90 μg/L, with the optimum separation being achieved through control of pH in the anaerobic cell. Another As removal process used during the purification of water included a chemical oxidation process followed by coagulation of As with Fe-

Mn oxidation or softening plants. In this process soluble As^{V} removal efficiency was primarily controlled by pH during coagulation by Fe^{II} oxidation and $Fe(OH)_3$ precipitation during Fe-Mn oxidation and by $Mg(OH)_2$ formation during the softening process.

6.2.4 Aquifer Oxygenation

Arsenic has been successfully removed from groundwater by injecting air or oxygen into the aquifer to precipitate arsenate (As^{V}) in the aquifer at a confidential Superfund site; therefore, aboveground treatment might be entirely avoided (279). This might be an attractive approach for treatment in certain geologies since capital and operational costs are relatively low. However, this approach requires extensive site-specific geological and hydrogeological investigations to confirm the effectiveness of the approach, and to design the system.

6.2.5 Low-Cost Emerging Technologies for the Developing World

Several low-cost remediation options have been suggested for the amendment of high-As groundwater, such as: (a) *autoattenuation*; (b) *use of geological material as natural adsorbents* for As, viz. laterite or Fe-rich oxisols; (c) *artificial recharge* to the aquifers following aeration; and (d) *bacterial iron oxidation*, where natural microbial activity is used to remove iron and As from groundwater. In the following section, the possibilities of each of these remedial techniques and their possible applications in the affected segments of the BDP are discussed.

Autoattenuation. The principle of autoattenuation is one of the lowest-cost and convenient methods to remediate groundwater containing high concentrations of As and iron (269,280). The method is simple to adopt at the rural household level and needs collected groundwater from wells and to stand for a few days. Most groundwater in the BDP is rich in dissolved iron, which readily oxidizes upon aeration and forms ferric precipitates. The autooxidation of Fe^{II} to Fe^{III} generates a favorable substrate with surface-reactive sites for the adsorption of both anionic As^{V} and uncharged As^{III} species. Studies on autoattenuation of As were carried out on several pairs of groundwater samples from Bangladesh. In situ filtered and acidified samples, from two adjacent wells in the Harian village, Rajshahi District, indicated initial Fe and As concentrations of 9–9.5 mg/L and 92–120 μg/L, respectively. Filtered, unacidified samples from the same wells were analyzed after a residence time of 15 days following refiltration and acidification and indicated Fe and As concentrations in the range of 53–47 μg/L and 23–36 μg/L, respectively, thus removing 70–75% of As. However, a similar set of water samples from different depths at Ujalpur in the Meherpur District revealed contrasting results. Shallow groundwater from a depth of 30 m with initial Fe and As concentrations of 9.2 mg/L and 104 μg/L, respectively, were

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reduced to 20 μg/L Fe and As after a similar period indicating removal of 88% As (Fig. 16). In deeper wells (75 m) where the initial Fe concentration in groundwater was low (2.7 mg/L) only 30% As was removed by autoattenuation, although the concentration of Fe decreased by ca. 95% (M-2, Fig. 16). The differential behavior is related to the variations in the hydrochemical characteristics, particularly the groundwater redox level ($E_H = -0.4$ V), Fe/As ratio, and the presence of other terminal electron acceptors. These observations clearly indicate that autoattenuation is one of the promising methods for the amendment of high-As groundwater and needs to be investigated further.

Use of Geological Materials as Natural Adsorbents. Laterite has been tested as an adsorbent and proved to be a promising low-cost remedial technique to safeguard high-As drinking water (281–283). Laterite occurs as a red-colored, vesicular, clayey residuum abundantly in tropical regions. Laterite is an acidic soil with a typical pH between 4 and 5. The major components of laterite are hydrous oxides of iron and aluminum, with minor proportions of manganese and titanium. Both hydrous iron and aluminum oxide components in laterite have a pH_{zpc} (zero point of charge) at 8.5–8.6 (284,285). The typical pH_{zpc} of the tested laterite samples from Medinipur in West Bengal was found to be around 8.1 (R. Bhattacharyya, personal communication, 2001). Under natural conditions they

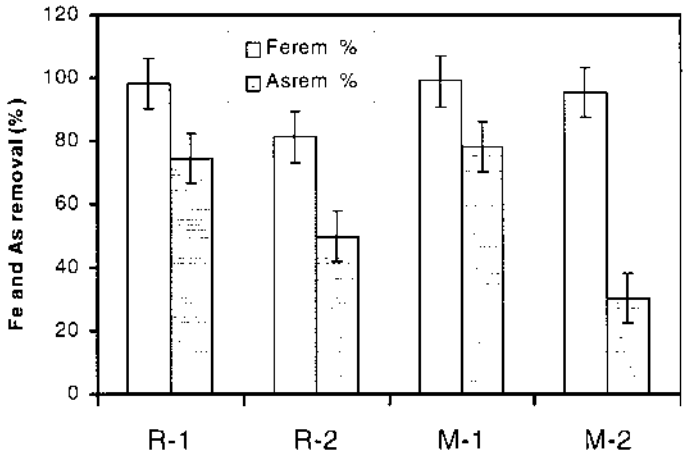

FIGURE 16 Results of some autoattenuation tests on groundwater in Bangladesh. R-1: Harian, Rajshahi (27 m); R-2: Harian, Rajshahi (27 m); M-1: Ujalpur, Meherpur (27 m); M-2: Ujalpur, Meherpur (75 m). The staple bars represent ±5% SD for the analytical measurements. (Data from ref. 280.)

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are characterized by net positive surface charge and capable of adsorbing several anionic contaminants at a wide pH range (105,286).

Laterite could either be used in a filter column or directly mixed with water in the water vessel where the soil particles would act as adsorbent during sedimentation. Adsorption batch experiments on high-As groundwater from Ghetugachi village in Chakdaha of Nadia district, West Bengal indicate a considerable decrease in As concentration after mixing with varying amounts of laterite. The efficiency of As removal varied between 50 and 90% for 5 g of added laterite per 100 ml water under a reaction time of 20 min (Fig. 17a,b). The maximum effective adsorption was achieved during the first 10 min and remained more or less constant with time (Fig. 18). The fine-grained laterite indicated highest adsorption due to available reactive surface area. Amendment or pretreatment of laterite also affects the adsorption capacity due to the increased specific surface area (Fig. 19).

Artificial Recharge. Artificial recharge has been used to augment the groundwater availability. The technique has been used to improve the ground-

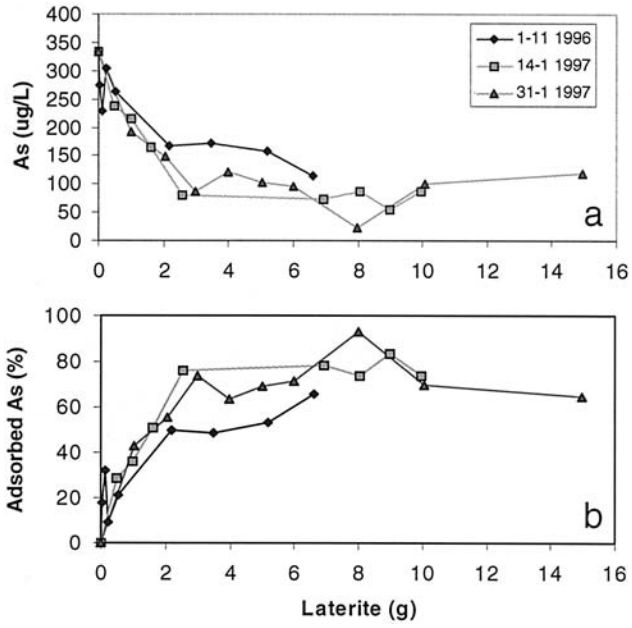

FIGURE 17 Arsenic adsorption on laterite using groundwater samples from Chakdaha Block, Nadia district, West Bengal, India. (a) Remaining As in water and (b) amount of As adsorbed on laterite. (Data from ref. 281.)

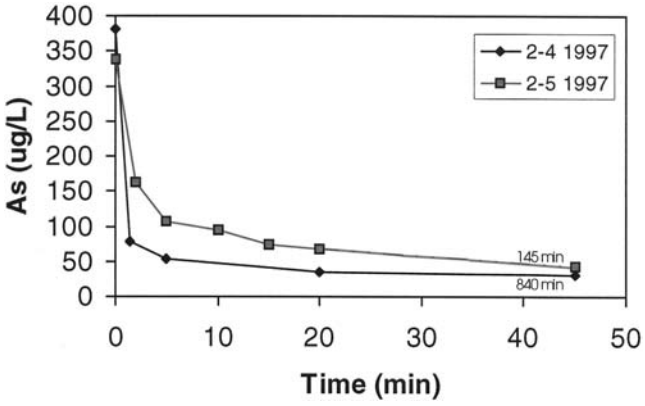

FIGURE 18 Reduction in As concentration in groundwater with increased residence time of laterite. (Modified from ref. 282.)

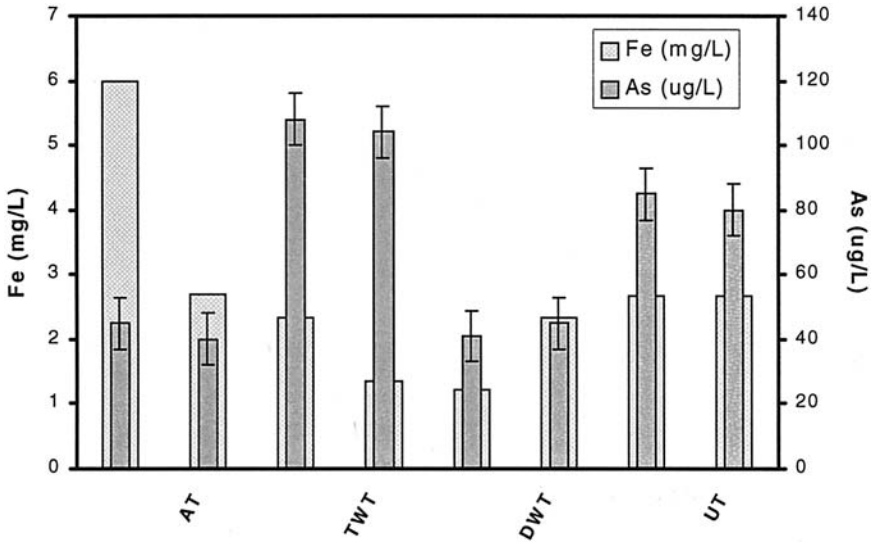

FIGURE 19 Behavior of As and Fe in groundwater with treated laterite. AT: acid treated; TWT: tap water treated; DWT: distilled water treated; UT: untreated. The staple bars represent ±5% SD for the arsenic analyses. (Data from refs. 281–283.)

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water quality to a large extent in Finland and in Sweden to remove iron from the groundwater. Removal of nitrate from groundwater in Denmark was tested by recharge through straw beds supplying organic matter for denitrification. Groundwater recharge has been used in India to decrease the fluoride content of groundwater. Evidence from within the BDP reveal that the As is mobilized in groundwater from an adsorbed pool of As-rich ferric oxides through reductive dissolution. The basic purpose is to elevate the redox status of the aquifer to prevent the transformation of Fe^{III} to soluble Fe^{II} forms. Atmospheric O_2, NO_3^-, and H_2O_2 (which is being used widely in the United States) are the three practical oxidants used in aquifers. Oxygen has a limited solubility at high ambient temperature in the wells of the area, thus well infiltration of oxygenated groundwater may help as compared to pond recharge where growth of algae and their subsequent microbial degradation may consume oxygen rapidly.

On the other hand, denitrification requires three conditions: the presence of nitrate, anaerobic conditions, and degradable organic matter (195,272). Nitrate can be added in the anoxic aquifers, but the key factor is the degree of degradability of the organic matter. The organic matter must also be readily available as a substrate for denitrifiers. It may not be a disadvantage if the organic matter is a bit recalcitrant as that would imply that the effect of recharge will be undisruptive to water pathways by avoiding local clogging with ferric precipitates. Ferrous iron in the blue clay layers that are sandwiched within the postglacial Danish aquifers can chemically reduce nitrate. Whether nitrate could be used as an oxidant could be evaluated by analysis of the $^{15}N/^{14}N$ ratio in the traces of nitrate occurring in the groundwater. Denitrification discriminates ^{15}N and causes accumulation of the isotope in the residual nitrate. The applicability of using nitrate as oxidant should then be tested in batch tests with sediment samples, preferably nonoxidized, core sediments. Applicability of recharge pits or ponds could be tested in areas where the surface sediments and the upper aquifers are permeable so that recharge water reaches the groundwater table directly. Simple and reliable recharge wells have been designed by Vivekananda Research and Training Institute in Gujarat, India, which have been in use for 13 years and are based on sand filtration on the top of the recharge well (287).

The Central Ground Water Board (CGWB) (288) has carried out experimental studies on artificial recharge of the shallow aquifers yielding high As in the North 24–Pargana district of West Bengal, India. A recharge pit ($3.1 \times 2.8 \times 2$ m) was constructed and water from the recharge pit was allowed to recharge the shallow aquifers, through a properly designed exploratory well (A. Ray, CGWB, personal communication, 2001) placed at a depth of 17 m. The design of the exploratory well with proper sealing and packing avoided inflow of As-rich water from overlying aquifers and aquitards (Fig. 20). It was observed that within a span of 90 days, groundwater As concentrations indicated a decrease from 128 μg/L to 1 μg/L (288). Based on the success of the study on experimental artificial

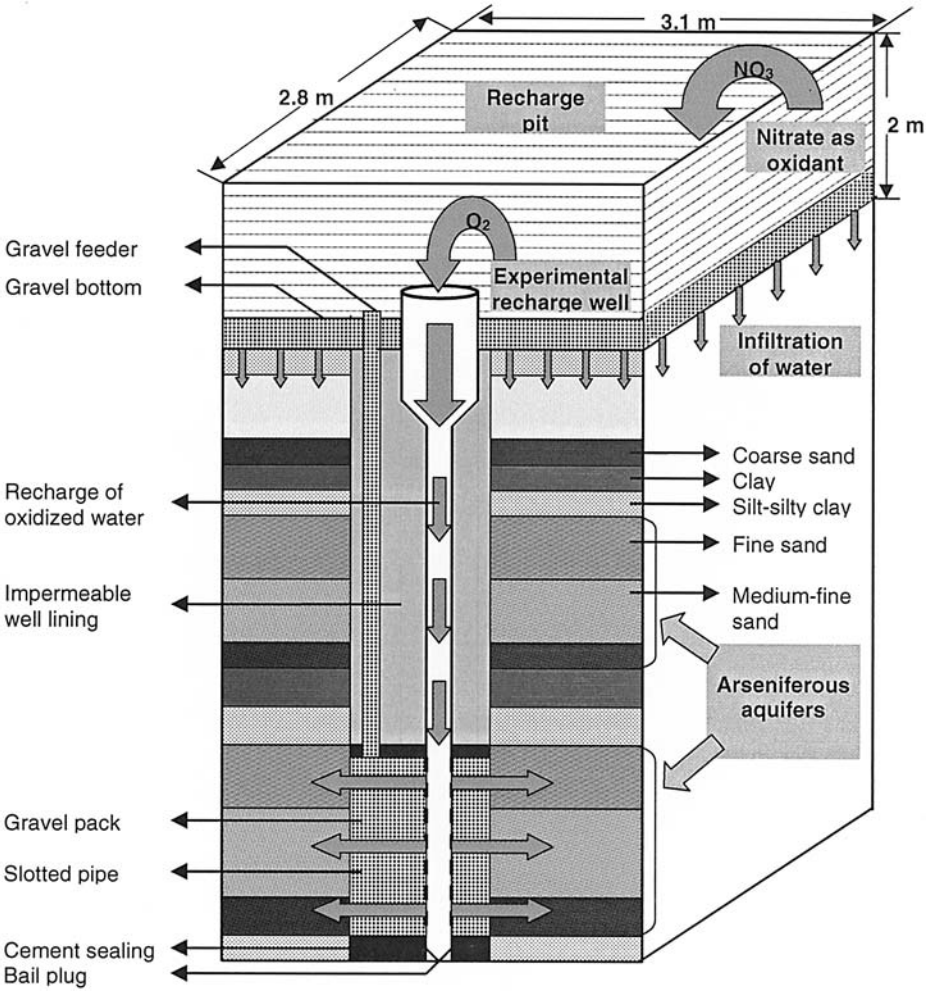

FIGURE 20 Schematic diagram showing the model for artificial recharge developed by the Cental Ground Water Board (288) and design of the recharge wells (A. Ray, personal communication, 2001) for the remediation of high-As groundwater.

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recharge, further studies are presently being conducted in other As-affected areas of West Bengal. Since the solubility of oxygen is fairly low (6 mg/L) at the high ambient temperatures, there can be risk of rapid depletion of oxygen owing to the growth of algae and other biota that may accumulate at the bottom of these recharge pits. To circumvent this problem, an oxidant, such as nitrate, can be added to these recharge pits in low concentrations. Nitrate can act as an electron acceptor, which can inhibit lowering of the redox to the Fe^{III}/Fe^{II} stage. Artificial recharge of groundwater would be one important strategy to elevate the redox status of the aquifers, where in situ oxidation of Fe^{II} offers the advantage that generation of large amounts of As-laden ferric sludge can be avoided.

Bacterial Iron Oxidation. The process of bacterial iron oxidation employed at some well sites in the United Kingdom and France seems to be a very useful method for the removal of iron from groundwater (289,290). The naturally occurring bacterial population in the well environment is carried to such filter beds by groundwater where the biogeochemical processes trigger the oxidation of ferrous iron to ferric iron. The biogenic filter rich in Fe^{III} precipitate may consequently adsorb the dissolved As species.

7. CONCLUSIONS

Like most elements, As is cycled both through natural processes and by human activities. However, the emissions to the atmosphere are totally dominated by anthropogenic emissions, which account for nearly 78 gigagram/year while the natural emissions are about 12.2 gigagram. Beneficiation of sulfide ores, combustion of coal, and incineration of preserved wood products are the major sources of anthropogenic As emissions.

In the earth's crust, As is incorporated in sulfide minerals and the distribution of As follows roughly that of sulfur. Among rocks, basic rocks and shales show the highest contents. Arsenopyrite is the major source of arsenic in sulfide ores and the mineral from which most of the arsenic used by humans is derived. The major uses of arsenic are for wood preservation and as a pesticide in agriculture. The latter use has declined drastically but many soils still bear the memory of past use. While As has limited use in the present society, the unintentional exposure of humans and other organisms to the element is of increasing concern. Emissions from smelters have decreased through strict control of the processing. However, coal combustion still emits huge quantities of arsenic to the atmosphere especially in developing countries.

The quantities of As in sulfide ores in excess of what has been used in the society have left huge amounts of As in the mine tailings. The mobilization to water from such material as well as from natural rocks and sediments is a key factor in assessing the risk for As poisoning of organisms. The mobility of As

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in natural environment is governed by the redox conditions. In oxygenated environments the As in arsenopyrite is oxidized to the trivalent or pentavalent state. Arsenite (As^{III}) is undissociated at neutral pH values making it more mobile than arsenate (As^{V}). Adsorption of arsenic species occurs predominantly on ferric and aluminum oxides and hydroxides. These components have a pH-dependent charge making them more efficient anion adsorbents at lower pH values.

Human exposure to As is mainly from groundwater. As is mostly mobilized from geogenic sources. Two main mechanisms have been identified, one through oxidation of pyrite or arsenopyrite, the other through reduction of ferric hydroxides carrying adsorbed As. The former type of As contamination of groundwater occurs in connection with mineralized zones and is found in Mexico and at several places in the Andes such as in Chile. The other type of groundwater As occurrence has affected large parts of the Bengal Delta Plain in Bangladesh and India and several other sedimentary aquifers elsewhere. Arsenic adsorbed onto ferric hydroxides has been deposited in the sediments along with organic matter from vegetation. Microbial degradation of the organic matter under anoxic conditions has led to the reduction of the ferric hydroxides to soluble ferrous iron, simultaneously releasing the As. A similar mechanism is inferred for a large area in northern China.

Remediation of As-contaminated soils has so far generally been done by chemical fixation of the As by applying oxyhydroxides that adsorb the As under oxygenated conditions. However, electroremediation, biotransformation, and especially bioremediation are promising measures. Biotransformation through stimulation of microbial oxidation of arsenite to arsenate renders the As less mobile. Another common process is biomethylation, which transforms inorganic As to a volatile methylarsine phase. Phytoremediation is another technique in which certain plants with a high uptake of As are used to cleanse the soil from As. Chemical remediation of soils using reducing agent such as oxalate releases the adsorbed As^{V} and As^{III} oxyanions by reductive dissolution of the poorly ordered and noncrystalline secondary hydrous oxides of Fe and Al as well as the ITM materials in the podzolic soils. This has been tested on a bench scale and shows strong promise for application to remediate As-contaminated sites.

The permissible limit of As for safe drinking has recently been lowered from 50 μg/L to 10 μg/L by the WHO and USEPA as a result of epidemiological investigations. Many countries have followed suit. Commonly ferric or aluminum hydroxide precipitation is used for the treatment of groundwater with excessive As content, sometimes combined with an oxidant to bring the As to the As^{V} state. As many of the occurrences of high-As groundwater are in developing countries with poor infrastructural facilities, there is a need for simple and cheap methods for removal. Autoattenuation when the groundwater contains abundant ferrous iron is one possibility. Use of locally available geological materials such as later-

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ite is another option. Artificial recharge affecting the underground redox conditions has been tested with promising results.

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7

Environmental Aspects of Arsenic Toxicity

J. Thomas Hindmarsh
The University of Ottawa and the Ottawa Hospital, Ottawa, Ontario, Canada

Charles O. Abernathy
U.S. Environmental Protection Agency, Washington, D.C.

Gregory R. Peters
Philip Analytical Services Inc., Bedford, Nova Scotia, Canada

Ross F. McCurdy
InNOVAcorp, Dartmouth, Nova Scotia, Canada

1. INTRODUCTION

Arsenic is the 52nd most common element in the earth's crust with an average natural abundance of approximately 1.5–3 mg/kg. It is ubiquitous in the environment, occurring from both natural and anthropogenic sources, and both may pose a threat to human health. Numerous control mechanisms for arsenic exist in the environment but the natural cycling of this element throughout the various envi-

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ronmental compartments (air, water, soil, and biota) is complex and poorly understood. The main sources of human exposure to arsenic are from the drinking water supply and food.

A decade ago, it seemed that chronic arsenic poisoning was a rare and diminishing problem. However, since then it has again emerged in parts of Asia with unprecedented fury where tens of millions of people are exposed to toxic levels in their drinking water, probably as a consequence of increased agricultural irrigation. In recent years the toxic potential, both carcinogenic and noncarcinogenic, of arsenic in drinking water has been intensely studied. However, further research is needed to determine the toxic threshold for this element as well as to understand the mechanisms governing the release of soluble arsenic into the various environmental compartments and how this can be modified.

The health effects of environmental arsenic and its geochemistry have recently been reviewed (1–3).

2. CHEMISTRY AND TOXICITY

Arsenic is a metalloid belonging to group 15 (old group 5) of the periodic table (N, P, As, Sb, Bi). It exists predominantly in nature as the oxyanion with an oxidation state of either (+3) or (+5); however, the (−3) state also exists in other arsenic species. Arsenic binds covalently with most metals and nonmetals, and it also forms stable organic compounds.

An important difference between arsenic and phosphorus (its neighbor in the periodic table) is the stability of their esters to hydrolysis. Adenosine triphosphate (ATP) is relatively stable whereas the corresponding compound formed with arsenate is easily hydrolyzed thereby uncoupling oxidative phosphorylation; this accounts for the toxicity of arsenates in oxidative phosphorylation. Trivalent arsenic compounds have an affinity for sulfur and this probably accounts for their inhibition of a variety of enzymes such as pyruvate oxidase and 2-oxoglutarate dehydrogenase. It has been proposed that the tumorigenic potential of arsenic compounds may be related to the ability of some of them to form free radicals (4).

Generally, trivalent arsenic compounds are more toxic than their pentavalent counterparts and inorganic arsenic compounds are more toxic than organoarsenicals. Elemental arsenic is the least toxic form. Arsenobetaine and arsenocholine (fish arsenic) are apparently virtually nontoxic. Arsenosugars are found in marine algae and seaweeds (5). Some aryl-arsenicals have been used extensively in the past as growth promoters for farm animals although these have been largely replaced by antibiotics more recently. Melarsoprol is still used to treat trypanosomiasis in humans. The formulae of some common arsenic compounds are shown in Table 1.

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TABLE 1 Arsenic Compounds Relevant to Human Toxicity

Arsenic trioxide	As_2O_3
Arsenous acid	H_3AsO_3
Arsenite	$H_2AsO_3^{1-}$, $HAsO_3^{2-}$, AsO_3^{3-}
Arsenic pentoxide	As_2O_5
Arsenic acid	H_3AsO_4
Arsenate	$H_2AsO_4^{1-}$, $HAsO_4^{2-}$, AsO_4^{3-}
Arsanilic acid	$C_6H_4NH_2AsO(OH)_2$
Arsenobetaine	$(CH_3)_3As^+CH_2COO^-$
Arsenocholine	$(CH_3)_3As^+CH_2CH_2OH$
Dimethylarsinic acid	$(CH_3)_2AsO(OH)$
Methylarsonic acid	$CH_3AsO(OH)_2$

Figure 1 outlines the global arsenic cycle, illustrating the cycling of arsenic through the various environmental compartments.

3. NATURAL SOURCES

Arsenic occurs naturally in many minerals with FeAsS being the most common. Although it is very stable and water insoluble as the arsenopyrite, this will readily oxidize when exposed to air to yield compounds that are water soluble.

Little is known about the release of arsenic compounds into the atmospheric compartment. Natural weathering and microbial action in the soil may release volatile species into the air; also, volcanic activity may release some volatile species and particles. However, these amounts are usually relatively small.

In soils, arsenic may exist in several forms. Soil has some self-cleansing properties in that adsorption and coprecipitation of inorganic arsenic occurs onto clay particles. Also, it forms insoluble precipitates with sulfur and soil cations, particularly iron, as arsenopyrites.

In the water compartment, arsenic can be naturally introduced as a result of erosion and weathering of rocks. Whereas anthropogenic sources can contribute significantly to the content of surface water, groundwaters are, not surprisingly, less commonly contaminated from this source and are more commonly contaminated by the natural weathering of arsenio-bearing minerals. In surface water, arsenic can undergo a number of reactions, which include oxidation/reduction, adsorption/precipitation, and methylation. Most surface water supplies have Eh and pH levels (acid and oxidizing) that favor arsenate precipitation. Surface water therefore has a self-cleansing action for arsenic as arsenite and more particularly

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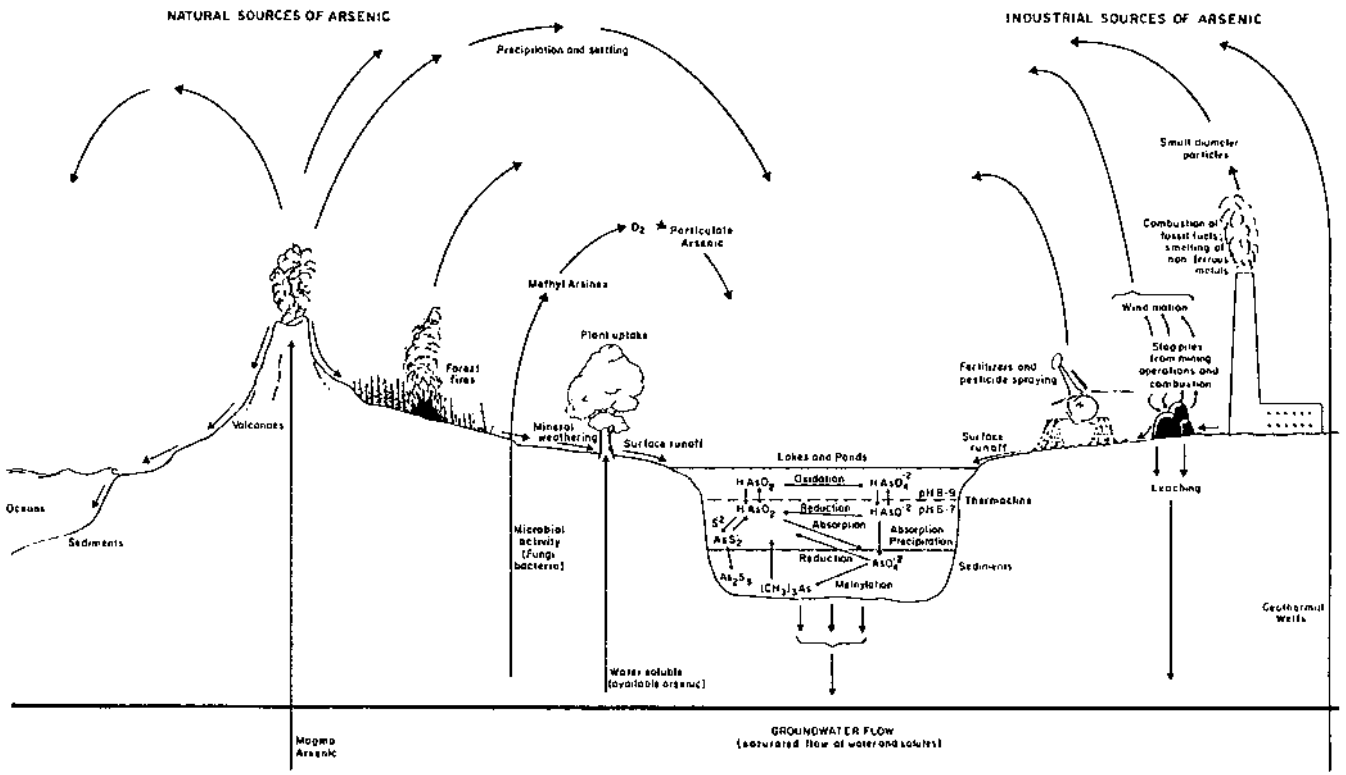

FIGURE 1 The global arsenic cycle. (From WT Piver. Biological and environmental effects, BA Fowler, ed., Top Environ. Health 6:1, 1983. With permission.)

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arsenate form insoluble salts with dissolved or suspended cations (usually iron) and these generally settle out in the sediments. Thus, much of the arsenic content of surface water is usually present as insoluble particulates and sediment (6,7). The relative significance of biomethylation in the surface water compartment is uncertain.

In groundwater, the arsenic cycle has important toxicological implications. Here, the more toxic reduced form, arsenite, is more prevalent (1,6). Unlike surface water, deep groundwater generally has higher pH and low Eh levels and this promotes the solubilization of arsenic released by weathering. As in surface water, groundwater also can be self-cleansing. However, iron salts, the principal agent with which arsenic combines, are often deficient because the increased pH reduces their solubility.

The extensive use of groundwater for human consumption has led to massive outbreaks of severe chronic arsenic poisoning in South East Asia (Bengal, Bangladesh, China, Taiwan) (8–11). In West Bengal, the most severe contamination was found in wells between 35 and 46 meters deep (12).

4. ANTHROPOGENIC SOURCES

The largest contributor to arsenic release in the environment is the mining and smelting of nonferrous metals. The burning of fossil fuels follows next in significance. The use of chromated copper arsenate as a wood preservative and the, now substantially reduced, agricultural use of arsenic are lesser sources of environmental contamination.

Arsenic is present in lead, copper, and gold ores and the smelting of these releases arsenic as a gaseous emission with arsenic oxides as by-products. Although these emissions account for 50–60% of total global contamination, their effects are localized to regions around the smelters.

Although mining is not a major contributor to global contamination, significant local contamination may occur from the arsenic-rich waste-rock tailings when they become weathered and oxidized, and arsenic can then leach into the soil and surface and groundwaters. The widespread use of these mine tailings as fill around houses and for road construction can spread the contamination beyond the limits of the mines. Surface runoff of the soluble arsenic species from waste-rock tailings and from contamination of soils with arsenic-containing pesticides can enter the surface water, but the self-cleansing action of this will often remove it by precipitation as insoluble iron salts or by adsorption with clays, provided the appropriate conditions prevail.

The arsenic content of North American coal is quite low (13). However, the soft coals of eastern Europe can have very high arsenic contents and can produce significant contamination in the fallout zones where this is burned (14). The arsenic content of petroleum fuels is much lower than that of coal. However,

because of the sheer quantity of these fuels that are burned, petroleum and oil burning contributes substantially to global pollution.

5. HUMAN TOXICOLOGY

The following discussion will describe the toxic effects of the long-term consumption of small amounts of arsenic by mouth derived from the drinking water supply or from medications such as Fowler's solution (1% potassium arsenite). The carcinogenic effect (lung cancer) of the chronic inhalation of arsenic trioxide dust is well established. Little or no arsenic can be absorbed through the intact skin.

5.1 Chronic Arsenic Poisoning—General Effects

Arsenic interferes with enzyme action, DNA transcription, and metabolism. Therefore, it is not surprising that its effects upon the body are protean. These include chronic weakness, general debility and lassitude, loss of appetite and energy, loss of weight, and sometimes a degree of dementia. Anemia, probably due to bone marrow suppression (normochromic and normocytic), is common and leukopenia may also occur. Basophilic stippling of the erythrocytes may be present and megaloblastic changes have been reported as have disorders of heme synthesis (1,6).

Noncirrhotic (presinusoidal) portal hypertension is a rare and relatively specific hepatic manifestation of chronic arsenic exposure (15), which may be the consequence of arsenic-induced vascular endothelial injury (16).

5.2 Dermatological Effects

The skin manifestations of chronic arsenic poisoning are hyperpigmentation and hypopigmentation, progressing to palmar/plantar hyperkeratoses in which skin cancer often subsequently develops (6). The hyperpigmentation develops around hypopigmented macules: the characteristic raindrop pattern. Diffuse pigmentation is more pronounced in the axillae and groin. The hyperkeratoses develop on the palms of the hands and the soles of the feet and, often, raised wart-like keratoses project from the surface or from the sides of the fingers. Hyperpigmentation may first appear 6 months to 2 years after onset of exposure in excess of 0.04 mg/kg/day; lower exposure rates can take longer. Palmar and plantar hyperkeratoses take several years to develop (1).

5.3 Cardiovascular Effects

Chronic arsenic exposure is associated with an increased prevalence of peripheral vascular disease, which in extreme examples can produce peripheral gangrene,

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the consequence of thromboangiitis obliterans, in small-limb vessels (blackfoot disease). Also, there appears to be a similar but less pronounced association between arsenic exposure and hypertension and cardiovascular disease.

5.4 Neurological Effects

Chronic arsenic exposure commonly produces central and peripheral nervous system impairment, and histological examination of the peripheral nerves in such cases reveals a sensorimotor axonopathy (17). The peripheral neuropathy is often largely confined to the arms and legs and is usually more pronounced distally. Paresthesia is often troublesome. Sensory impairment is commonly more pronounced than motor effects, and the legs are often more severely affected than the arms. The features are often severe and slow to recover (17,18). There is convincing experimental work in animals supporting the concept that arsenic has an immunomodulating effect (19,20) and this may explain the effectiveness of arsenic-containing medications in treating asthma in humans. There is also animal evidence that it is teratogenic and mutagenic (1).

Inorganic arsenic is detoxified in the human by methylation to monomethyl arsonic and dimethyl arsenic acids (the latter the most prevalent) and these are excreted in the urine. This process is impaired, at least in rabbits whose diets are deficient in methyl donors (methionine, choline) and protein (21). Thus, deficient diets have the potential to aggravate arsenic toxicity.

5.5 Cancer Effects

The association of chronic arsenic ingestion and skin cancer (intraepidermal carcinoma, Bowen's disease), squamous cell carcinoma, and superficial multicentric basal cell carcinoma is long established. Also associated are bladder cancer and angiosarcoma of the liver, and probably also renal carcinoma. The relationship between arsenic inhalation and lung cancer is well known but there now seems to be a clear association between arsenic ingestion (from the drinking water supply) and the increased prevalence of lung cancer in humans (1).

The lack of an animal model has hampered investigation of potential mechanisms of the tumorigenic action of arsenic. A recent study in mice reported that the administration of arsenate (500 μg/L) induced tumors of the gastrointestinal tract, lung, liver, spleen, bone, skin, reproductive tract, and eye (22). However, more work is needed prior to accepting this model for studying arsenic-induced carcinogenicity. Although there is no accepted mode of action for arsenic, it has been established that arsenic does not directly react with DNA and cause point mutations in bacterial or mammalian cells (1,23). There are several potential ways that arsenic could cause cancers. For example, administration of arsenic or dimethylarsinic acid (DMA, a metabolite of inorganic arsenic) can increase oxidative stress, a putative mechanism for induction of cancer (1,4).

Other authors have reported that arsenic can alter gene expression by altering gene methylation: Mass and Wang (24) found that arsenic caused a dose-response-related hypermethylation in human lung adenocarcinoma cells. On the other hand, Zhao et al. (25) reported that arsenic could transform a rat liver epithelial cell line into one that could cause tumors in mice and concluded that hypomethylation of DNA was a potential mechanism for arsenic-induced cancer. Since alterations in methylation patterns could affect DNA metabolism such changes could affect gene expression. In addition, arsenic can also affect cell proliferation (26). Although each of the above mechanisms could induce cancer, more work is necessary prior to accepting these or other theories on the mechanism of arsenic-induced cancers.

6. SOURCES OF HUMAN EXPOSURE

Apart from persons working in nonferrous metal smelters and those living near these (and also near electricity-generating stations burning heavily arsenic-contaminated soft coal), the major exposure of the general public to arsenic is from ingestion from their drinking water and food supply. Arsenic is found in large amounts in some soils and rock formations but is relatively inert, and unless it enters the drinking water supply, it is harmless.

6.1 Exposure from Food

As a generalization, provided the drinking water supply is uncontaminated, then the risk of eating vegetables grown in arsenic-contaminated water seems to be small; there is no well-documented evidence that they cause a risk. In root vegetables and fruits, much of the arsenic tends to migrate to the outer surface and is removed in washing and peeling. However, this is an underresearched area and is the subject of active investigation at the present time. It has been recently reviewed (1,27–29).

Of concern is the amount of arsenic ingested from rice and other foods in the diet, grown in the heavily arsenic-contaminated waters of parts of southeast Asia. Few data are available on this but two studies from Taiwan (30,31) report rice arsenic contents of 0.15 mg/kg and 0.7 mg/kg, the former diet providing a calculated daily intake of approximately 19 μg/ (plus 31 μg from yams). This intake would be the equivalent of ingesting 1 L of drinking water containing arsenic at the widely accepted standard of 50 μg/L. Speciation of arsenic in food has repeatedly shown it to be predominantly inorganic (arsenate and arsenite).

Large amounts of arsenic are found in fish and shellfish (arsenocholine, arsenobetaine) but these are apparently nontoxic and are mostly excreted un-

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changed in the urine (32). Thus, when using urine arsenic measurements to assess exposure, it is necessary to fractionate the arsenic species (33).

6.2 Drinking Water

Most large-scale episodes of chronic arsenic poisoning have resulted from arsenic contamination of drinking water. However, none have been so large as the current outbreaks in southeast Asia (Bengal, Bangladesh, China, Taiwan). The data from Taiwan and from Mexico, Argentina, and Chile on large numbers of people subjected to high levels of drinking-water arsenic have provided opportunities to delineate the risks of excess drinking-water arsenic. The largest study reviewed 40,421 persons using contaminated water, compared with 7500 controls (11,34,35).

The toxic threshold, if one exists for humans, is heatedly debated. Stöhrer (36) has reviewed the extensive data from Taiwan and elsewhere and has concluded that skin cancers, internal cancers, and noncancerous effects of arsenic have approximately the same ''threshold'' and that these decrease sharply when the intake falls below 400 μg/day, and that the disease potential above this level is well established. Other researchers have reported data from human studies that suggest that adverse health effects occur below 400 μg/day. In Utah, Lewis et al. (37) examined the effects of arsenic in drinking water at concentrations under 200 μg/L. They found increases in mortality from prostate cancer, hypertensive heart disease, nephrosis, and nephritis in males and in hypertensive heart disease and in all other heart disease categories in females. In their review of epidemiological evidence for a threshold, Smith et al. (38) state that most human studies do not provide any data supporting a threshold for arsenic. The problem associated with establishing a threshold for arsenic is that the current epidemiology studies are ecological in nature (no individual exposure data are provided) and, as such, are poorly suited for this task. Although the in vitro studies all indicate that arsenic may mediate its effects through mechanisms that would give sublinear curves (1), there is no accepted mode of action for the deleterious actions of arsenic. Studies examining the effects of arsenic in drinking water at concentrations of 10–200 μg/L or acceptance of mechanism(s) of action for arsenic are necessary to resolve this question. Thus the risks of ingesting water with a content that provides an arsenic intake of less than 400 μg/day are unresolved and have been the target of extensive study by the U.S. Environmental Protection Agency and the U.S. National Research Council, who have used the above data in an attempt to determine the upper limit of acceptable arsenic content for drinking water for the United States (39–41).

The U.S. National Research Council has constructed several models to assess toxicity at low concentration in drinking water including extrapolation of

the dose-response curve to the left, assuming it is linear. Their deliberations have recently resulted in a major report, a detailed description of which is beyond the scope of this chapter (1). In general, allowing for the difference in water intake and weight between Taiwanese and U.S. residents, their conclusion was that each microgram of inorganic arsenic per liter in the drinking water might increase the lifetime risk of skin cancer by three to seven additional cases per 100,000 persons, or approximately one to five additional cases per 1000 persons consuming water with an arsenic content of 50 μg/L. However, the authors recognize the limitations of this information, which is based on ecological studies, and also that the dose-response curve below the level of observed effects may not be linear and that the risk may well be less than that implied by linear extrapolation. It should also be remembered that skin cancer is an eminently treatable disease. The risk data on other cancers is more complex.

The purpose for recommending or instituting regulatory limits for a chemical in drinking water is ideally to prevent or at least decrease the occurrence of adverse health effects after consumption of water containing that chemical. One of the first considerations is whether the chemical causes adverse human health effects. The World Health Organization (WHO), Canada, and the U.S. EPA have all classified arsenic as a human carcinogen. At the present time, WHO and Canada have published recommendations for arsenic in drinking water, while the United States is in the process of proposing a regulatory level. WHO (42) proposed an arsenic guideline value of 10 μg/L based on occurrence of skin cancer and analytical techniques, whereas Canada set an interim maximum arsenic concentration of 25 μg/L based on estimated lifetime cancer risk, the practical quantitation limit (PQL), and practical treatment technology (43). In its arsenic proposal, the U.S. EPA selects the most appropriate health effect and establishes a PQL, considering the costs of treatments, the water system size, and the numbers of persons exposed to various concentrations of arsenic (44). The U.S. EPA used the bladder cancer analysis from the NRC report (1999) (1) for the health effect. They calculated a 1% effective dose of approximately 400 μg/L. Since there is no accepted mode of action for arsenic, a maximum contaminant level goal (MCLG) of zero was selected for arsenic. The MCLG is a health goal and is nonregulatory in nature. After considering various analytical techniques, a PQL of 3 μg/L was selected. The PQL is the value that can be measured in a commercial analytical lab and sets the lowest value for the maximum contaminant level (MCL—an enforceable regulatory value). The treatment technique and cost of implementation will depend on the size of the drinking water system. After considering the health effects, PQL, costs of treatment, and stakeholder input, the U.S. EPA proposed an MCL for arsenic of 5 μg/L and will consider comments on 3, 10, and 20 μg/L. In January 2001, the U.S. EPA was scheduled to promulgate a final MCL after considering the comments of the stakeholders on the proposal.

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7. CONCLUSION

Arsenic is ubiquitous and a potent environmental hazard to humans if it enters the drinking-water supply. Further work is necessary to determine the factors governing its movement through the various environmental compartments, and to delineate the possible toxic effects of low intakes.

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8

Cadmium

Monica Nordberg
Karolinska Institute, Stockholm, Sweden

Gunnar F. Nordberg
Umeå University, Umeå, Sweden

1. INTRODUCTION

This chapter on cadmium (Cd) provides a review of pertinent literature of present knowledge on Cd toxicology. A synopsis of current opinion related to this field is presented in the chapter. It includes and pinpoints aspects on future trends in Cd toxicology in the form of outlined hypotheses to be explored based on opinions by researchers in the field.

Special emphasis is put on health effects in humans of Cd exposure and molecular mechanisms explaining such effects. It defines the critical effects and includes a risk estimate. Attention is paid to occurrence of exposure and health effects, historical and geographical endemic areas, exposure and dose levels giving rise to health effects, and vulnerable groups. Experimental studies performed on cellular systems, laboratory animals, and epidemiological studies constitute background information for risk estimation and recommendations of importance for prevention. The important role of metallothionein in modulating Cd toxicity is emphasized. A review of Cd toxicity based on organs and effects is presented. Methods for detection of adverse effects of Cd are brought to attention.

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2. PHYSICAL AND CHEMICAL SPECIES

Cadmium was discovered in 1817 by the German chemist Friedrich Strohmeyer. It is a soft, silver-white metal and is similar in appearance to zinc, but is softer, and is to some extent used in a similar way as zinc. Cadmium originates from the Latin word cadmia, which means ''calamine,'' that is, zinc carbonate. The Greek word ''kadmeia'' has the same meaning. Cadmium was found as an impurity of zinc carbonate, which upon heating changed color owing to impurities of cadmium. Cadmium does not have a defined taste or odor. Location in the periodic table is in group IIB. Atomic number is 48 and atomic mass is 112.411. Naturally occurring isotopes are 106 (1.22%), 108 (0.88%), 110 (12.9%), 111 (12.75%), 112 (24.07%), 113 (12.6%), 114 (28.86%), and 116 (7.5%) (1). Many radioactive isotopes of Cd, e.g., 109 and 115m, are well recognized in experimental toxicology. Melting and boiling temperatures are 320.9°C and 765°C, respectively.

3. OCCURRENCE AND USES

Cadmium is an element with an average distribution of 0.1 mg/kg in the earth's crust. High concentrations are found in sulfide ores. Many inorganic compounds are soluble in water, e.g., chloride, sulfate, and acetate while oxides and sulfides have a low solubility; in fact, they are regarded as nonsoluble species of Cd. Knowledge about solubility in biological media is limited. Cadmium forms complexes with sulfur groups, e.g., thiocarbamate. The high affinity for such groups has been the basis for many analytical methods.

Cadmium is usually found associated with zinc. Cadmium occurs naturally in the geosystem. Particularly high concentrations occur in some sulfide ores, but many soils and rocks, coal, and mineral fertilizers contain some Cd. Cadmium is widely dispersed in the environment. Human exposure to low levels occurs as a result of natural processes as well as human activities such as mining, smelting, fossil fuel combustion, and industrial use. Owing to the natural occurrence in the geo-environment some farming products including tobacco could be high in Cd. Sometimes Cd is a by-product in the production of metals such as zinc, lead, and copper. However, Cd is mostly found as chemical compounds of elements, such as oxygen, fluorine, chlorine, and sulfur. Chemical compounds, e.g., Cd bromide and iodide, are used in photography and photoengraving; Cd sulfide (Cd-yellow) is used in high-quality paints, glazes, and inks and in artists' pigments. Negative plates (electrodes) of nickel-Cd storage batteries are made of Cd oxide.

Cadmium is used in plating in order to protect steel, iron, copper, brass, and other alloys from corrosion. Cadmium does not corrode easily. Alloys of Cd are valuable, e.g., in internal-combustion engines as resistance to high speeds

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and high temperatures increases. Cadmium also strengthens the copper used in electric wires and other commercial products.

In the environment Cd is present in air due to incineration of household wastes, through emission from industry including mining, and from energy production based on coal combustion. Cadmium particles can be transported in air long distances and thus the ground and water could be contaminated far from the emission source. Cadmium remains in the soil and water strongly bound to other compounds. The United States now produces less than one-tenth of the world's production and imports the metal from Canada, Australia, and Mexico (2).

4. METHODS OF ANALYSIS

Concentrations of Cd in samples from biological tissues varies from nanogram to microgram depending on the kind of sample. In air and water only a few nanograms might be present in a sample intended for analysis. Thus it is necessary to have proper analytical equipment, sampling technique, and control of contamination during sampling. A common way of performing analysis of samples containing nonradioactive Cd is by atomic absorptions spectrophotometry with graphite oven. Inductively coupled plasma mass spectrometry (ICP-MS) is a more modern tool for performing analyses. Methods for analyzing cadmium in biological tissues and in environmental samples have previously only been possible to use for total Cd concentration. By new inventions such as ICP-MS coupled to HPLC or FPLC it is possible to analyze according to isotope and also to chemical species of Cd compound in the sample (2). Newly developed techniques can also improve the analysis further. For example, in samples with protein-bound Cd also the amino acid composition of the Cd-bound compound can be detected. Cadmium in tissues can also be determined in vivo by X-ray fluorescence (3).

5. EXPOSURES

Occupational exposure mostly takes place by inhalation in the workplace. Exposure in battery manufacturing, metal soldering, or welding is the most prominent. In most countries threshold limit values are set for exposure (see below). In the general environment exposure takes place via food and drinking water. Foodstuff contains Cd with the highest concentrations in liver, kidney, Cd-contaminated rice, and shellfish.

5.1 Food and Water

Very high intake of Cd via heavily contaminated food and drinking water irritates the stomach and can give rise to vomiting and diarrhea (4). Cadmium is present

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in food as a natural component. In Sweden major sources of Cd from food are those food items that are most frequently consumed, i.e., cereals and potatoes, corresponding to 48 respectively 19% of total Cd intake calculated on the basis of a medium consumer. During recent years there has been an increase in Cd concentration in carrots and potatoes in Sweden, probably explained by the ongoing acidification of soil. Wheat flour contributes 65% of the Cd intake from cereals. Durum wheat flour, which also is used for pasta, contributes 17%. Those figures have been reported by Swedish regulatory agencies and are based on Cd analyses performed by the regulatory agencies (5). The concentration in foodstuff, e.g., shellfish, liver, kidney, certain mushrooms, and cacao, often contains more than 100 μg Cd/kg (6). Beans, sprouts, lentils, and various seeds have a concentration of Cd that is above 100 μg Cd/kg. Meat and fish are examples of foodstuff with low Cd concentration, mostly below 5 μg Cd/kg. Cereals, however, have a higher concentration.

Flour has been reported to have a mean concentration of 25 μg Cd/kg in a study of 55 samples. Those figures can be compared to the data in Table 1 compiled in 1988. Foodbaskets collected in 1987 in Sweden containing 60 different foodstuffs showed a daily intake of 12 μg Cd in Sweden (7). The intake of one crab per year will contribute the increase (8) in daily intake of around 2 μg (6,9).

Studies on seafood and shellfish have shown high intake of Cd (10). It was also shown (10) that the chemical species of Cd vary between species of oysters. Different Cd-binding proteins have been identified in foodstuff (11). The chemical species of Cd is of importance (see below) in the toxicity of Cd.

Reported values for Cd in various foodstuff are shown in Table 1 (12).

The contribution of Cd from foodstuff has been calculated in Sweden by the National Board of Food Safety to give a daily intake in Sweden of 12 μg/day. This is based on an assumption of the following concentrations of Cd in foodstuff: meat, fish, and fruit, 1–5; cereals, potatoes, and root fruits, 10–50; bran, 150; and kidney and liver, 100–400 μg Cd/kg.

TABLE 1 Examples of Various Foodstuff and Cd Concentration (2,12)

Food	Cd (mg/kg) wet weight	Food	Cd (mg/kg) wet weight
Potatoes	0.01–0.06	Beef kidney	0.2–1.3
Wheat grains	0.005–0.08	Beef meat	0.005–0.02
Rice Noncontaminated areas	0.008–0.13	Fish meat, other than crab	0.004–0.1
Milk	0.00017–0.002	Spinach	0.043–0.15
Oysters	0.1–4.7	Carrots	0.016–0.030

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Ysart et al. (13) also studied the intake of Cd with the double-basket technique. A comparison (14) on Cd exposure via intake of Cd in food in Japanese women between 1977 and 1981 with a daily intake of 37.5 μg and between 1991 and 1997 with a daily intake of 25.5 μg resulted in a decrease by 12 μg Cd/day. The contribution of Cd from intake of rice was 11.7 μg/day, constituting about 40% of intake. The current levels from environmental exposure of these groups are still high compared to other countries. Analyses were performed on Cd in food, blood, and urine that was corrected for creatinine. It should be mentioned that analyses were performed by ICP-MS, a fairly new analytical technique.

In 1994–95 within the framework of the Scientific Cooperation Project (SCOOP) estimated intake of Cd in Europe (15) was found to vary between the countries. From Greece and Portugal a daily intake of Cd was reported to be 50–60 μg, which is 70–80% of what JECFA (WHO and FAO, Joint Expert FAO/WHO Committee on Food Additives) (16) recommended as the highest tolerable daily intake. Belgium and Italy reported a daily intake of 20–30 μg Cd/day, and other countries reported an intake of around or below 20 μg Cd/day.

5.2 Air

Ambient air is usually low in Cd concentration. Weekly mean concentration of Cd has been reported to be around 5 ng/m^3 in Stockholm compared to rural areas with about 0.9ng/m^3. Air concentration in certain occupational activities is limited to the threshold limit values for each country (see below). Cigarette smoking contributes to air concentration of Cd. One cigarette can contain up to 2 μg of Cd (17).

6. METABOLISM AND KINETICS

6.1 Uptake via Inhalation

Inhalation of Cd occurs when smoking cigarettes and in occupational exposures in smelters and in operations where Cd fumes in welding may be inhaled. Occupational exposure to Cd has decreased markedly in industrialized countries due to improved work environment. Previously concentrations as high as 10000 μg/m^3 have been reported (2) (in the 1950s) compared to today's exposure levels of less than 10 μg/m^3 in some countries (see below). Uptake of Cd via inhalation is dependent on particle size, aerodynamic diameter, and in vivo solubility. Pulmonary absorption of CdS might be lower compared to uptake of CdO. After inhalation of Cd aerosol Cd is taken up via alveoli or after deposition on bronchial epithelium and mucociliary transport to pharynx, where it is swallowed into the gastrointestinal tract. While up to 100% of Cd reaching the alveoli is transferred to blood, only 5% of Cd reaching the gastrointestinal tract is taken up into blood. The proportion of an inhaled aerosol that reaches the alveoli varies with particle

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size. Maximal uptake in blood (35%) will occur at a particle of 2 μm, while particles with an aerodynamic diameter of 10 μm will only be taken up to an extent of 5% i.e., totally transferred to the gastrointestinal tract (12). Uptake of Cd to blood after inhalation is between 5 and 35% of the inhaled amount due to mentioned factors. An average of about 10% (18,19) of Cd in cigarettes is inhaled during smoking. Assumption of 50% uptake of the inhaled Cd gives a daily contribution of 1 μg Cd for 20 cigarettes.

6.2 Uptake via Gastrointestinal Tract

Absorption of Cd from a single oral dose in animal experiments has been shown to be 1–6%. The proportion that is absorbed depends on dietary composition and on dose (18). For humans similar data are 4.6–7%. However, for humans with low iron stores up to 4 times higher absorption is reported compared to humans with normal iron stores (20) (see below).

Conditions influencing the increased uptake of Cd via the gastrointestinal tract are low intake of protein, vitamin D, calcium, iron, zinc, and copper (21). However, a high intake of fibers can result in a lower intestinal absorption of cadmium.

6.3 Toxicokinetic Aspects of Transport of Cd to the Kidney

The kinetics of Cd is most likely dose-dependent and also possibly route-dependent. With regard to transport and distribution of Cd in mammals, a basic detailed description (18) constituted the background for the considerations concerning these aspects of Cd toxicology given by WHO in 1992 (2) and recently updated (22). The kinetics of Cd are described in Scheme 1 and can be summarized as follows: Immediately after uptake of cadmium from the gastrointestinal tract or the lungs, Cd is mainly bound to albumin and other larger proteins in blood plasma. There is, however, only limited information on the variation of binding with time, dose, and route of administration. Available evidence indicates that there is a pattern with proportionally more of plasma Cd in a low-molecular-weight form (probably mainly bound to metallothionein, MT) when low doses of Cd are given by the oral route compared to when large doses are given by injection. There is also a time dependence of plasma binding, with a larger proportion of plasma Cd being bound to low-molecular-weight plasma proteins at longer time intervals after a single administration.

Cadmium bound to albumin is to a large extent taken up by liver, where the complex is split and Cd can cause toxicity to liver cells (at relatively high doses, particularly by injection). Cd also induces the synthesis of metallothionein in liver cells and gradually an increasing proportion of liver Cd is bound to MT.

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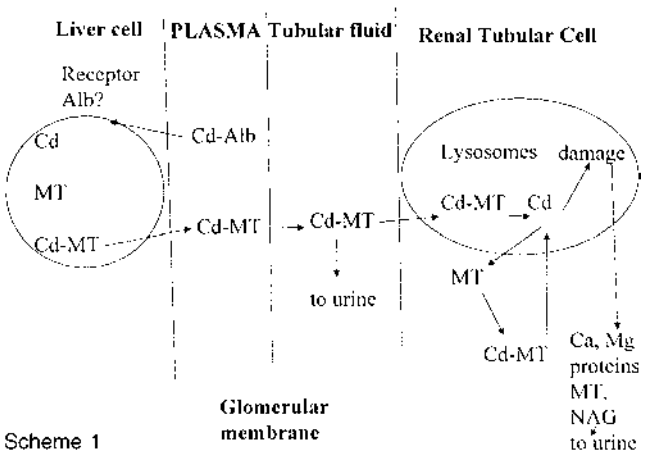

Scheme 1

In the early phase after a single administration of Cd (particularly if injected), plasma Cd is mainly bound to albumin and uptake of Cd by the kidney is limited.

In previous studies (18,23,24) Cd has been shown to be excreted in bile mainly bound to glutathione. Biliary excretion of Cd was more recently studied (25) in mutant Eisai hyperbilirubinuric (EHB) rats and normal Sprague-Dawley (SD) rats. The EHB rats have a near absence of biliary excretion of glutathione. Biliary excretion of Cd in EHB rats was found to be only one-fortieth of that in SD rats (25). This finding gives further confirmation of the previous conclusion that Cd excretion in bile is related to glutathione.

Long after a single exposure, or in long-term exposure, a considerable proportion of plasma Cd is bound to metallothionein. CdMT (26), is of small molecular size, and is efficiently filtered through the glomerular membrane in the kidneys and taken up by renal tubular cells. Uptake of CdMT may be more efficient in cells preexposed to Cd compared to non-pre-Cd-exposed cells (27). In long-term exposure there is a slow release of CdMT from the liver to blood. This transport phenomenon has gained more support by studies where Cd-containing livers were transplanted to non-Cd-exposed animals, which demonstrated a gradual uptake of Cd in the kidney (28). After uptake of CdMT into renal tubular cells via pinocytosis, MT is catabolized in lysosomes releasing Cd ion. Metallothionein binding in plasma and tissues thus has been considered to be of considerable importance for Cd distribution after uptake.

Uptake in the gastrointestinal tract has been considered to be to some extent related to MT synthesis in the intestines (29). However, higher basic MT concentrations in tissues of transgenic mice had no appreciable effect on the concentration of Cd in tissues compared to controls with normal tissue concentrations of

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MT (30). The only exception was that the transgenic mice given the very high dose of Cd orally (300 μmol Cd/kg) had twice the tissue Cd concentrations of controls. These observations were considered to shed doubt on the role of MT in Cd toxicokinetics. However, as described previously, uptake and distribution of Cd occurs mainly in the initial phase in a form where Cd is bound to albumin in plasma. It should not be expected that this phase would be influenced by different basic levels of MT. In another study of transgenic mice (31), lacking metallothionein-I and-II (MT-null mice) it was found that the elimination of Cd was much faster in MT-null mice than in control mice. This confirms a role of MT in tissue retention of Cd. The Cd concentration in the kidney continued to increase with time in control mice but not in MT-null mice, confirming an important role of MT in transport of Cd to the kidney (31).

6.4 Biological Half-Life

It has been estimated that the biological half-life of Cd in the kidney is in the order of 20 years in humans. Such a long biological half-life explains why Cd accumulates constantly up to approximately 50 years of age in humans. Cadmium accumulating in the kidney is probably largely bound to MT that is synthesized de novo. This process may be responsible for the long biological half-life of Cd in the kidney and its accumulation in long-term exposure (18).

6.5 Excretion/Elimination

Excretion and elimination of Cd has been summarized (2). Urinary excretion of Cd has been demonstrated in a number of experimental studies in laboratory animals to represent about 0.01–0.02% of the total body burden upon long-term exposure. In many mammalian species it has been demonstrated that urinary excretion increases slowly upon exposure to Cd, and after renal damage has developed a marked increase of excretion of Cd is manifested. For humans it has been estimated that approximately 0.01% of the body burden is excreted in urine (2). Urinary excretion, like the body burden, of Cd is age dependent. If tubular proteinuria occurs there is an increased Cd excretion. High excretion of Cd without proteinuria may occur in short-term high-level exposure. Cadmium is excreted in urine bound to MT.

Since it is not possible to distinguish net gastrointestinal excretion from unabsorbed Cd in feces, it is very difficult to study net fecal excretion of Cd. In oral Cd exposures the major part (approximately 95%) of fecal Cd represents unabsorbed Cd. Measurements of fecal Cd can be used as an indicator of oral intake. Based on studies of injected Cd in experimental animals, it was found that initially the fecal excretion is higher than urinary excretion calculated on a percentage basis. This is probably explained by a contribution from the bile. Reported data on fecal excretion of Cd in humans are almost nonexistent. It

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should be taken into consideration that excretion of Cd via urine or feces is greatly dependent on route of exposure.

7. MT—DNA AND GENE STRUCTURE AND ITS DISTRIBUTION AMONG TISSUES

Metallothionein, often related to toxicokinetics of metals such as Zn, Cd, Hg, and Cu (32), has been extensively studied in relation to Cd toxicity, as described in other sections of this chapter. Metallothionein is known to play an important role in the toxicokinetics of Cd (33,34). Metallothionein concentration can vary among organs and within theses organs. Metallothionein has been found in most human tissues and concentration of MT in blood and urine is generally considered a good measure of exposure to Cd, which forms clusters with MT.

Tissue levels of MT in humans (normal concentrations) are shown in Table 2.

Metallothionein is a family of proteins with molecular weight of approximately 6500 Da, rich in cysteine, and with seven metals distributed in two domains, the α- and β-clusters. The dominating metals are Zn, Cd, Hg, and Cu, with increasing stability of binding in the order mentioned. The definition of the MT superfamily follows the criteria for polypeptides, which have features in common with equine renal MT (38,39). The MTs consist of four major groups. The best-studied MTs are mammalian MT-1 and -2. MT-1 exists in many isoforms and together with MT-2 is present and expressed in almost all tissues. MT-3 is present in brain and MT-4 is specific for squamous epithelium and expressed in keratinocytes.

Mechanisms of importance for protecting cells from toxic insults are known to only a limited extent. Expression of MT and heat shock proteins can be used

TABLE 2 Tissue Levels of Metallothionein in Humans

Method	Media	Concentration (ng/ml)	Status	Ref.
RIA	Sera (human)	0.01–1	Normal	35
RIA	Sera (human)	>2	Abnormal[a]	35
RIA	Urine (human)	1–10	Normal	35
RIA	Urine (human)	>10	Abnormal[a]	35
ELISA	Liver (rat)	18 μg/g		36
ELISA	Kidney (rat)	30 μg/g		36
ELISA	Kidney (rat)	35 μg/g		37

[a] Occupational exposure.

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as a biomarker related to survival of the cell and to metal exposure that induces the synthesis of these proteins. Metals, among which Cd is the strongest, and glucocorticoids can induce MT-1 and MT-2. However, MT-3 has not, so far, been shown to be induced and the concentration in the central nervous system (CNS) appears to be unchanged regardless of metal exposure.

Human MT genes are localized on chromosome 16. Of the 14 genes coding for MT six are functional, two are not, and six have not been characterized. Whether that number of genes on the same chromosome reflects coding for various functions and reflects gestational age remains to be demonstrated as the newborn is almost free from Cd. The level of expression of the genes coding for MTs varies during gestational and developmental age and among different organs. Genetic polymorphism for MT would be of interest with regard to the kinetics of Cd. Potential effects and related health effects of translocation of the genes coding for MT are not clear. Induction of MT-1 and -2 is under regulation of Cd. MT-1 and -2 have 61 amino acids. A comparison of the amino acid sequences shows that MTs have been conserved through evolution, with fundamental similarities such as low molecular weight, around 6500 Da, 30% cysteine residues, and very few aromatic or hydrophobic residues. Pure MT can contain up to 10% of Cd (w/w).

MT-3 resembles the other MTs in its cysteine number, alignment, metal composition, and metal-binding characteristics. At the N-terminal region of MT-3 an additional threonine is inserted and acidity is increased and charge surface is changed, which facilitates the interaction of MT-3 with other biological constituents. The C-terminal region contains six more amino acids consisting of glutamic acid and alanine. Alanine is also found in MT-1 and MT-2 at the C-terminal. The characteristic short repeating sequences of cysteines with either one or other amino acids in between are still seen.

The MT-4 gene is located separately from the gene for MT-3 on chromosome 16 in humans. MT-4 contains an additional glutamate compared to MT-1 and -2 and consists of 62 amino acids. The isoforms of MT have structural similarity with the same number of cysteine residues and high metal-binding affinity but differ in their total charge because of differences in certain amino acids other than cysteine (40). It has been shown (41) that mRNA for MT-4 is expressed in stratified squamous epithelia associated with oral epithelia, esophagus, upper stomach, tail, footpads, and neonatal skin. Tongue epithelia contains MT with Zn and Cu. Rats showed epithelia parakeratosis during zinc deficiency. In situ hybridization showed expression of MT-1 predominantly in basal proliferative layer while MT-4 mRNA was found in the differentiating spinous layer of cornified epithelia. MT-4 is suggested to be involved in Zn metabolism during differentiation of stratified epithelia.

The MT-4 gene is restrictedly expressed in keratinocytes of skin and the

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upper parts of the digestive tract (42) and maternal deciduum (43). Its modulation by toxic agents still needs to be explored (22).

8. HEALTH EFFECTS

8.1 General Aspects, Short-Term Versus Long-Term Exposures, Factors Influencing Tissue Sensitivity

Cadmium may cause health effects upon both acute and long-term exposure. Epidemiological studies concerning adverse health effects in humans have been reported to an increasing extent during recent years, e.g., Cadmibel and PheeCad (44,45). Cadmium is a metal that accumulates in the body with age and has an extremely long biological half-life. Because of its long biological half life, long-term toxicity has attracted particular attention. However, there are also some important aspects of short-term toxicity, which will be briefly described in the following section. Acute toxicity after ingestion of drinks with more than 15 mg/L of Cd has been described in children exposed to Cd via a soft drink machine (4). Symptoms of acute toxicity are nausea, vomiting, and abdominal pain. High Cd concentrations can occur when acid food comes in contact with Cd-plated utensils.

Acute toxicity by inhalation may occur in workers welding Cd-containing materials. Pulmonary edema and pulmonary respiratory distress (2) characterize acute toxicity after inhalation of fumes containing Cd.

Skin contact or Cd exposure via the skin is not known to cause health effects in humans or animals. Metallothionein-4 is present in the squamous epithelium of the skin and may have a protective role against development of skin effects.

Pollution of the general environment by Cd has as yet been related to the development of human disease only in some special situations, such as itai-itai disease in Japan and renal dysfunction and increased occurrence of osteoporosis in Belgium and in China.

Long-term exposure to Cd in air, food, or water increases Cd concentration in the kidneys and gives rise to kidney disease. Other effects due to Cd exposure are lung damage, bone effects, liver dysfunction, and reproductive toxicity, which will be described in the following sections.

8.2 Lung

In laboratory animals exposed to Cd dust in some studies also containing other metals such as Fe it is reported that Cd causes emphysema, interstitial pneumonitis, and lung fibrosis after intratracheal installation or inhalation (2). A series of

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studies (46) showed increased incidence of lung cancer in laboratory animals, which will be described below.

In previous times Cd concentration in the work environment usually was high and gave rise to adverse effects on the respiratory tract. When increasing knowledge was gained, the threshold limit values were decreased to avoid such health effects. High-level occupational exposure to Cd causes chronic obstructive airways disease. IARC (47) has classified Cd as a human carcinogen belonging to group 1 agents. It is, however, difficult to completely support the notion that Cd gives rise to lung cancer from the observations in exposed workers (2). The relationship between smoking habits and lung cancer is well recognized and it is probable that Cd may be a contributing factor since tobacco is rich in cadmium.

8.3 Liver

After high-level short-term exposure to Cd in experimental animals and in humans with such exposures, liver toxicity is a prominent feature, while influence on this organ is less common in long-term exposures. This is considered to be due to the induction of MT synthesis in the liver at longer time exposures. As described earlier, one molecule of MT can bind up to 7 atoms of Cd and thereby sequester this toxic metal from interference with important cellular targets such as enzymes and membranes. The first study demonstrating a protective role of MT against Cd toxicity to the liver was performed by one of the present authors (48). As described in detail earlier, Cd occurring in blood plasma in a form bound to albumin is taken up in the liver. After release from albumin nonbound Cd is free to cause toxicity to liver cells. This happens at high Cd exposure when liver MT levels are not high enough to handle all nonbound Cd ions. Genetic differences with regard to capability to induce MT were shown to be related to tissue toxicity (49) in MT-1 and -2 knockout (MT-null) mice. Studies of MT in Cd-induced hepatotoxicity and nephrotoxicity and in Zn-induced protection showed that MT-null mice were more sensitive to i.p. $CdCl_2$ hepatotoxicity than normal mice. Zinc pretreatment by subcutaneous injection increased hepatic MT 80-fold in control mice but not in MT-null mice and prevented $CdCl_2$ hepatotoxicity in control mice only. These findings confirm a role of MT in protecting the liver from Cd in medium-term and long-term exposures (49).

8.4 Kidney

8.4.1 Tubular and Glomerular Dysfunction

Renal damage caused by long-term Cd exposure is characterized by proteinuria with increased excretion of low-molecular-weight proteins as well as ions such as Ca, Mg, and Cd. Effects can also be seen on the glomerular function with increased excretion of albumin and in some cases also with reduced glomerular

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filtration rate. In long-term exposure to Cd the kidney is the critical organ, i.e., the organ (50) suffering damage or dysfunction at relatively low exposure. The critical concentration of Cd giving rise to tubular dysfunction in 10% of persons in the general population was previously estimated at 200 μg Cd/g wet weight cortex (2). More recent data show that tubular dysfunction can occur at much lower concentrations of Cd in the kidney cortex. A concentration of 50 μg/g wet weight has recently been estimated as the lowest concentration of Cd causing renal dysfunction among the most sensitive individuals (22).

Cadmium can give rise to tubular and less frequently to glomerular effects on the kidney. Whether the renal dysfunction is progressive or irreversible has often been debated. In a five-year follow up study in Belgium of a subcohort of the Cadmibel study it was concluded that subclinical renal effects related to increased Cd body burden most likely represents nonadverse effects, as they were not associated with progressive dysfunction of the kidney (45). Humans in China were studied for *N*-acetyl-beta-D-glucosaminidase (NAG) and isoenzymes in urine in an area contaminated with Cd by industrial wastewater from an adjacent smelter. Cadmium-polluted wastewater was discharged into a river used for the irrigation of rice fields. Reported Cd concentrations in rice were 3.70, 0.51, and 0.07 mg/kg. Concentrations of Cd in urine were above 5 μg/L in most of the subjects living in the area with highest Cd concentration in rice. Dose-dependent increase in NAG and NAG B was prominent and concentration in urine was related both to Cd concentration and to the calculated Cd uptake. This shows that urinary NAG and isoenzymes could be used as a biomarker of early renal dysfunction in Cd-exposed populations (51).

The following section deals with mechanistic information of importance for an understanding of how renal damage results from Cd exposure.

8.4.2 Cellular Targets for Cd Role in Membrane Damage and Protective Cd-Binding Proteins

As mentioned, in long-term exposure to Cd both in experimental animals and in humans, Cd continuously accumulates in the liver and kidneys. CdMT is efficiently transported through the glomerular membrane and taken up by the renal tubular cells. The first reports on renal damage after injection of CdMT to experimental animals were published by Nordberg (52), Nordberg et al. (53), and Cherian et al. (54). Nordberg et al. (52,53) interpreted their data as indicative of renal damage occurring subsequent to the dissociation of Cd from CdMT, which is compatible with the view later developed by Fowler and Nordberg (55). Cherian et al. (54), on the other hand, discussed their data in relation to a possible direct effect of Cd on the brush border membrane, such membrane damage causing the cellular damage that subsequently developed in these animals. The hypothesis involving release of Cd from MT and subsequent attack by the ''free'' Cd on various intracellular targets has been considered the most valid explanation for

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the toxicity. However, the hypothesis of direct membrane damage has recently gained some support (56). It was shown that in MT-transgenic mice, which have high levels of MT in their kidneys (10-fold over control mice), similar increases in protein and glucose excretion were observed as in control mice after injection of CdMT (0.1–0.6 mg Cd/kg i.v.) (56). Zinc-induced protection was also indicated to occur in the absence of MT induction (see next section). These findings are not readily compatible with the hypothesis implying release of free Cd ions from CdMT for induction of renal toxicity, because such ions would be expected to be picked up by the higher MT concentrations in the cells of the transgenic animals. It is presently unclear how these findings can be reconciled with other findings. They would, however, be compatible with the hypothesis by Cherian et al. (54) of a direct damage on the brush border membrane from the CdMT complex.

Evidence supporting the hypothesis that dissociation of the CdMT complex is first required and released Cd gives rise to cellular membrane damage has been provided by Nordberg et al. (57). One group of rats, which had preinduced MT synthesis by pretreatment with Cd, was compared with a group of nonpretreated rats with low cellular MT concentration. Both groups were given a s.c. challenge dose of radiolabeled CdMT. A considerably larger proportion of the radiolabeled Cd in the subcellular membrane fraction was bound to a high-molecular-weight component in the nonpretreated animals than in the pretreated ones. In the latter group, a larger proportion was bound to fractions corresponding to MT and possibly other low-molecular-weight proteins in the membrane (57). Cadmium bound to non-MT sites in cellular membranes is thus of decisive importance for the elicitation of the toxic effects of Cd on the kidney. The animals that were pretreated with Cd were protected against toxic effects of CdMT, whereas nonpretreated animals later developed kidney damage. Another observation that may be of importance when discussing mechanisms of Cd nephrotoxicity is the early pertubation of Ca metabolism preceding the development of proteinuria after CdMT injection (58). It is also interesting to note that in studies of uptake and binding of Ca to membranes isolated from the renal cortex of CdMT-exposed animals, there is a considerably lower binding and uptake in exposed animals than in controls. This is true both in luminal and particularly in basolateral membrane vesicles (58,59). Thus, it is likely that the basolateral Ca pumps constitute a primary target for Cd. When brush border membrane vesicles, isolated from kidneys of experimental animals were exposed to Cd chloride in vitro, there were effects on the uptake of L-glutamate (60,61) and citrate (61). In a cell line (LLC-PK1) it was shown that uptake of Cd occurred by carrier-mediated transport involving Na^+ and energy-dependent processes (62).

These observations were made in vitro, or in membranes or membrane vesicles obtained from animals in single-dose experiments. In such experiments the disturbances of renal tubular function were reversible. In long-term exposures,

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or when repeated doses of CdMT are given with short intervals (63), irreversible changes in the urinary excretion of calcium and a longer pertubation of protein excretion in urine are seen. The last-mentioned experimental models of Cd-induced nephropathy reproduce more of the features of human Cd-induced renal dysfunction than single-dose experiments. Studies by Sudo et al. (64) demonstrated renal damage after repeated subcutaneous high doses of Cd chloride. In these studies it was indicated that Cd occurring bound to cellular membranes in the kidneys was involved in the manifestation of renal injury (64).

As mentioned, cellular Ca metabolism is perturbed in Cd-induced renal dysfunction. Since apoptosis may be induced by pertubation of cellular Ca metabolism, it seems likely that this type of cell death would occur in Cd toxicity to the kidney. Apoptotic cell death was observed in rats exposed to subchronic Cd intoxication by repeated subcutaneous injections (65). Apoptosis has also been observed in human kidney cell lines. DNA fragmentation was found after exposure of kidney cells to Cd in vitro leading to apoptotic cell death (66). Cells exposed to relatively low concentrations of Cd chloride displayed apoptosis, particularly when exposed to a Cd-containing protein complex with characteristics similar to those of MT (67). Liu et al. (68) described cytotoxicity of Cd to renal proximal tubule cells and showed that cells from rats pretreated with Zn were less sensitive to $CdCl_2$ toxicity. In cultured kidney tubule cells Cd inhibited Na glucose cotransport, while CdMT did not give this effect (68,69).

Although there is some evidence, as described earlier, that a direct interference of Cd with Ca transport in renal membranes may be responsible for the toxicity of Cd to the kidney, there may also be a component of the membrane toxicity caused by lipid peroxidation. Such effects have been demonstrated in the kidneys of rats exposed to Cd (70). Increased lipid peroxidation was also seen in several tissues including the kidney of rats given Cd intraperitoneally (71).

Based on available evidence, a model for the mechanism by which Cd exerts its effect on the renal tubule has been described (24). It is assumed that the rate of influx of CdMT into the renal tubular cell compartment and the rate of de novo synthesis of MT in this compartment regulate the pool of intracellular ''free'' Cd ions that can interact with cellular membrane targets in the tubules (see scheme 1). When there is efficient MT synthesis, and influx of CdMT into the lysosomes is limited, the free Cd pool is limited and no membrane damage occurs. Calcium transport in the cell is normal. When CdMT influx into the lysosomal compartment is high and de novo synthesis of MT is deficient, the free Cd pool is sufficiently large to interact with membrane targets to block Ca transport routes and there is deficient uptake and transport of Ca through the cell. This gives rise to an increased excretion in urine of Ca and proteins. It is possible in animal models, to inject CdMT and induce nephrotoxicity by a high influx of CdMT into the renal tubule compartment, thus overloading the sequestering

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mechanism of de novo cellular synthesis of MT. In the most acute models it seems possible also to cause a direct toxic effect on the brush border membrane by CdMT. Such acute toxicity does not occur in exposure of humans, which takes place by oral or inhalation routes, which can only provide a limited flow of CdMT.

In exposure situations similar to those occurring to humans, renal toxicity is not expressed until Cd concentration in the renal cortex is between 50 and 300 μg/g. Cadmium that is released intracellularly from MT is partly delivered from CdMT via plasma from other organs in the body. Another part is derived from the comparatively large amounts of CdMT accumulated intracellularly in long-term Cd exposure. The reason why renal tubular dysfunction occurs in various individuals at different total concentrations of Cd in the kidney may have various explanations, but one important source of such variation may be the variable ability among individuals to synthesize MT and other protective components. Such an interpretation has received support by findings of a relationship between MT expression in lymphocytes in peripheral blood (see below) and development of renal toxicity in Cd workers. Nevertheless, these hypotheses require further confirmation before they can be considered fully established.

8.4.3 Influence of Zn and Cu on Cd Nephrotoxicity

Early data on the effects of Zn or Cu on Cd toxicity have been summarized (72). Experiment on rats (73) showed that proteinuria caused by CdMT injection could be more efficiently reduced by pretreatment by Zn injections than by Cu injections. Excessive Ca in urine and renal cortex, on the other hand, could be more efficiently reduced by Cu than by Zn. It was shown that Cd retention was markedly reduced in renal cortex and increased in liver by Cu pretreatment while the urinary excretion of Cd was significantly lower in these rats (37). The levels of endogenous Zn in renal cortex and liver increased significantly in rats pretreated with Cu. Copper induced the production of MT in liver and renal cortex more efficiently than Zn (37). The efficient protective effect of Cu against calciuria can thus be explained by both increased MT induction and less accumulation of Cd in the renal cortex (37). Renal tubular cells isolated from Zn-treated rats studied in vitro (68) displayed not only increased MT concentrations but also increased expression of low-molecular-weight Cd binding heat shock proteins (HSP). These proteins may serve an important protective role in addition to MT. In studies on MT-1 and -2 knockout (MT-null) mice (68) it was shown that such mice were more sensitive to i.p. $CdCl_2$ hepatotoxicity than normal mice. Zinc pretreatment by subcutaneous injection increased hepatic MT 80-fold in control mice but not in MT-null mice and prevented $CdCl_2$ hepatotoxicity in control mice only. Zinc increased renal MT in control mice only; however, it protected against CdMT-induced renal injury in both control and MT-null mice. The authors sug-

gested that MT plays less of a protective role in CdMT-induced nephrotoxicity than in $CdCl_2$-induced hepatotoxicity. They also stated that Zn-induced protection against CdMT-induced nephrotoxicity did not appear to be mediated through MT. Considering that renal tubular cells from rats treated in vivo with Zn were shown to have an increased level of several heat shock proteins in addition to MT (68), this may explain the lack of difference between normal and MT-null mice.

8.4.4 Cadmium-Induced Renal Dysfunction—Increased Sensitivity in Diabetics

There is epidemiological evidence indicating that diabetics may have an increased susceptibility to the development of Cd-induced renal dysfunction (44).

Data from animal experiments support the possibility of an increased susceptibility for Cd nephropathy in diabetics. When discussing this possibility, it is of interest to consider animal models in which the metabolic situation simulates type I and other animal models in which type II diabetes is simulated.

Streptozotocin (STZ)-induced diabetes in animals is similar to insulin-dependent diabetes or type I diabetes in humans. In experiments performed during short time intervals on STZ diabetic rats, an increased resistance to CdMT nephrotoxicity was demonstrated (74). Increased binding of Cd to MT occurred in the liver of the STZ-injected animals 24 h after the injection of CdMT and parallel to an increased resistance to nephrotoxicity. Induction of MT by STZ thus protects against nephrotoxicity.

In long-term experiments (75,76) rats with STZ-induced diabetes given Cd in drinking water were compared with similar animals without diabetes. Animals with diabetes developed more prominent nephropathy compared to nondiabetic animals. Another diabetes model is the Umeå obob obese mice, which are similar metabolically to type II diabetes in humans. It was shown (77) that there is an increased susceptibility to development of CdMT-induced proteinuria and calciuria in Umeå obob mice when compared to normal mice (77).

Another experiment (78) indicated that CdMT nephrotoxicity is increased in genetically diabetic as compared with normal Chinese hamsters.

8.5 Bone Effects

The issue whether Cd causes bone effects or not has been of interest for many years (79). Early observations of bone fractures were made in our own studies on laboratory animals exposed for a long time to Cd in order to study renal dysfunction. Changes in bone mineral content were also observed (80). There are also several other animal studies demonstrating a relationship between renal tubular dysfunction, urinary losses of Ca, changes in vitamin D metabolism, and decalcification of the skeleton in Cd-exposed animals (2).

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Bone effects are prominent features in humans suffering from itai-itai disease, an extreme form of chronic Cd poisoning by the oral route, which will now be briefly described.

After World War II, Dr. Hagino, a general practitioner, discovered a number of patients suffering from a bone disease with multiple fractures and deformities of the spine and the long bones occurring in a village (Fuchu) in Toyama prefecture in western Japan. The patients suffered severe pain and complained ''itai-itai'' (''ouch-ouch'') and the disease was therefore called itai-itai disease. The patients lost body height and had deformities of the spine, a result of multiple vertebral compression fractures. In the long bones, characteristic pathological fractures with osteoid formation (Milkman's pseudofractures) occurred. This latter finding is typical of osteomalacia. By analysis of urine from cases and noncases in the endemic area and from persons from nonendemic areas, it was demonstrated that there was a considerably increased excretion of Cd in urine, particularly in the cases in the endemic area. Kidney damage with proteinuria occurred in these patients. It was thus obvious that itai-itai disease was a form of renal osteomalacia, kidney damage being of basic importance for the development of bone effects. The main factor explaining this disease to the kidney is the excessive Cd intake from Cd contamination of rice, which occurred as a result of Cd-containing wastewater from a smelter being discharged into a river that was used for irrigation of rice fields. The disease occurred almost exclusively in postmenopausal women. The relatively low Ca content of Japanese food may have been a contributing factor. Also the tradition of dressing so as to screen away from the sunshine gave the women in this area only a low contribution of vitamin D synthesized by the action of ultraviolet light on the skin.

Characteristics of itai-itai disease are osteomalacia, osteoporosis, renal tubular dysfunction, malabsorption, and anemia (2,81).

The frontier interest in health effects caused by Cd focuses at present on bone effects. In industrialized countries of the world an increasing incidence of osteoporosis occurs and a high number of such patients are smokers. Since tobacco contains Cd there may be a link here.

A suggested relation between Cd dose and decreased bone mineral density and also between Cd dose and osteoporosis has been reported (19,82) in humans occupationally exposed to Cd almost 20 years before the study was performed. Also, recent results from China (83,84) indicate bone effects at cumulative doses somewhat lower than those giving rise to the classical itai-itai cases in Japan. A relation between concentration of Cd in rice, blood, and urine and number of cases with low bone density was found.

In Belgium bone effects at much lower exposure levels of Cd than those giving rise to itai-itai disease have been observed. A prospective population study (85) shows that environmental exposure to Cd is related to an increased risk for fractures and causes changes in the forearm bone density. The conclusion by

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the last-mentioned authors is that Cd can promote skeletal demineralization with increased fragility of the bones, leading to increased risk of fractures even when exposure level to Cd is low.

It is interesting to investigate whether bone mineral disorders due to oral intake of Cd can be related to the estrogen receptor, since it is known that itai-itai disease occurred almost exclusively in postmenopausal women. It has been reported that polymorphism for the estrogen receptor alpha is related to reduction of bone mineral density in postmenopausal women in Japan. The genetic distribution of the receptor was, however, the same in itai-itai disease subjects (86). However, nothing has been reported for the estrogen beta-receptor.

Studies on the occurrence of low bone density in China (87) among humans environmentally exposed to Cd via rice show a relation between body burden of Cd and low bone density particularly in postmenopausal women.

8.6 Blood Pressure

The relationship between blood pressure (BP) and metal exposure has been debated for decades (2). Under special conditions, increased BP in animals has been reported. In environmentally and occupationally exposed humans; observation of increased BP has also been reported. In some early studies, a relationship was shown between BP and Cd in animal studies (88). However, studies reporting similar relationships in humans (89) did not give information on smoking habits.

In a study (90) of an aged population it was found that Cd concentration in blood was related to diastolic BP in nonsmoking and nondemented individuals but no correlation was found between blood Cd concentration, age, and cognitive function. There were no differences in Cd levels in blood between Alzheimer disease (AD) sufferers and nondemented persons. Observed differences in Cd concentration in blood were related to smoking habits (90) and the relationship to BP may be explained by smoking.

This was also further supported in a study (91) on a well-defined cohort of 804 aged Swedish subjects. By age +77 different multiple regression models found no relation between Cd concentration in blood and BP. Both systolic BP and diastolic BP were tested. Regression analyses were also performed with and without subjects treated with antihypertensive drugs but no association between Cd concentrations and BP (systolic or diastolic) was seen. Results were also corrected for smoking habits.

The PheeCad (Public Health and Environmental Exposure to Cadmium) study group investigated a random sample consisting of 692 subjects in a large age span, 20–83 years, to see how environmental exposure to Cd influenced BP and the incidence of hypertension. Blood pressure was measured by conventional sphygmomanometry with 15 readings in total and also 24-h ambulatory BP monitoring. Systolic/diastolic BP was on average 128.4/77.3 mmHg. The baseline

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blood Cd concentration was of 11.1 nmol/L and urinary Cd excretion of 10.2 nmol/24 h (both geometrical means). BCd and UCd declined by 29.6% and 15.2%, respectively, over a 5-year follow-up period. The systolic BP decreased 2.2 mmHg in men and remained unchanged in women. The diastolic BP increased by 1.8 mmHg in both genders. It was concluded (92) that environmental exposure to Cd was not associated with higher conventional sphygmomanometry (CBP) or 24-h ambulatory blood pressure (ABP) or with increased risk of hypertension. The mechanism behind a possible influence of Cd on the BP was not discussed. A toxic impact of Cd on BP may only be possible to see during ongoing exposure, e.g., in relation to nitrogen oxide signaling in the bloodstream. If that is the case it will be most difficult to relate changes in BP to Cd exposure on a population basis. During ongoing exposure to Cd equilibrium is likely to occur.

8.7 Cadmium and the Central Nervous System

There is only limited data from animals and humans that exposure to Cd can give rise to adverse effects on the CNS (2,79). Oral exposure of pregnant or lactating rats to low-level Cd exposure caused alterations in brain serotonin levels in the offspring (93).

A direct toxic effect of Cd on primitive nervous tissue appears to be possible only in embryonic tissues at early stages of gestation, before the blood-brain barrier is established (94). At later stages of pregnancy and in adult animals, the blood-brain barrier protects the CNS from Cd as shown by autoradiography in a study with mice injected i.v. with Cd (95). One explanation why Cd does not pass the blood-brain barrier might involve its binding to MT in blood plasma (see below). The blood-brain barrier is considered to keep Cd outside the CNS. Reported neurotoxic effects of Cd during development are thus likely to be secondary to an interference of Cd with Zn metabolism or other factors of importance for the development of the CNS, e.g., hormones, and not a direct effect of Cd on brain cells. It is of interest to investigate whether neurotoxicity induced by Cd can be related to mechanisms involving MT-3 in brain. Brain MT is rich in Zn and an interference by Cd with Zn, MT-3, MT-1, and MT-2 cannot be excluded as a possible mechanism. However, for substances not passing the blood-brain barrier other routes of uptake in the brain have been suggested. Active axonal transport via the olfactory bulb has been studied in laboratory animals intranasally exposed to metals, e.g., Cd (96).

Available evidence on Zn and MT in the brain will only be mentioned briefly. MT-3 has been shown to be present in brain and was first identified as growth inhibitory factor (GIF) because of its inhibitory role on the growth of cultured neurons (97). Involvement of GIF in a number of neurodegenerative disorders, e.g., Alzheimer's disease (97), established its neurophysiological and neuromodulatory role. A role of MT in neurotoxicity is mostly focused on MT-

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3 even if both MT-1 and -2 are expressed in brain tissue (42,98). However, MT-3 differs from MT-1 and -2 as it does not seem to be inducible by Cd and other metals. By immunoreactivity technique, localization of MT in brain in sheep (99) was shown and also a shift of expression of MT-1 and -2 during development of the brain. Expression of MT-1 and -2 at the mRNA level is found in proliferating ventricular zones followed by expression in radial glial cells, oligodendrocytes, and astrocytes in many regions in the brain, particularly cerebral cortex. However, in the adult brain MT is expressed in astrocytes but not in oligodendrocytes.

MT-3 is expressed mainly in large neuronal cell bodies, whereas MT-1 is predominant in regions rich in glia (100). The highest expression of MT-3 is found in the dentate gyrus of hippocampus and the olfactory bulb. The distribution of MT-3 expression is remarkably related to that of histochemically reactive Zn, which suggests that MT-3 is expressed mainly in neurons that sequester Zn in synaptic vesicles, the so-called Zn-ergic neurons. It also was found that the expression of all three forms of MT is relatively high in the olfactory bulb (101). This suggests that MT might play a protective role in the CNS, because olfactory bulb provides a direct route of entry into the CNS via the nasal epithelium for agents that are regarded as not passing the blood-brain barrier.

The location of MT-3 in Zn-ergic neurons suggests a relation to Zn-related metabolism and physiological function. To investigate the functions of MT-3, transgenic mice that cannot synthesize MT-3 and overexpress human MT-3 (hMT-3) have been produced (102). It was found that MT-3 might participate in the utilization of Zn as a neuromodulator (100). It was shown that Zn or Cd toxicity was unchanged in MT-III-/-, or hMT-III mice (102). Although MT-III changes the total brain Zn content, it does not affect the synaptic pool of Zn (102). It is clear that further studies are needed to draw sound conclusions of the ultimate function of MT-3 and possible involvement in Cd neurotoxicity.

8.8 Endocrine Effects

It was shown by Parizek and Zahor (103) that Cd exposure by injection in animals could give rise to damage to the testicles, which leads to decreased androgen action. There has been a long-standing discussion concerning the role of MT in modulating the effects of Cd on hormone production in the reproductive tissues. Early observations by Nordberg in 1971 (52) of a low-molecular-weight Cd binding protein in the testicles of rats, considered to be MT, demonstrated protection of the interstitial tissue from toxic damage from Cd. Such protection allows continued testosterone production by these cells at doses of Cd normally giving rise to cessation of testosterone production. Other authors have claimed, based on early molecular biology techniques, that MT is not expressed in testicular tissues. However, recent evidence using quantitative RT-PCR techniques (81) as well as by others (104) has shown that both MT-1 and MT-2 are expressed in testicular

tissue, and particularly MT-2 expression at the mRNA level is increased upon Cd exposure in vivo.

The role of Cd and diabetes was discussed earlier.

Regarding Cd effects on female ovaries see below. The possibility of an effect of Cd on the hypothalamus and the pituitary related to changes in brain Zn levels and related MT binding is an interesting possible explanation of some of the endocrine effects of Cd.

8.9 Blood

Because of its low molecular weight and related efficient glomerular filtration in the kidney, binding of Cd to MT in blood plasma serves as an explanation for the efficient transport of Cd from liver and other tissues to the kidney (105). Toxicological implications of such uptake in the kidney are described above in the section on kidney. Metallothionein binding in plasma and tissues thus plays an important role for Cd distribution after uptake. Studies in mice found no difference in Cd distribution between transgenic mice with increased tissue concentration of MT compared to normal mice, and these observations were considered (30,31) to shed doubt on the role of MT in Cd toxicokinetics. However, uptake and distribution of Cd in the initial phase occurs mainly in a form where Cd is bound to albumin in plasma (see section on transport to the kidney, above), and this phase would probably not be influenced by different basic levels of MT. Another study of transgenic mice deficient in genes coding for MT-1 and -2 (MT-null mice) showed that elimination of Cd was much faster in MT-null mice than in control mice, confirming a role of MT in tissue retention of Cd. It was also observed that the Cd concentration in the kidney continued to increase with time in control mice but not in MT-null mice, confirming an important role of MT in transport of Cd to the kidney (31,49).

Studies on the expression of MT gene in human peripheral lymphocytes (PBLC) indicate that such MT expression might be useful as a biomarker of Cd exposure and tissue sensitivity to Cd (106). Cadmium could induce MT gene expression in PBLC in vitro in a dose-dependent pattern. PBLC as such (basal) and PBLC with Cd added to culture medium (induced) were examined. Concentration of Cd in blood and MT basal and induced gene expression in PBLC were compared in a study on workers. MT basal expression level was significantly correlated with Cd-B, but induced MT expression level was not. Basal MT expression in PBLC thus can be an indicator of Cd exposure. It was also observed in the studies by Lu et al. (106) that workers with low MT induction in PBLC displayed renal dysfunction at lower urinary Cd concentrations than those with high induction of MT. MT expression in PBLC can thus be used as an indicator of tissue sensitivity to Cd in the kidney.

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8.9.1 Anemia and Erythropoietic System

Animal experiments showed that hemolytic anemia may develop upon long-term and heavy exposure to Cd (107) with increased urinary excretion of Cd and involvement of MT (18). In humans (20) increased uptake of Cd was related to Fe status and such increased uptake is likely to occur also in anemia subjects.

Cadmium is also recognized as a potent inducer of MT synthesis. A study on the relationship between Fe deficiency and MT-1 concentration in blood and tissues of rats showed that Fe deficiency increased the concentration of MT-1 in bone marrow of rats with hemolytic anemia. On the other hand, unchanged concentrations in liver of MT-1 were related to decreased concentration of MT in kidney. The data were interpreted to mean that MT-1 in blood might reflect erythropoietic activity (108). The change of MT-1 in bone marrow might also explain why Fe deficiency increases absorption of metals, not only Cd but also Pb and Ni.

Erythrocyte MT has been suggested to be used as an index of Zn status in pregnant and nonpregnant women (109); however, data were not conclusive. That is probably due to changed blood volume and important change in trace element metabolism observed during pregnancy.

Discussions about a possibly increased sensitivity to Cd exposure among humans suffering from hemochromatosis, i.e., an Fe overload disease, have been going on. The disease is under the control of a gene that encodes for a protein HLA-H and is described as a histocompatibility complex (MHC) class-1-like protein. The mechanism of how HLA-H defect affects the Fe metabolism needs further evaluation (110) but may be related to genetic changes in the regulation of transferrin receptor in duodenal cells (111,112) and other iron transporter genes (113).

8.10 Reproductive and Developmental Effects

Reviews have previously been presented on reproductive and developmental effects of cadmium (2,114–116).

8.10.1 Male Reproductive System

Effects on Experimental Animals. Injection of soluble cadmium salts ($>10\mu$mol/kg) to experimental animals gives rise to acute necrosis of the testicles, first reported by Parizek and Zahor (103) and Parizek (117) and later confirmed by many authors. Also inunction of soluble Cd-salts on the skin of the scrotum or oral ingestion of Cd in high concentrations may give rise to this effect (115,116).

Indications of effects on testosterone production (118) and related effects on accessory genital organs in mice were found (119) after long-term Cd treat-

ment. Several more recent studies (120) have shown effects on androgen levels and accessory male genital organs including the prostate in rats. A decreased reproductive capacity has been found in some long-term studies (121) but not in other studies (53).

Since the first reports in the 1970s, a considerable number of publications have discussed the involvement of MT as a protective agent against tissue damage from Cd in reproductive tissues. In the first study performed by one of the present authors (52) it was demonstrated that in mice pretreated with small, not testicle-damaging doses of Cd a normally testicle-damaging dose did not cause testicular damage. Compared to nonpretreated animals, a larger proportion of testicular Cd in pretreated animals was bound to a low-molecular-weight protein assumed to be MT (52). Subsequent studies by others have questioned the existence of MT in the testis and the prostate and this has been the subject of considerable controversy. Some authors (120) consider that the lack of expression of MT in these tissues would explain their sensitivity to development of cancer subsequent to Cd exposure.

In our own studies in rats, apoptosis was indicated by DNA elecrophoresis 48 and 72 h after a single injection of 5–10 μmol/kg of $CdCl_2$. Expression of MT-1 occurred to a similar extent in controls and Cd-treated animals according to RT-PCR studies (122). Expression of the p53 gene in testicular tissue decreased markedly with increasing doses of Cd. In the ventral lobe of the prostate a slight increase in MT-1 expression was noted, while p53 remained largely unchanged. In subsequent studies, extending the survival time to 96 h (123), similar findings were found and (123) the c-jun protooncogene was also expressed.

An increased expression of both MT-1 and MT-2 in the testicle of rats has been observed by Suzuki et al. (104) and in our own (unpublished) studies, providing additional evidence that MT actually is expressed in testicular tissue.

Effects on Humans. Since humans are not exposed to Cd by injection, acute effects similar to those seen in animals are not likely to occur although some acute poisoning cases in human males have displayed histological damage to reproductive organs including the tests (115,124). The few human studies that have been published in recent years have not detected any clearly adverse effects.

No difference was found in fertility between male Cd workers and an unexposed population ($n = 138$) when assessed by birth experiences of their wives (125). Concentrations of Cd in semen of two workers with occupational exposure to Cd (mean concentration 3.3 μg/L) were higher than those in semen samples of 174 men without Cd exposure (mean concentration 0.38–0.44 μg/L) (126). No correlation was found between Cd concentrations and fertility status. Mean Cd concentrations in seminal plasma were slightly higher in smokers (0.54 μg/L) compared to non-smokers (0.42 μg/L). However, no difference was found in another study (127).

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Concentrations of Cd in reproductive organs of men who had died suddenly revealed the highest concentrations in epididymides, seminal vesicles, increasing with age (128).

8.10.2 Female Reproductive System

Effects on Animals. Since the first observations by Kar et al. (129) that in prepubertal rats, ovaries underwent morphological changes after injection of Cd chloride, several subsequent studies have shown that ovarian hemorrhages occurred in rats after injection of Cd salts. Similar effects have also been shown in mice. In prepubertal rats, endothelial damage in the blood vessels of the uterus and ovaries was reported and normal ovulation was inhibited in hamsters. These effects occurred at injected doses higher than 2.3 mg/kg body weight (20 μmol/kg). Long-term exposure to lower daily doses (0.036 and 0.18 mg Cd/kg body weight) was reported to give rise to changes in the blood vessels of the myometrium of the uterus in female rats (115,116).

When pregnant rats were injected (up to 5 mg Cd/kg), normal steroidogenesis was affected with changes in serum estradiol concentrations and ovarian estradiol production (130).

Uptake of Cd into the fetus is dependent on a number of conditions such as the dosage and stage of gestation. Cadmium passage through the placenta into the fetus is limited after closure of the vitiline duct (94,131,132).

Since the first studies on the teratogenic effects of injected Cd by Ferm and Carpenter (133) on golden hamsters, there have been several subsequent studies showing fetal death as well as severe malformations. A factor that may be of importance is Zn. Maternal Zn deficiency increases fetal susceptibility to Cd. Maternal Cd injections inhibit the transport of Zn from the mother to the fetus (115,134). Injections in pregnant rats of 2.4 mg Cd/kg body weight in the late stage of pregnancy (17–20 day) gives rise to vascular changes at sites of oestrogen biosynthesis and destruction of the placenta and this can subsequently cause fetal death (135). Also toxicity to the dam occurs—a condition similar to ''toxemia of pregnancy'' (136). Some data on long-term exposures also indicate that lower doses can increase the barrier between maternal and fetal circulation. High peroral exposure in pregnant animals may also result in reduced fetal weight and in some experiments also malformations (115,124).

Prenatal exposure to Cd can affect brain development in rats: conditioned avoidance reactions were more frequent compared to controls in rats whose dams had been injected with $CdCl_2$ (137).

Gupta et al. (138) gave dams 20 mg/L of Cd in the drinking water from day 0 of pregnancy. In the pups, there were changes in brain enzymes and lipid peroxidation in relation to controls. Considerable concentrations of Cd have been found in the brains of pups from similar experiments (34 μg/g) (139).

Rats exposed during lactation (dams) or postweaning to 5 μg/g of Cd in

drinking water had undetectable levels of Cd in their brains but brain levels of serotonin and 5-hydroxyindoleacetic acid were reduced (93).

Differences in MT isoforms in livers of both prenatal and neonatal rats were shown depending on Cd or Zn injection (140). Another study on Cd-treated rats made pregnant showed that in the pregnant rats there was a faster decrease in hepatic Cd levels and a faster increase in kidney Cd levels compared to nonpregnant rats. The pregnant rats also had higher plasma Cd levels (and MT levels) than nonpregnant rats. These observations were further supported by the studies of Chan et al. (141), in which it was shown that basal levels of MT were increased already at day 8 of pregnancy in rats not treated with cadmium. This observation is in accordance with tentative increased demand for metals, e.g., Zn and Cu, during pregnancy.

Effects on Humans. Clarkson et al. (114), in their overview of reproductive effects of Cd, discussed the possibility of the placental vasculature as a critical tissue in long-term Cd exposure, and pointed out that there was insufficient evidence at that time to consider effects on that tissue as a critical effect.

WHO (2), in a section dealing with fetal effects, pointed out that maternal hypertension and decrease in birth weight have been associated with elevated levels of Cd in the neonate. In addition, it is well established that babies of mothers who are cigarette smokers are smaller at birth than are those of nonsmokers. However, neither the placenta nor the fetus was considered as a critical organ in the further discussions in the WHO document (2). In the following text some more recent data will be reviewed. Eisenmann and Miller (142) studied the toxicity of Cd to the isolated human placenta using a perfusion technique. Cadmium (20 nmol/ml) produced decreased production and release of human chorionic gonadotropin (hCG).

Cadmium (10^{-9} M) inhibited spontaneous contractile activity in vitro and changed effects of Ca^{2+} and oxytocin in myometrial strips from term pregnant women (143). Pregnancy complications were recorded in a group of women residing in the vicinity of a Cu smelter (144). In patients with threatened spontaneous abortion, toxemia and anemia, biochemical changes suggestive of increased lipid peroxidation and decreased antioxidant protection, were found. Increased Cd concentrations in blood were associated with a decrease in reduced glutathione in blood (144). Changes in glutathione protection were related to placental As and Cd and tobacco smoking (145).

Low maternal Fe stores and high intake of fiber increased the accumulation of Cd in the placenta in Swedish women (146).

In placental tissue from 55 mothers from Ontario, Canada, who were not smoking, Cd concentration was 32 ng/g. There were strong positive correlation between Zn, Cu, and MT concentrations in placenta, but a negative correlation between MT and Cd concentration (147).

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In female Ni-Cd battery workers mean placental Cd concentration was 21 ng/g. No effect of Cd exposure on birth weight was detected, but a statistically significant effect on birth weight of smoking during pregnancy was observed (148). Elevated placental Cd (0.73 nmol/g dry weight) was reported in a smelter area, similar to the level found in smokers but there was no association between placental Cd and birth weight. The authors therefore concluded that the effects of smoking on birth weight are not mediated through Cd (149).

Lagerkvist et al. (150) found higher blood Cd values (10 nmol/L) in smoking pregnant women than in non/ex-smokers. There was a tendency in non/ex-smokers toward increasing blood Cd levels during pregnancy, reaching a blood Cd of 7 nmol/L at delivery, while an opposite tendency appeared for smokers. The decrease of blood Cd in smokers was due to decreased smoking during pregnancy. The increase in nonsmokers may be related to a mobilization of Cd from liver to blood during pregnancy. Such an interpretation is supported by animal data by Chan and Cherian (140,141). Lagerkvist et al. (151) found the following Cd concentrations in placenta samples of women in northern Sweden: GM = 5.0, 3.6, and 2.6 ng/g wet weight in smokers, ex-smokers, and nonsmokers, respectively. It was noted that placental Cd concentrations were four and six times higher than maternal and umbilical cord blood Cd, respectively (151).

8.10.3 Summary and Conclusions Concerning Reproductive and Developmental Effects

Cadmium injection in a single dose can cause acute necrosis of the testicles of experimental animals. In long-term exposure to Cd such effects are not seen but there may be other disturbances of the male reproductive system involving changes in male sex hormones. However, with the possible exception of prostate cancer, presently available evidence is not sufficient to state that such effects occur at low exposures and male reproductive effects are not considered as critical effects in humans.

In female rats hemorragic changes in ovaries, changes in ovarian hormonal production, endothelial changes in blood vessels with fetal death, and placental necrosis have been documented. Similar Cd doses given early in pregnancy can give rise to teratogenic effects in animals. Long-term exposure may give rise to reduced fetal weight and in some experiments to malformations. Changes in brain development and changes in brain enzymes have been reported in pups from rats treated orally with Cd during pregnancy and lactation. A factor contributing to the embryotoxicity and teratogenicity of Cd is that the transfer of Zn to the fetus is reduced by Cd exposure.

In human placentas exposed to Cd after delivery, pathological changes can be induced. In humans, elevated levels of Cd in the neonate of smoking mothers have been associated with decreases in birth weight. In female Cd workers and in women exposed to environmental Cd, there were elevated Cd levels in placen-

tas but no effect on birth weight. It is therefore considered unlikely that smoking-related increases in Cd levels would cause effects on birth weight.

There are no convincing observations of teratogenic or embryotoxic effects in humans. Reproductive, teratogenic, and developmental effects are not considered critical effects in humans at present.

8.11 Carcinogenicity

Whether Cd can give rise to cancer in humans has been much debated and mostly focused on cancer in the prostate and the lung. Data up to 1992 have been summarized in Nordberg et al. (50). IARC classified Cd as a human carcinogen belonging to group 1 (47). This was based on data showing that Cd can give rise to lung cancer in industrial workers. An interesting observation is that tobacco can be high in Cd and that the body burden of Cd is elevated in smokers. Cadmium could be one important agent in tobacco that contributes to the causation of the lung cancer in smokers.

The U.S. Department of Health and Human Services (DHHS) has determined that Cd and Cd compounds may reasonably be anticipated to be carcinogens. The evidence for this is based on increased lung cancer in humans caused by inhalation of Cd.

The assessments by these organizations were partly based on data summarized in Nordberg et al. (50) with an emphasis on the occurrence of lung cancer in an American cohort of exposed workers (152,153). These findings have been criticized (154). In a reanalysis of the data from the U.S. cohort (155) it was shown that a significant trend for lung cancer risk was found only for the combined exposure to As and Cd and there appeared to be no independent effect of Cd. In a study of Cd-exposed workers in the United Kingdom (156) no excess of lung cancer could be demonstrated. Early observations of an increased occurrence of prostate cancer in Cd workers (157) have not been confirmed in some later studies on Cd workers (158) and more recent epidemiological studies have reported variable results (159,160).

Evidence for carcinogenicity by Cd in animals has been available since 1960 (161). Injection site sarcomas and tumors in the male reproductive organs of rats and mice have been repeatedly reported (47,162). Oral Cd exposure produced dose-related increases in the incidence of leukemia, interstitial cell tumors of the testis, and proliferative lesions of the prostate in male rats in one study (163) but not in another study (164). Prostate carcinogenesis is dependent on an intact testicular production of testosterone. The use of Cd doses causing testicular degeneration (see above) may explain the lack of tumorigenesis in the prostate in some experiments. A possible involvement of Cd-induced changes in expression of MT, p53, and protooncogenes, such as c-jun in the development of pros-

tatic and testicular tumors in rats, has recently been indicated (122,123). With regard to lung cancer in animals, a dose-related increase in such tumors was shown (46) after inhalation of Cd in rats.

9. RISK ASSESSMENT BASED ON CRITICAL EFFECTS AND DOSE-RESPONSE RELATIONSHIPS

Risk assessment in environmental and occupational medicine serves as a basis of preventive action for avoidance of adverse health effects in populations exposed to chemicals. Concepts that are useful in risk assessment are critical effect, critical organ (tissue), and critical concentration (50).

When a group of individuals is exposed to high concentrations of a toxic substance, clinical disease or death will occur in a proportion of the exposed individuals that is related to dose. Similarly, at a lower dose range, there will be a dose-response relationship for less severe effects. In occupational and environmental health, it is important to prevent adverse effects on human health and dose-response relationship and the adverse effect that occurs in the lowest dose range is therefore of particular interest. This effect is critical for preventive action and is termed the ''critical effect.'' The organ or tissue whose damage gives rise to the critical effect is the critical organ (50). At a certain concentration in the critical organ the earliest adverse effect will occur in an individual. This is defined as the critical concentration for that individual. In many cases the effects are irreversible. Whether the damage that occurs upon exposure to an agent is reversible or irreversible is of importance in determining whether an effect is to be considered adverse, i.e., whether it should be classified as a critical effect. To prevent suffering and influence on quality of life, it is important to find indicators for early detection of effects in the critical organ.

The critical effect of Cd exposure in both the general environment and the work environment is considered to be renal tubular dysfunction. Recent evidence indicates that renal damage occurs at much lower Cd concentration than previously estimated. Based on evaluations of several recent studies (19) in the general population, it has been concluded that an average urinary Cd level of 2.5 μg/g creatinine is related to an excess prevalence of renal tubular dysfunction of one or a few percent. A Cd concentration of 50 μg/g cortex wet weight, which is reached after decades of Cd intake of 50 μg/day, gives rise to the mentioned urinary Cd excretion of 2.5 μg/g creatinine. This is a higher risk of tubular damage than indicated by earlier estimates. These risk estimates are made for a population group in total. However, estimates have to be made for certain identified groups with increased risk for development of renal tubular dysfunction. Groups that have been identified as constituting such risk groups are subjects with low Fe stores and smokers. It has been estimated that a small percentage (1–5%) of

such groups may develop renal tubular dysfunction at an oral intake of about 15 μg/day. Glomerular dysfunction is not considered to occur below the Cd concentration in renal tissue where tubular dysfunction is manifested.

At present data on bone effects of Cd are not sufficient to perform a risk estimate in the human population. It should, however, be stressed that such data might be available in the near future. At that time a reevaluation of critical effects and exposure levels has to be performed.

IARC concluded in 1993 (47) that there was sufficient evidence to classify Cd as a human carcinogen, thus belonging to the class 1 agent group according to the IARC classification system. However, recent reanalyses of data on Cd and lung cancer have not been confirmatory. The lack of sufficient data on Cd and lung cancer means that it may not be possible at present to draw a conclusion that Cd is a human carcinogen. It would at present perhaps be more appropriate to classify Cd as a 2A chemical, i.e., a probable human carcinogen.

Based on the risk estimates presented in the foregoing paragraph, a risk characterization of Cd exposure in the general population can be as follows:

A daily intake of 30 μg Cd over a lifetime will give an increase of 1% renal dysfunction in the adult population and in the high-risk groups up to 5% of such groups will display such an effect.

With the present Provisional Tolerable Weekly Intake (PTWI) of 70 μg/day via food up to 7% of the population will be expected to develop tubular dysfunction and the figure for the risk groups will be higher, i.e., 17%.

When discussing risk characterization it has to be kept in mind what characteristics are set and which cutoff level is chosen.

Dietary intake of cadmium has been estimated to be 12–50 μg/day on average in various countries, as mentioned earlier.

10. DIAGNOSIS OF CHRONIC POISONING

Chronic Cd poisoning subsequent to long-term (years-decades) exposure to industrial air with Cd concentrations considerably above present exposure limits is characterized by renal tubular (and, in some rare cases, glomerular) damage, lung disorders, and, in some rare cases, osteoporosis. Lung disorders with obstructive and restrictive components are seen only in persons exposed to high levels in the past when occupational exposure limits were high. If industrial monitoring data are lacking, past exposure can be estimated from current biological monitoring data either by general approximate relationships or more precisely using a model (165) to calculate cumulative dose and body accumulation in various organs.

Concentration of Cd in blood reflects either ongoing or recent exposure to Cd. Cadmium in urine reflects the body burden of accumulated Cd. There is an approximately linear relationship between urinary Cd and Cd in the renal cortex.

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At urinary Cd concentration of 5 μg/g creatinine (19) the renal cortical level is approximately 100 mg/kg, corresponding to approximately 5% excess risk of low-molecular-weight proteinuria (see also preceding section on critical effects and dose-response relationships). Various biomarkers, such as β_2-microglobulin, protein HC, and activity of NAG A and B with a preference for NAG B in urine, can be analyzed to evaluate whether signs of renal dysfunction are present.

11. PREVENTION OF Cd POISONING, NATIONAL AND INTERNATIONAL RECOMMENDATIONS

11.1 International Evaluations and Recommendations

Cadmium has been repeatedly evaluated by the International Agency for Research on Cancer. The latest evaluation was made in 1993 (47). Cadmium and its compounds was classified as a human carcinogen (Group 1). Health-based limits for occupational exposure to Cd fumes and respirable dust have been proposed by the World Health Organization (166): 250 μg/m^3 for short-term exposures; provided the recommended time-weighted average (40 h/week) of 10 μg/m^3 is respected. Cadmium levels in urine and blood of individuals should not exceed 5 μg/g creatinine and 5 μg/L of whole blood, respectively.

A drinking-water guideline value of 5 μg/L has been set (167).

The joint FAO/WHO Expert Committee on Food Additives and Food Contaminants has set a provisional tolerable weekly intake (PTWI) of Cd at 400–500 μg for adult persons (168), corresponding to approximately 1 μg/kg body weight for each day of the week.

Oral intake of Cd in high single dose via food or drink gives rise to gastrointestinal symptoms (see above). The PTWI is set to allow a certain variation of intake during a week provided the weekly intake is not exceeded. The extent to which amounts above the PTWI can be allowed during various time intervals was discussed by Nordberg (169). A single dose of 4–14 μg/kg body weight or a dose of 3 μg/kg body weight over periods of months was considered tolerable without gastrointestinal symptoms when PTWI is temporarily exceeded. There should be a period of compensatory intakes lower that the PTWI to avoid the risk of renal dysfunction. For children and pregnant or lactating women daily intake should not exceed 1 μg/kg body weight.

11.2 National Regulatory Rules and Other Legislation

To protect human health, the government in each country makes recommendations and sets regulatory rules regarding human Cd exposure. The U.S. Environmental Protection Agency (EPA) (170) allows 5 parts of Cd per billion parts of drinking water (5 ppb). Limits for how much Cd can enter lakes, rivers, waste

sites, and cropland are also under EPA regulation. The EPA does not allow Cd in pesticides.

The U.S. Food and Drug Administration (FDA) has set a limit on the amount of Cd in food colors to 15 parts of Cd per million parts of food color (15 ppm) (15 mg/kg).

The U.S. Occupational Safety and Health Administration (OSHA) has set a threshold limit value for occupational exposure via air of 100 μg Cd/m^3 as Cd fumes and 200 μg Cd/m^3 as Cd dust. The intention is to limit all Cd compounds to either 1 or 5 μg/m^3. The National Institute for Occupational Safety and Health (NIOSH) currently recommends keeping inhalation of Cd as low as possible (170).

In Sweden certain industrial uses of Cd (electroplating, pigments) are banned. The National Board of Occupational Health regulates the exposure to Cd in the work environment. A hygienic limit value for respirable dust has been set at 10 μg/m^3 and for total dust 50 μg/m^3 (171). Cadmium is listed as a carcinogenic compound. Medical checkup is compulsory for employed workers with periodic determinations of blood Cd.

Cadmium is regulated in the United States by the EPA and in some states under the Clean Water Act's National Pollutant Discharge Elimination System and General Pretreatment Regulations. EPA offices overseeing regulations and guidelines applicable to Cd include the Offices of Air Quality Planning and Standards; of Drinking Water; of Toxic Substances; of Solid Waste; of Pesticide Programs; and of Emergency and Remedial Response. Cadmium is listed as a toxic chemical under the Emergency Planning and Community Right-to-Know Act; estimates of Cd releases into the air, water, or land must be reported annually and entered into the national toxic release report (172,173).

Sweden lacks threshold limit values for Cd in food but is most active in the international work to develop such a concentration. On the national level a recommendation regarding restricted intake of liver and kidney exists. Depending on the age of the animal, an intake of 1–2 meals per week is recommended or no consumption at all (174).

12. SUMMARY

Cadmium is a silver-white metal. It occurs naturally widely dispersed in the environment and is produced as a by-product in the production of other metals. Human exposures occur as inhalation of Cd-containing dust in industry and orally as Cd-containing food in the general population. Uptake of Cd via inhalation is 5–35% depending on particle size. Oral uptake is approximately 5% but may be higher in persons with Fe deficiency. After uptake Cd in blood is initially taken up by the liver and subsequently slowly redistributed to the kidney because of its binding to the low-molecular-weight protein MT. Cadmium bound to this

protein in blood selectively accumulates in the kidney. In long time exposures Cd gradually accumulates particularly in the kidney but also in other tissues. When concentration in the kidney cortex reaches 50 mg/kg or higher, damage to renal tubular cells may occur in the most sensitive persons. This gives rise to a decreased ability of kidney to reabsorb proteins with increased concentrations of low-molecular-weight proteins and Ca in urine. As a result of long-term Ca losses and other metabolic changes related to the renal damage, osteoporosis and, in severe cases, osteomalacia (itai-itai disease) occur. Cadmium has been shown to cause damage to reproductive organs and carcinogenesis in laboratory animals. Long-term human exposures have given rise to lung cancer in industrial workers and Cd is considered carcinogenic to humans, but there is some uncertainty in this assessment based on recent data. Because of its toxicological properties, legislation implying considerable restrictions in its use has been passed in some countries. Industrial exposures as well as exposures in the general environment have to be kept below recommended exposure limits in order for adverse health effects to be avoided.

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9

Chromium and Cancer

Montserrat Casadevall and Andreas Kortenkamp
The School of Pharmacy, London, England

1. INTRODUCTION

More than a century ago, David Newman published a case report on a chrome pigment worker who suffered from carcinoma of the upper respiratory tract. This report (1) marked the beginnings of systematic research into the carcinogenicity of chromium compounds. Since then, a multitude of epidemiological studies have appeared. The link between inhalation of chromium(VI) compounds and the causation of cancers of the airways and lungs is now well established (2). Although neoplasms of the respiratory system are the most prominent effect of chromium(VI), other cancers have also been associated with exposure to these metal compounds (3).

Inhalative exposure to chromium(VI) occurs in many working environments, including the primary production of chromates, chromium plating, chromium(VI) pigment manufacture, and stainless steel welding. The major source of chromium(VI) exposure in the construction industry is via cement. People not themselves engaged in handling chromium compounds at their workplaces may also come into contact with these carcinogens. Prominent examples include residential populations living near ferrochromium smelters and other industrial installations involving chromium use, or sites where highly toxic chromium-con-

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taining wastes were dumped (e.g., New Jersey, U.S.; Glasgow, U.K.; Ruhr area, Germany). Chromium(VI) is also used as an anticorrosive agent for water-cooled installations such as cooling towers or pumping stations. In one instance, chromium(VI)-containing water from a pumping station operated by the Californian utilities company Pacific Gas and Electric reached the wells of residents who used it as drinking water (3).

Toxicology and epidemiology have played their roles in identifying working environments where chromium(VI) is a hazard. The focus is now shifting to questions concerning the early detection of signs of malignancies in exposed individuals. Here, the biological monitoring of exposed workers has raised hopes, not only as a means of verifying compliance with existing health regulations but also as a tool that might allow further refinements of risk estimations. Another challenge is to establish whether exposures close to current occupational exposure limits of around 50 $\mu g/m^3$ in workplace air pose cancer risks. This question has, of course, wider implications: is there a ''safe'' exposure level for chromium(VI) or is it necessary to regard any exposure as potentially hazardous?

In this chapter, we will present an overview of environmental settings where exposure to chromium occurs. In view of the overall hazards, we will concentrate on workplace environments and will only briefly discuss scenarios of (residential) environmental exposure. A short update on recent relevant epidemiological studies will be followed by considerations of the toxicokinetics of chromium(VI) upon inhalation. A discourse on the molecular mechanisms underlying the carcinogenicity of chromium(VI) will set the scene for an in-depth consideration of approaches to the biological monitoring of chromium(VI)-exposed subjects.

2. EXPOSURE TO CHROMIUM(VI)

2.1 Occupational Settings

The metallurgical industries are the most important users of chromium. Eighty percent of the total mined chromium is used as an alloying agent in the production of chromium steels. The remainder of the output of primary chromium production goes into the manufacture of chromium chemicals that are used in pigments, leather tanning, wood preservation, and metal-finishing processes (4,5). Millions of workers worldwide are exposed to fumes, mists, and dust containing chromium. The highest exposures occur during chromate production, chromium plating, pigment production, ferrochromium production, spray painting, stainless steel welding, and cement finishing.

Workers engaged in the production of chromate are exposed to dusts containing chromium(III) (derived from chromite ore) and chromium(VI) (sodium, potassium, calcium, and ammonium chromates and dichromates). The relative

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amounts of the two chromium oxidation states in the air vary along the chromate production line, with workers involved in chromite and lime mixing being exposed mainly to trivalent chromium and those involved in the following steps, roasting, filtering, and shipping, exposed to a mixture of both.

Of the three methods of stainless steel welding, i.e., manual metal arc (MMA), metal inert gas (MIG), and tungsten inert gas (TIG), the MMA method produces welding fumes containing the largest amounts of chromium(VI). In MMA stainless steel welding, oxidation of metallic chromium to chromium(VI) occurs in close proximity to the electrodes and is followed by an immediate reaction with alkali oxides from the electrode coatings. These processes give rise to the formation of sparingly or readily water-soluble alkali chromates including potassium dichromate and calcium chromate. In shield gas welding (MIG, TIG), the inert gases largely prevent metallic chromium from being oxidized to chromium(VI), although not completely. As a result, the predominant oxidation state of chromium in MIG and TIG welding fumes is thought to be chromium(III), with chromium(VI) being present at relatively low levels. Another important factor is that alkali oxides capable of reacting with chromium are not used in shield gas welding processes. The total fume concentration in the breathing zone of MMA welders may be as high as 100 $\mu g/m^3$, with the level of chromium(VI) reaching 4 mg/m^3 in extreme cases (6).

The manufacture of chromium pigments begins with solutions of sodium chromate or bichromate to which water-soluble salts of, for example, lead are added to form precipitates of lead chromate. Similarly, zinc chromate is formed by reaction of zinc oxide with sodium chromate or bichromate. Once the precipitates have formed, they must be separated, dried, milled, and packed. Exposure to lead or zinc chromate is therefore greatest in the latter stages of the process, the ''dry'' department, where the conditions are very dusty. Exposure to sodium chromate is highest at the beginning of the process, in the so-called ''wet'' department. Thus, simultaneous exposure to more than one chromium compound occurs frequently (4,7).

Chromium platers are exposed to mists of chromium(VI) trioxide, which are generated during electrolysis in plating baths containing chromium trioxide, sulfuric acid, and various organic additives. The mists are formed when bubbles of oxygen and hydrogen arise from the electrodes and burst at the liquid surface of the plating bath. The use of surfactants or floating balls, combined with local exhausts, can substantially lower exposure to chromium(VI) trioxide. Around baths equipped with local exhausts chromium(VI) air levels are around 10–30 $\mu g/m^3$, but rise to 120 $\mu g/m^3$ without exhausts (4).

The production of ferrochromium steel involves the electrothermal reduction of chromite ore with coke in furnaces. Workers near these furnaces are exposed to fumes containing mostly trivalent chromium, but also chromium(VI) trioxide. The exposure patterns peculiar to the ferrochromium manufacturing in-

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dustry were used to investigate whether exposure to forms of chromium other than chromium(VI) would cause cancer.

2.2 Nonoccupational Exposure

Exposure of the general population to chromium occurs through air, food, and water, but the levels are usually much lower than those found in occupational settings. Anthropogenic activities are responsible for the presence of chromium in the environment.

In the United Kingdom the main sources of chromium emissions into the atmosphere are waste incineration, fuel combustion, and industrial processes such as iron and steel production. In 1995, 60 tonnes of chromium were emitted in the United Kingdom (8). The concentration of chromium in the air of U.S. cities with chromium-related industries is higher than the national average. Coal-fired plants also contribute to the amount of chromium in the air owing to the release of chromium that is present in coal. An additional source of chromium are cement-producing plants.

The presence of chromium in water is the result of mineral-weathering processes. Other contributory factors are soluble organic chromium compounds and the mobilization of chromium compounds from sediments. In addition, surface waters and groundwater can be contaminated with wastewater from electroplating, leather tanning, or waters laced with chromium(VI) as an antirusting agent. Solid waste from the chromate production processes or municipal incineration can also find its way into drinking water if not properly disposed of (4).

Solid waste disposal from industrial activities is now controlled and concentrated in restricted landfill sites. Previous practice, however, when residues were indiscriminately used as landfill material, has resulted in large-scale contaminations of land. Representative examples are those of Glasgow in the United Kingdom where the world's largest chrome producer was in operation from the nineteenth century until 1967, and the Aberjona River basin in Massachusetts where chemical manufacturing and leather-tanning activities have left a legacy of environmental contamination. The area now contains two of U.S. EPA's Superfund sites and 20 state-identified hazardous waste sites (9–11). In southeast Glasgow, U.K., chromite ore-processing residues were used routinely as landfill material; a reported 60–70 tonnes of waste was dumped daily between 1960 and 1966 (9). Annual mean chromium levels in watercourses passing through this contaminated land are around 3920 μg/L as opposed to the 0.02 μg/L reported as background levels (12). Similar contaminated sites exist all over the Ruhr area and to the north of Cologne in Germany.

In residential settings near to or on waste-dumping sites containing chromium-contaminated soil exposure is mainly via accidental ingestion of soil and inhalation of dusts (4,13).

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3. THE CARCINOGENICITY OF CHROMIUM(VI)

3.1 Epidemiological Studies in Occupational Settings

The carcinogenicity of chromium(VI) compounds in various occupational settings is well documented, and the interested reader is referred to various excellent in-depth reviews of the topic (2–4,7,13,14). Here, we will only present an overview of the most important findings.

Epidemiological studies conducted in many different countries have consistently demonstrated an increased risk of developing lung cancer in the primary chromate production. The risk of lung cancer increases with duration and severity of exposure.

Similar studies carried out in the pigment production industry have also shown an excess risk of lung cancer. Workers engaged in pigment production inhale dusts of calcium, zinc, and lead chromate. Zinc chromate is reported to be a particularly potent human carcinogen. The available epidemiological data do not provide strong evidence for the carcinogenicity of lead chromates in humans. However, the data are not sufficient to rule out the possibility of such an association.

In a recent survey among chromium platers Sorahan and colleagues (15) were able to show that lung cancer mortality and nasal ulcerations were correlated with duration of chrome bath work. Crucially, their results suggest that a working life at the current U.K. maximum exposure limit for chromium(VI) of 50 $\mu g/m^3$ (time-weighted average) may present unacceptable risks.

Comparatively few studies have addressed the issue of lung cancer in workers of the ferrochromium industry. Where an increase in lung cancers could be demonstrated, the presence of chromium(VI) in the work environment was shown. In other investigations no excess of lung cancer was observed. Finally, the carcinogenicity of chromium was investigated in industrial settings such as stainless-steel welders where a relation between exposure to chromium and increased incidence of lung cancer could be confirmed.

3.2 The Types of Cancers Observed After Exposure to Chromium(VI) by Inhalation

Squamous cell carcinoma of the lung is the most frequent type of lung cancer observed after exposure to chromium(VI) compounds by inhalation. However, other types of cancer are also detected, and the kind of lung cancer appears to depend on the nature of the chromium compound, the duration of exposure, and the smoking habits of the exposed individuals. As a general rule, exposure to increasingly refined chromium(VI) compounds with lower levels of chromium(III) results in squamous cell carcinoma as the dominant type of cancer, whereas heavy exposure to mixed chromium compounds, especially chromi-

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um(III) and chromium(VI), leads to the formation of both squamous cell carcinoma and small cell carcinoma (13).

The occurrence of rare sinonasal cancers was reported in the epidemiological studies of workers in the chromium pigment production conducted by Langard and Norseth (16). Of the cancer cases described later by Langard and Vigander (17), one patient had small cell carcinoma, three had epithelial carcinoma, one had oat cell carcinoma, and one had adenocarcinoma.

Squamous metaplasias of the bronchial epithelium are also frequently observed in lung cancer patients with a history of exposure to chromium(VI) (18–20).

Epidemiological studies of the health experience of workers exposed to chromium have produced suggestive evidence that chromium(VI) is also capable of producing nonrespiratory cancers, including malignancies of the digestive system, stomach, nasal, larynx, pleura, kidney, prostate, and bladder (3).

3.3 Health Effects in Residential Populations Exposed to Chromium(VI)

A few studies suggest adverse health effects due to exposure to environmental chromium, primarily for people living near chromium-related industries or in areas where solid waste from mining has been used as a landfill (3,4,10). In Woburn, located in the Aberjona River basin, a fourfold increase in childhood leukemia was attributed to the possible consumption of water with chromium(VI) levels above the standard (4,10). However, recent studies, during which hair analyses for metal exposure were conducted, could not confirm the suspected chromium and arsenic exposure due to consumption of contaminated drinking water (11).

Epidemiological studies of chromium exposure and incidence of lung cancer have also been performed. A Swedish study analyzing the incidence of lung cancer in a population living near a ferrochromium smelting plant did not find elevated cancer mortalities relative to the general population. Examinations of two populations in New Jersey, living in properties containing chromite ore-mining residues concluded that there were no significant increases in carcinogenic or noncarcinogenic effects (4,13).

One of the main conclusions of a comprehensive review on the impact of chromium in environment and general population published by the Canadian Government reads:

> It has also been concluded that the group of hexavalent chromium compounds as a whole is entering the environment in a quantity and concentration or under conditions that may constitute a danger in Canada to human life or health, while the group of trivalent chromium compounds as a whole is not entering the environment in a quantity and concentration or under conditions that may constitute a danger in Canada to human life or health (21).

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It is clear that the possibility of adverse health effects due to environmental chromium exposure is of concern. However, the number of published studies is too small to reach clear-cut conclusions. In most cases human health risk cannot be properly assessed because there are insufficient data on population exposure. Furthermore, there is a lack of understanding of the long-term effects of chromium(III) accumulation in the body.

3.4 Noncancer Effects

Exposure to chromium can result in toxic effects other than malignant neoplasia. Chromium dermatitis and skin ulcers have been consistently reported in various occupations with exposure to chromium compounds, including the manual handling of cement, leather, plastics, dyes, textiles, paints, printing inks, cutting oils, photographic materials, detergents, wood preservative, anticorrosion agents, and welding rods (4,13). Perforations and ulcerations of the nasal septum and bronchial asthma are frequent results of inhalation of chromium(VI), particularly among chromium platers. With reference to ulcerations among platers Sorahan and colleagues (15) quote Her Majesty's Factory Inspectorate as stating in 1967 that ''ulcers . . . on the fingers or hands . . . are usually the outcome of lack of personal measures of protection. . . . Nasal ulceration is, in contrast, usually due to airborne mist or spray . . . and most commonly denotes a failure of plant control.''

4. TOXICOKINETICS OF CHROMIUM(VI) FOLLOWING EXPOSURE BY INHALATION: ''HOT SPOTS'' OF CHROMIUM ACCUMULATION IN THE LUNG

4.1 Deposition in the Lung and Site-Specific Carcinogenesis

A characteristic feature of carcinogenesis following exposure by inhalation is the site-specific formation of neoplasms. The carcinoma developing after exposure to inhaled carcinogenic agents usually appears in central, rather than peripheral, regions of the lung. Studies using hollow casts of the respiratory system have shown that particulate matter is preferentially deposited near bifurcations of the conducting airways (22). Judging from experiences with other carcinogens, considerable amounts of chromium should be deposited in the lungs of exposed persons and sites of neoplasia should coincide with sites of enhanced deposition.

Postmortem analyses of lung tissue obtained from chromium workers who died of lung cancer have indeed revealed that chromium(VI)-containing particles stay in the lung for very long periods of time (23,24). Even years after cessation of exposure most of the chromium can still be found in the respiratory tract. Only relatively small amounts reach liver and kidneys via the bloodstream. The total

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amount of chromium residing in the lungs of Japanese chromium(VI) production workers was found to be as high as 30–70 mg, while the amounts found in the liver and kidney were 3.8 mg and 0.8 mg, respectively (25). In comparison, the lungs, livers, and kidneys of decedents with no occupational chromium exposure contained 0.08–1.2 mg, 0.3 mg, and 0.07 mg, respectively. [These figures were calculated from the data in Kishi et al. (25) using published reference values for human organ weights.]

Raithel et al. (26) were able to demonstrate that the lungs of stainless steel welders showed chromium levels 10–30 times higher than those found in unexposed control subjects (Table 1). The distribution of chromium within the lungs was heterogeneous, with the upper lung lobes frequently containing higher amounts of chromium than the lower lobes.

Ishikawa and co-workers (27) have systematically addressed the issue of local distribution of chromium-containing materials in the lung. In analyses of autopsies from the lungs of ex-chromate workers in Japan they observed long-term retention of chromium in the bronchial walls. There were ''hot spots'' of chromium deposition at airway bifurcations. The accumulation of chromium became more pronounced with increasing tracheobronchial branching. The chromium concentrations at these sites were in the millimolar range. A direct relationship between chromium hot spots and neoplasia was observed.

Table 1 shows a compilation of data on chromium levels in the lungs of occupationally unexposed referents. Kollmeier et al. (28) observed an age-dependent increase in lung chromium levels and found that men on average showed levels twice as high as those in women. These authors were even able to demon-

TABLE 1 Chromium Levels in the Lungs of Occupationally Exposed and Nonexposed Individuals

Study population	Cr in lungs (μg/g dry weight)	Ref.
Chromium(VI) production		
Case 1	397	25
Case 2	1467	
SS welders	30–86	26
Referents	0.31	25
Referents	1.37	26
Referents, smokers	4.3	29
Referents, ex-smokers	4.8	
Referents, nonsmokers	1.3	
Referents, industrial area	2.14	28
Referents, nonindustrial area	0.57	

strate differences due to environmental factors. The lung chromium content of people living in a heavily industrialized conurbation (the Ruhr area) was significantly higher than that of individuals living in a city where occupations are mainly associated with trade and administrative services (Münster).

In summary, the bulk of chromium(VI), once inhaled, stays in the lung for very long times. Only a relatively small fraction of the total inhaled amount enters the systemic circulation, to be distributed to liver, kidney, and urine.

4.2 Biological Activation of Chromium-Containing Materials Deposited in Lungs

The nature of the processes following deposition of chromium-containing materials in the lung is relatively ill-defined. This is perhaps not surprising, considering that these events are difficult to study experimentally. However, we can conceive of three processes that govern the biological activation of chromium(VI) in the lung: solubilization of chromium(VI), cellular uptake of chromium(VI), either as the soluble chromate anion or as particulate matter, and extracellular reduction of solubilised chromium(VI) by constituents of pulmonary epithelial lining fluids.

Elias and co-workers (30,31) have provided evidence that the biological effects of particulate chromium(VI) result from extracellularly solubilized chromate. In their hands, internalized particles did not play a role in the transformation of cells to malignancy. In experiments with calcium, strontium, and zinc chromates, Elias and colleagues showed that the yield of transformed cells increased with the amount of chromium present inside cells. Within 7 days, even poorly soluble compounds such as the chromates of zinc and lead liberated sufficient amounts of chromate anions into the culture medium to cause biological effects.

The groups of Landolph (32) and later Patierno (33,34) have obtained results that indicate an involvement of particulate chromate in cell transformation and elastogenicity. These workers favor the idea that such effects arise from particle-cell interactions, without any involvement of solubilized chromium(VI); however, no attempts were made to measure the levels of dissolved chromate.

Levy and co-workers (35) have analyzed the processes following deposition of chromium-containing materials in the lungs of animals by using intrabronchial pellet implantation techniques. Briefly, a metal wire basket or pellet containing the test material was surgically implanted into the left bronchus of an anesthetized rat. The metal mesh acts as a framework in and around which the test material, mixed with cholesterol, is suspended and from which it leaches. A selected zone of bronchial epithelium is exposed to chromium compounds for a long period. Factors identified as determining the response of the rat lung were the amount of chromate contained in the pellet, the rate of release of chromate ions to the target tissue, and the lipid/water interactions and lipoprotein penetration at the cell membrane.

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The observations made in these studies can be explained in terms of the aqueous solubility of chromium(VI) compounds. Very poorly soluble compounds such as lead chromates hardly induced any carcinogenic effects. It is conceivable that these compounds leached out too slowly from the implanted pellet, resulting in far too low concentrations of chromate ions in the exposed area of the lung for carcinoma to be formed. Similarly, highly soluble chromates failed to produce severe effects because they leached out too rapidly from the pellet for local concentrations of chromate to build up in the target tissue. Malignant neoplasias could only be induced within the duration of the pellet implantation bioassay, when the lung tissue was chronically exposed to an optimal concentration of chromate ions. The zinc and calcium chromates provoked strong effects in this assay because they delivered an optimum amount of chromate anions to lung tissues.

Once solubilized, the chromate anion is effectively taken up by mammalian cells via the sulfate anion carrier system (36). However, chromium(III) compounds cannot easily penetrate cell membranes (37). For this reason, the extracellular reduction of chromium(VI) can prevent its cellular uptake and thus afford a certain degree of protection. It is well established that pulmonary epithelial lining fluids in humans contain ascorbate and glutathione at high concentrations, both effective reductants of chromium(VI) (38). However, the stores of ascorbate and glutathione may be rapidly exhausted, particularly in hot spots of chromium(VI) deposition. Therefore, the protective effects of epithelial lining fluids and other respiratory tissues with chromium(VI)-reducing capacity has probably been overemphasized (38).

4.3 The Intracellular Reduction of Chromium(VI)

Once inside cells, the chromate anion is rapidly reduced to chromium(III) complexes. Owing to the impermeability of the cell membrane to chromium(III) complexes, there is always a concentration gradient favoring uptake of chromate anions into the cell. The inevitable result is an accumulation of chromium inside cells and cell organelles. Sehlmeyer et al. (39) have reported millimolar cytosolic and intranuclear chromium concentrations after treatment of V79 cells with low levels (10 μM) of chromium(VI).

The in vitro studies of Connett and Wetterhahn (40) have helped to establish the important role of thiols, especially glutathione (GSH), in the intracellular reduction of chromium(VI). In view of its abundance in the cytosol of mammalian cells (concentrations in the millimolar range) and the rapid formation of a chromium(VI)-GSH thioester followed by a slow reduction step, the authors argued that GSH may well prolong the lifetime of chromium(VI) inside cells, thereby increasing the likelihood of interactions with cellular macromolecules.

Suzuki and co-workers (41–43) were the first to provide experimental evidence that ascorbate (AsA), under physiological conditions, is more reactive toward chromium(VI) than GSH. Since then, further investigations have confirmed the role of AsA as an important, if not the principal, chromium(VI) reductant in a variety of tissues including lung, liver, and kidney (44,45).

Enzyme systems with chromium(VI)-reducing capacity include the cytochrome P-450 systems (46) and complexes I (NADH–ubiquinone oxidoreductase) and IV (ferrocytochrome c–oxygen oxidoreductase) of the electron transport chain (47). Interestingly, even small concentrations of oxygen (1%) can effectively inhibit the reduction of chromium(VI) by cytochrome P-450 (48,49). Given the need to exist in an aerobic environment, the importance of cytochrome P-450 in the reduction of chromium(VI) appears to be negligible for most cells. Similarly, mitochondrial enzymes are not likely to play a major role in the activation of chromium(VI) to genotoxic species since most of the reaction products remain trapped in mitochondria, unable to reach the cell nucleus (50).

Much recent research has focused on hydrogen peroxide as a chromium(VI) reductant, in particular in the context of the formation of radical species that have the potential to damage DNA (51,52). Although the significance of hydrogen peroxide in chromium(VI) reduction appears to be negligible in view of its low estimated steady-state levels inside cells (in the order of 1–100 nmol/L) (53), there is the possibility of hydrogen peroxide being formed during reductions of chromium(VI) by GSH or AsA, subsequently leading to the formation of reactive species with DNA-damaging potential.

In summary, GSH and AsA appear to dominate the reductive conversion of chromium(VI) inside cells. These processes play a crucial role in the conversion of chromium(VI) to DNA-reactive species.

5. MECHANISMS UNDERLYING CHROMIUM(VI) GENOTOXICITY

Chromium(VI) compounds are genotoxic and various forms of genetic damage have been observed in bacteria, cultured mammalian cells, and laboratory animals, including chromosomal aberrations, sister chromatid exchanges, and DNA lesions (4). It is this genetic damage that is thought to mediate the mutagenicity and carcinogenicity of chromium compounds.

5.1 Mutagenicity

Chromium(VI) compounds are well-established mutagens in bacterial, yeast-based, and mammalian assay systems (4,13,54–56).

In *Salmonella typhimurium* base substitutions are frequently observed in the Ames strains with mutations predominantly in A-T rather than G-C se-

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quences. In oxidation-sensitive *Salmonella strains* such as TA 102 the mutations induced by potassium dichromate were totally dependent on the presence of molecular oxygen (57).

Data from studies with mammalian cells show that chromium(VI) mutagenicity is influenced by the nature of the chromium compound and the cell line used as well as by the genetic loci selected for analysis. Studies on the specific mutations caused in mammalian genomes by chromium(VI) compounds showed conflicting results. A predominance of mutations in A-T-rich gene sequences of the *hprt* locus was observed with Chinese Hamster Ovary cells (58), whereas three prominent hot spots at G:C base pairs were found in exon 3 of the *hprt* gene from human lymphoblasts (59).

Chromium(III) compounds, on the other hand, have yielded negative results in the majority of mutagenicity tests (4,13,54,56). Some chromium(III) complexes were found to be mutagenic in the Ames test with the *Salmonella* strains TA98, TA100, and TA92 when complexed to certain organic ligands that facilitate their uptake into cells, e.g., 2,2′-bipyridyl or 1,10-phenanthroline (4,54,55). The mutations induced by the chromium(III) complex with 2,2′-bipyridyl in the oxidation-sensitive *Salmonella* strains TA2638 and TA102 were oxygen dependent (57).

5.2 Intracellular Chromium(VI) Reduction as a Prerequisite for Formation of DNA Damage

A wide variety of DNA lesions including DNA-protein cross-links, DNA interstrand cross-links, single-strand breaks, alkali-labile sites, and chromium-DNA adducts have been described as being caused by chromium(VI) in mammalian cells. Do these lesions arise from chromium(VI), intracellularly accumulating chromium(III), or are they caused by reactive intermediates that are formed during the reductive conversion of chromium(VI)?

It was found that chromium(VI) itself, in the absence of reducing agents, is totally unreactive toward isolated DNA or cell nuclei (60–62). This initially surprising finding has prompted extensive research into the role of cellular constituents in the reductive conversion of chromium(VI) and their influence on the patterns of DNA damage in cells.

A large body of evidence shows that glutathione and ascorbate are involved in the formation of chromium(VI)-mediated DNA lesions. Important clues came from studies that assessed the influence of antioxidants or of artificially altered intracellular levels of reductants on the patterns of DNA damage.

Cupo and Wetterhahn (63) demonstrated that increased levels of glutathione, induced by pretreatment of cultured chick embryo hepatocytes with acetylcysteine, led to a marked elevation of the number of single-strand breaks caused

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by chromium(VI). These changes were even more pronounced after pretreatment with isopentanol, which increases both glutathione and cytochrome P-450. Similarly, the depletion of cellular glutathione by using buthionine sulfoximine was associated with decreases in the level of single-strand breaks. Interestingly, the number of DNA-protein cross-links and interstrand cross-links was only marginally affected. These results suggest that distinctly different mechanisms are operating in the formation of strand breaks and cross-links.

A similar marked increase in the level of single-strand breaks was observed when the intracellular glutathione levels were raised by treatment with sodium selenite (64) or by cultivation in medium supplemented with glutathione (65).

Sugiyama et al. (66) found that elevated levels of intracellular ascorbate, induced by preincubating cells with ascorbate, led to decreases in the alkali-labile sites arising from chromium(VI), but caused increases of DNA-protein cross-links. In contrast, Capellmann et al. (67) failed to detect any marked influences of raised ascorbate on the formation of DNA-protein cross-links. Instead, increased levels of glutathione were associated with higher levels of DNA-protein cross-links.

5.3 DNA Strand Breaks and Alkaline-Labile Sites

The formation of strand breaks has been consistently observed in cultured mammalian cells upon treatment with chromium (65,68,69). DNA breakage was observed in normal human fibroblasts and in excision-deficient xeroderma pigmentosum cells after treatment for 4 h with 0.5 μM potassium chromate, indicating that the breaks observed were a result of the chromate treatment and not a consequence of the DNA repair mechanism (70). However, this type of DNA lesion is repaired very efficiently and may not contribute to chromium mutagenicity. Single-strand breaks induced in human diploid fibroblasts and in cultured chick embryo hepatocytes exposed to nontoxic doses of chromium(VI) were no longer observed 2 h after the removal of the metal from the media (65,68).

Evidence for the formation of alkaline-labile sites was obtained from alkaline elution studies using cultured mammalian cells exposed to chromate. Christie et al. (71) and Cantoni and Costa (72) observed that DNA from treated cells eluted with increasing rates at alkaline pH. The shape of the resulting elution curves differed markedly from those usually found with agents known to cause ''frank'' single-strand breaks. This elution pattern was attributed to the formation of alkaline-labile sites, a lesion that under the conditions applied during alkaline elution ultimately results in the induction of DNA breaks. Casadevall and Kortenkamp (73,74) showed that the alkaline-labile sites observed were very likely due to the presence of DNA abasic sites formed during the reduction of chromium(VI) (see below).

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5.4 Chromium-DNA Adducts

Chromium-DNA adducts were initially detected in liver cells of chick embryos after in vivo treatment with chromate. These adducts have been proposed to mediate the formation of interstrand cross-links and protein cross-links in liver cells of chick embryos. No DNA adducts or cross-links were detected in red blood cells of chick embryos upon in vivo exposure to chromium(VI) (75,76).

Extensive chromium-DNA binding was observed upon treatment of cultured mammalian cells with chromate for 2 h (77–81). The observation that bound chromium was extractable using the chelator EDTA suggested that chromium was bound to DNA mainly as chromium(III) (77). The majority of the chromium adducts in CHO-treated cells were detected in the nuclear matrix subfraction of the chromatin, in which a number of essential nuclear processes, including replication and transcription, take place. These adducts were very persistent, and they were observed even 48 h after the chromium(VI) treatment (78). Costa and co-workers have shown that as much as 50% of the DNA-bound chromium was cross-linked to glutathione or free amino acids. Cysteine, glutamic acid, and histidine were the major amino acids bound to DNA. Again, these cross-links dissociated in the presence EDTA, suggesting that GSH and amino acids are bound to DNA via a coordination complex involving chromium(III). There was no correlation between the intracellular levels of amino acids and their participation in cross-link formation, pointing to specific chemical reactions as being the cause of these lesions (80).

5.5 DNA-Protein Cross-Links

Cultured chick embryo hepatocytes exposed to sodium chromate for 2 h showed persistent DNA-protein cross-links, which were detectable even 40 h after the removal of chromate. In contrast, DNA interstrand cross-links and single-strand breaks were completely repaired after 12 and 3 h, respectively (68). Work by Costa and co-workers was important in establishing the nature of the proteins cross-linked to DNA after exposure of cells to chromium(VI). Using two-dimensional gel electrophoresis and immunoblotting, a protein with characteristics similar to actin was identified as one of the major constituents of chromium(VI)-induced cross-links. Another as-yet-unidentified acidic protein of 95 kDa was found to be complexed to DNA. The DNA-protein cross-links could be dissociated by 2-mercaptoethanol or EDTA, indicating that chromium(III) forms an integral part of these complexes. Interestingly, histone proteins were not cross-linked to DNA by chromium(VI) (82–84).

5.6 Chromium-DNA Interstrand Cross-Links

Wetterhahn and co-workers were the first to report the formation of DNA interstrand-cross links in rat kidney, liver, and lung and in chick embryo liver upon

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treatment of the animals with sodium chromate (61,75). However, this type of lesion had not been detected in chromate-treated mammalian cells using the alkaline elution technique, which is routinely employed to analyze breaks, alkaline-labile sites, and protein cross-links in mammalian cells. Recently, Patierno and co-workers have shown the presence of DNA-DNA interstrand cross-links in human lung cell fibroblasts treated with chromate using renaturing agarose gel electrophoresis (79). Further experiments (85) established that under the experimental conditions usually used during alkaline elution, chromium-DNA interstrand cross-links were disrupted. This observation may explain why the lesion has gone undetected in various studies. Chromium(III)-monoadducts are the precursor lesion of interstrand cross-links, where chromium(III) acts as a bridge between the two DNA strands, linking either two phosphate groups or DNA bases.

5.7 Mechanism of Formation of Chromium-Induced DNA Lesions

In vitro studies have been essential in providing insights into the types of DNA lesions formed after the reduction of chromium(VI) by various intracellular constituents and in establishing which reactive intermediate species may be involved in their formation.

5.7.1 Single-Strand Breaks and Abasic Sites: Reactive Species Derived from Chromium(VI)/Glutathione and Chromium(VI)/Ascorbate

The reduction of chromium(VI) by glutathione leads to the formation of a variety of reactive intermediates, including chromium(V) (86), chromium(IV) (87), and glutathione thiyl radicals (88). Chromium(V) species have also been detected in chromium(VI)/ascorbate mixtures alongside the ascorbate radical anion (89). Recently, chromium(IV) species as well as carbon-based free radicals were identified as intermediates in these reactions (90).

Kortenkamp et al. (91) have demonstrated that chromate and glutathione have the potential to generate intermediates that cleave isolated DNA. The effective concentrations of GSH crucially affect the ability of solutions of chromium(VI) and glutathione to cause DNA strand breaks. With constant levels of chromium(VI), the number of single-strand breaks initially increased with rising levels of GSH but started to decline again when the ratio of GSH : chromium(VI) exceeded 10 : 1 (91). At high levels of GSH (10–20 mM) strand breaks failed to occur even in the presence of relatively high concentrations of chromium(VI) (up to 2 mM) (87,92). Cruz Fresco and Kortenkamp (93) as well as Stearns and Wetterhahn (90) showed that DNA cleaving species also arise from chromate and AsA with an optimum number of breaks at a 1 : 1 molar ratio of chromium(VI)

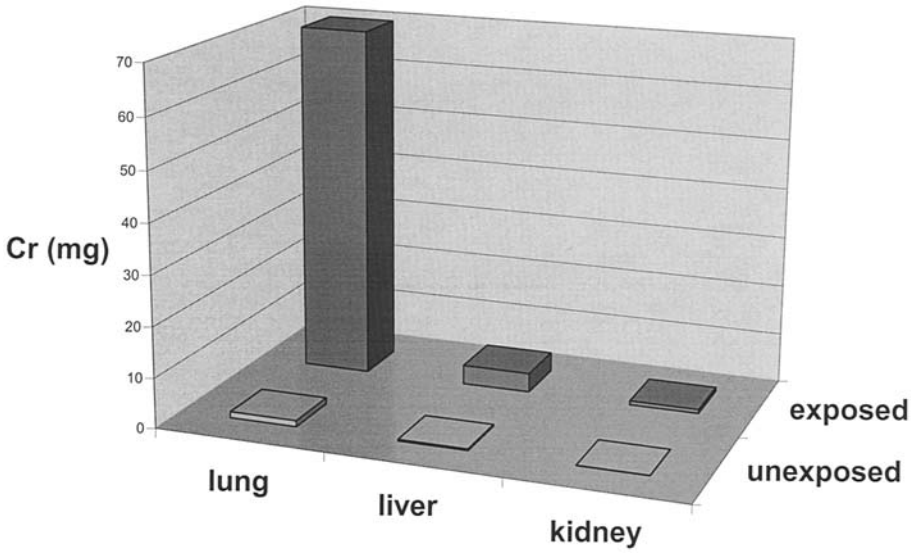

FIGURE 1 Chromium organ loads in chromium-exposed workers and unexposed subjects. Data are from lung cancer patients with history of employment in Japanese chromium(VI) production facilities (reference 25). Unexposed subjects were defined as those without occupational chromium exposure. Chromium organ loads were calculated from the original data reported by Kishi et al. (reference 25) by assuming average organ weights. It can be seen that the lung is by far the most important target organ for chromium deposition after inhalative exposure. This applies not only to chromium production workers. A similar picture has emerged from studies involving stainless steel welders, although their lungs show lower chromium loads than those found in Japanese workers (see Table 1).

to ascorbate. Taken together, these results suggest that depletion of glutathione and ascorbate stores in exposed cells may promote the formation of oxidative DNA damage by chromium(VI).

Casadevall and Kortenkamp (73,74) and da Cruz Fresco et al. (94) have explored the induction of abasic sites by the chromate/glutathione and chromate/ascorbate under experimental conditions similar to those that gave rise to the formation of DNA breaks. The formation of DNA breaks and abasic sites could be suppressed by adding catalase and was dependent on the presence of molecular oxygen. Both lesions, strand breaks and abasic sites, were formed by the same highly oxidizing reactive species. Hydrogen abstraction from C4′ of the deoxyribose moiety seems to trigger the events that lead to strand breaks and abasic

sites (95,96). Similar observations were reported by Sugden and Wetterhahn, who employed the chromium(V) complex $[Cr(V)O(ehba)_2]^-$ to mimic chromium(V) formation in situ by reducing agents in the cytosol (97,98).

The possible involvement of hydroxyl radicals in the formation of single-strand breaks has been extensively studied using various approaches (92,99). There is now sufficient evidence to rule out hydroxyl radicals as the species causing single-strand breaks or abasic sites during the reduction of chromate by glutathione or ascorbate (87,90,95,99,100).

Even so, reactive oxygen species (other than hydroxyl radicals) do play a role in the formation of DNA lesions in reaction mixtures containing chromium(VI) and reducing agents. Snyder (65) observed that strand breaks failed to occur in human diploid fibroblasts treated with chromium(VI) in the presence of catalase DNA. Superoxide dismutase exhibited a less pronounced protective effect, and hydroxyl radical scavengers such as manitol or potassium iodide did not affect the number of single-strand breaks induced by chromium(VI). Sugden et al. (57) published the results of a crucial experiment in which the mutation frequency in strains of *Salmonella typhimurium* exposed to chromium(VI) was determined under anaerobic and aerobic conditions. Chromium(VI) was able to induce reversions in strain TA102 only in the presence of oxygen, thus implicating molecular oxygen in the formation of the reactive species responsible for DNA damage. Observations by Kortenkamp and his co-workers ruled out chromium(V) intermediates as being able to cause DNA strand breaks directly, unless activated by molecular oxygen (95).

The ability of chromium(VI) and glutathione or ascorbate to form strand breaks and abasic sites seems to be the result of complex interactions between chromium in higher oxidation states and molecular oxygen. Lefebvre and Pézerat (101) have formulated a concept implying electrophiles arising from the activation of oxygen by chromate/ascorbate as the species causing primary DNA damage. Their idea is based on the observation that a formate-oxidizing intermediate produced by chromate/ascorbate could be prevented from occurring by the exclusion of molecular oxygen or the addition of catalase. Chromium(V) still appeared in the absence of oxygen, once again suggesting that chromium(V) or a chromium(V) ascorbate complex alone does not participate in the oxidation of formate. Lefebvre and Pézerat suspected a chromium(V)superoxo complex as the causative species in their system.

5.7.2 The Involvement of GSH and AsA in the Formation of Chromium-DNA Adducts

A series of studies by the Wetterhahn and co-workers (100,102,103) have shown that chromium-DNA adducts can be formed in isolated DNA during the reduction of chromium(VI) by glutathione. A 1:1 complex of chromium and glutathione with DNA was strongly resistant against removal by chelating agents, good evi-

dence for a chromium(III)-mediated DNA-peptide cross-link. Cross-links of this kind were recently observed in cultured cells treated with chromium(VI) (80). Ascorbate was also shown to be able to cause chromium-DNA adducts upon reaction with chromium(VI), but unlike glutathione, ascorbate was not cross-linked to DNA (104).

Attempts were made to identify the reactive species mediating the formation of chromium-DNA adducts in these systems. Based on correlations between

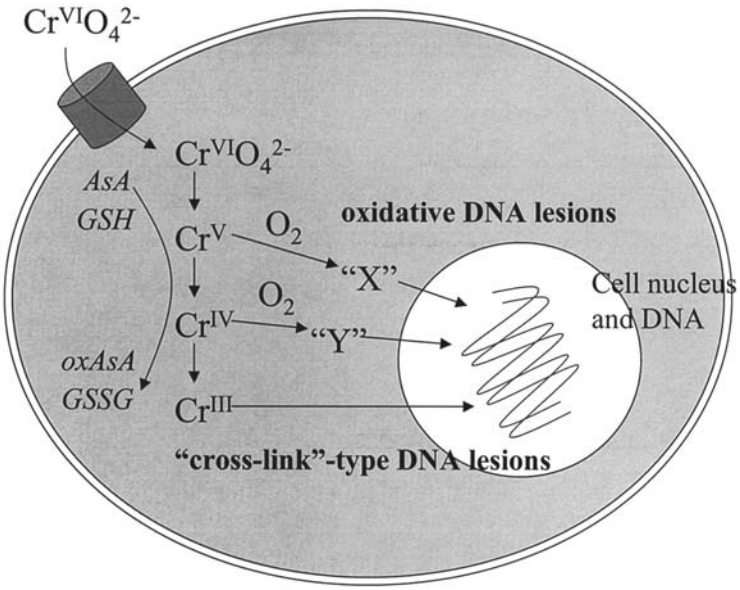

FIGURE 2 An extension and update of the uptake-reduction model originally proposed by Karen Wetterhahn (references 40, 46). The chromate anion enters cells through sulfate anion carriers. Once inside the cell, it is reduced by ascorbate (AsA), glutathione (GSH) and a number of other reductants (see text). These processes generate a number of highly reactive intermediate chromium species, including oxidation states V and IV. Ultimately, all chromium(VI) that has entered cells is converted to chromium(III). Crucially, chromium(VI) is unable to cause DNA damage. Some poorly characterized, highly oxidising species arising from chromium(V) and/or chromium(IV) are thought to cause DNA strand breaks and AP-sites (''oxidative DNA damage''). These oxidation states may also be involved in the formation of cross-link type DNA lesions (intra- and inter-strand DNA cross-links, DNA-protein cross-links). The chromium(III) species evolving from the reduction of chromium(VI) may directly contribute to cross-link-type DNA damage.

the level of chromium(V) intermediates and the number of chromium-DNA adducts, Borges and Wetterhahn (102) and Aiyar et al. (88) concluded that chromium-DNA binding is mediated by a chromium(V) species and ruled out a role for chromium(III) complexes in this process. In their studies of chromium(VI)/ascorbate systems, chromium(III) complexes were shown not to be involved in chromium-DNA binding. Upon addition of DNA to solutions of preconditioned chromium(VI)/ascorbate no chromium binding to DNA was observed, firmly ruling out chromium(III) as the species reacting with DNA, and pointing to an intermediate, possibly chromium(V) or chromium(IV), as being responsible for the formation of chromium-DNA adducts (104). However, in the case of glutathione, the evidence presented so far does not clearly rule out chromium(III) species as possibly involved in the formation of DNA-chromium cross-links.

To identify the nature of the molecular binding site of chromium adducts on DNA has been the subject of much work. In view of the more pronounced binding of chromium to guanine-cytosine-rich polynucleotides relative to polynucleotides of differing composition, Borges and Wetterhahn (102) suggested DNA bases, and in particular guanine, as the likely site of DNA-chromium-glutathione cross-links. Similar inferences were made for ascorbate in view of higher levels of binding to single-stranded than to double-stranded DNA (104). The results of NMR studies with ATP, chromium(III) complexes, and chromium(VI)/GSH have provided evidence for secondary sphere interactions of chromium(III) species with phosphate groups (105). This, however, did not rule out the possibility of binding to DNA bases. Crucially, Salnikow et al. (77) found that there was no chromium associated with the bases of DNA isolated from cells treated with chromium(VI), again pointing to DNA phosphate groups as the likely binding site of DNA-chromium cross-links arising from the reduction of chromium(VI) in cells.

In summary, the research into the mechanisms underlying the ability of chromium(VI) upon reduction to form DNA lesions has yielded the following insights: The formation of oxidative DNA damage, i.e., strand breaks and abasic sites, is the result of complex interactions of intermediate chromium oxidation states (V and/or IV), produced during reductive conversions, with molecular oxygen. Neither hydroxyl radicals nor chromium(III) complexes, the final products of intracellular chromium(VI) reduction, are involved.

On the other hand, cross-link-type lesions can arise from intermediate chromium oxidation states (V and/or IV) without the activation of molecular oxygen. The involvement of chromium(III) species in forming cross-links is worth serious consideration and has already been demonstrated with synthetic chromium(III) complexes. Complexes of chromium(III) are present at massive concentrations inside cells that have come into contact with chromium(VI), and their slow hydrolysis may present considerable hazards. However, to decide the question as to the extent of genotoxic risks it is important to consider which of the multiple

lesions caused by reducing chromium(VI) are likely to play a role in the causation of gene mutations.

5.8 The Mutagenicity of DNA Lesions Induced During the Reductive Conversion of Chromium(VI)

Despite extensive work on the mechanisms of formation of DNA lesions by chromium, very little is known about the relative mutagenicity of the various forms of DNA damage seen in chromium(VI)-exposed cells. A number of recent papers have begun to address this point, which is of great importance in biomonitoring strategies.

Snow (106) was able to demonstrate that chromium(III) present at low concentrations (5 μM) acts as a polymerase cofactor in the absence of magne-

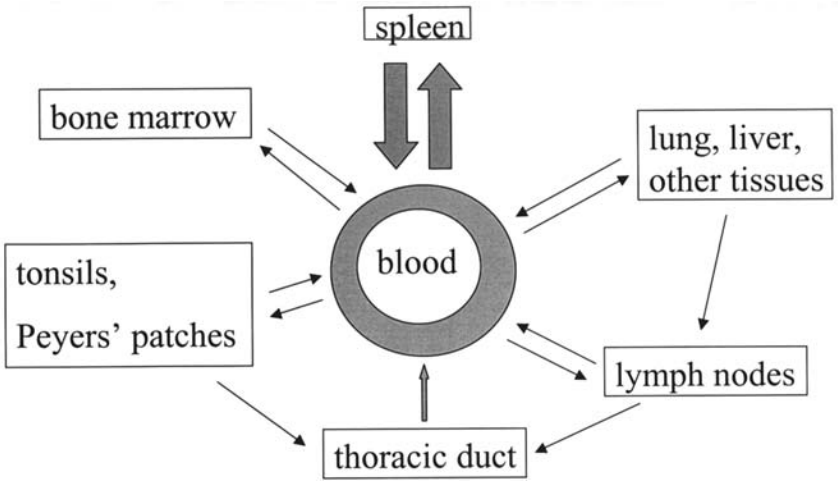

FIGURE 3 Lymphocyte traffic and the fundamental dilemma in chromium biomonitoring (graph modified from Westermann and Pabst, reference 139). Lymphocytes are produced in a multitude of organs, and are constantly released to the blood. Crucially however, the recirculation of lymphocytes from the lung back to the blood is negligible. This poses a fundamental problem for conventional biomonitoring strategies that rely on peripheral lymphocytes. These cells will have come into contact with chromium while residing in the blood, and are not representative of the processes giving rise to malignant transformation of lung cells. Thus, the patterns of DNA damage and mutations seen in lymphocytes bear no relation to the damage in lung cells. For purposes of chromium biomonitoring, lymphocytes are unsuitable, and cannot be viewed as surrogate tissue for the lung.

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sium, the primary metal cofactor for these enzymes. The presence of chromium(III) increased polymerase processivity in single-stranded DNA with a concomitant decrease in DNA replication fidelity. Alterations in DNA polymerase function also increased the rate of bypass of oxidative DNA damage induced in single-stranded DNA by $KMnO_4$. Higher concentrations of chromium(III) and/or the use of different polymerases led to inhibitions of the initiation and extension steps of DNA replication.

In work along similar lines, Patierno and co-workers (107,108) have shown that in vitro treatment of template DNA with either chromium(III) or chromium(VI)/ascorbate resulted in dose-dependent polymerase arrest. The specific pattern of arrests, one base prior to guanine residues, was similar in both systems, suggesting that the same lesion was responsible for the obstruction of DNA replication. Measurement of the chromium bound to DNA in relation to the number of arrests showed that only a small fraction of the adducts (18.5%) was involved. The authors suggested that DNA interstrand cross-links that were detected in both systems were very likely responsible for these effects. Experiments with different ratios of chromium(VI):ascorbate led to a decrease in polymerase arrest and cross-links but not in total Cr binding, further substantiating the idea that DNA cross-links are the polymerase-arresting lesion. These observations prompted further studies with the use of a more relevant system, normal human lung fibroblasts (79). Treatment of cultured cells with sodium chromate again resulted in polymerase arrest. Consistent with in vitro data, only guanine bases were involved. The results strongly indicated that DNA cross-links were the lesions responsible for blocking DNA replication; however, the involvement of DNA strand breaks and DNA-protein cross-links cannot be ruled out. The role of DNA cross-links in chromium carcinogenicity is as yet unknown.

Zhitkovich and colleagues (109) specifically investigated the promutagenicity of chromium(III)-mediated cross-links of glutathione and amino acids with DNA. To obtain the specific adducts a plasmid containing the target gene, *supF*, was treated with chromium chloride hexahydrate and glutathione or amino acids. Mutational frequencies due to the chromium(III) adducts could then be analyzed after replication of the plasmid in human fibroblasts. Chromium(III)-DNA cross-links with glutathione, cysteine, and histidine were found to be mutagenic, with chromium(III)-glutathione exhibiting the highest mutagenic potential. Binary chromium(III)-DNA adducts of the type likely to form interstrand cross-links were found to be only weakly mutagenic. Single-base substitutions were the predominant mutation, with substitutions at G: base pair accounting for $>95\%$ of all point mutations.

Kortenkamp and co-workers have begun to explore the mutagenic potential of DNA-damaging species formed in situ in solution of chromium(VI) and with AsA. The plasmid pUC19 was used for such purposes. It carries a fragment of the *lac Z* gene of *Escherichia coli* and also contains the ampicillin-resistance gene.

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The plasmid was treated with chromium(VI) and ascorbate and then used to transform *E. coli* TG1. Subsequent cultivation of the transformed cells on selective medium allowed the screening for clones harboring plasmids with nonfunctional *lac z* gene fragments (''mutants''). The treatment of pUC19 with chromium(VI)/ascorbate under conditions that caused single-strand breaks and abasic sites increased the prevalence of ''mutant,'' ampicillin-resistant, *lac Z*–deficient clones (Amp^r lac Z^-). The use of SOS-induced cultures of *E. coli* TG1 caused even higher mutation frequencies, as would be expected. These findings demonstrate that the in vitro modification of pUC19 with chromium(VI)/ascorbate inactivates the *lac Z* gene segment on the plasmid and provide good evidence that the DNA lesions caused in this system have the potential to induce gene mutations (95).

In view of the known mutagenicity of abasic sites (110) it is tempting to attribute the increased prevalence of ''mutant'' ampicillin-resistant, *lac Z*–deficient *E. coli* clones (Amp^r lac Z^-), to the formation of this type of DNA lesion. It is likely, however, that chromium-DNA adducts that are formed by chromium(VI)/ascorbate (104) are an additional cause of mutations.

Liu and Dixon used a shuttle vector construct to show that lesions induced by chromium(VI)/glutathione are mutagenic (111). Comparative mutagenesis studies, in which the same vector was transfected untreated to monkey kidney cells before treatment with chromate, showed no significance differences in the distribution of mutations (112). Mutations were induced by chromium(VI) in a dose-dependent manner and were widely distributed over the gene, primarily at G: base pairs. However, significant differences were observed when cells transfected with untreated plasmid were treated with H_2O_2 instead, indicating that the DNA lesions induced by the two systems are of different nature. Their results are in agreement with those of Chen and Thilly (59), who reported that the mutational spectrum induced by chromium(VI) on exon 3 of the *hprt* gene from human lymphoblasts differed markedly from that observed with hydrogen peroxide, molecular oxygen, iron(II) (113), or copper(I)/(II) (114)—all thought to be dependent on hydroxyl radicals or activated oxygen species. In discussing their results, Chen and Thilly concluded: ''Whatever the merits of the hypothesis that chromium(VI) operates via free radical generation or a chromium-DNA adduct, it seems clear it does not share the pathway of either hydrogen peroxide and oxygen or X-ray mutagenesis all of which have been suggested to proceed via oxygen free radical intermediates.''

In summary, the limited evidence available today suggests that cross-links of the monoadduct type are mutagenic, as are oxidative lesions such as abasic sites. As would be expected, interstrand cross-links primarily cause polymerase arrest, with subsequent cell death. They probably have a relatively low mutagenic potential.

Further progress in this field is hampered by the wide variety of different DNA lesions that are formed by chromium(VI) upon reduction. Another compli-

cation is the fact that the precise binding sites of chromium adducts with DNA are ill-defined. If these adducts were linked to DNA bases, strong mutagenicity is likely. Whether DNA adducts coordinated via DNA phosphate groups are mutagenic depends on their propensity to induce large-scale DNA helix distortions.

6. THE BIOLOGICAL MONITORING OF CHROMIUM(VI)-EXPOSED SUBJECTS

During the last two decades analytical techniques have become available that allow the monitoring of chromium(VI) in body fluids. Today, exposure to chromium(VI) at levels well below 50 $\mu g/m^3$ (the occupational exposure limit in a number of countries) can be reliably verified by determining chromium concentrations in urine or plasma. In contrast, experiences with cytogenetic surveillance techniques have been mixed. Studies undertaken in the early 1980s with relatively heavily exposed workers have produced evidence of chromosome damage, but the outcome of many of the more recent studies was inconclusive or negative. In this section we will review the state of the art of techniques for chromium(VI) biomonitoring and explore strengths and limitations of current approaches.

6.1 A Conceptual Framework for Carcinogen Biomonitoring

The various approaches and techniques in carcinogen biomonitoring can be conveniently grouped in terms of key stages and events in neoplasia. The aim is to measure parameters that reflect early events in the disease process (115). Thus, techniques of biomonitoring focus on analyses of internalized carcinogen doses, monitoring of biologically effective doses, and determinations of early biological effects.

To exert their effects, carcinogenic agents have to enter the bodies of exposed subjects. Depending on their biological availability they reach systemic circulation and are then distributed to other tissues. Measurements of the level of carcinogens in body fluids such as blood or urine can give indications of the *internalized dose*, i.e., the amount of the agent or its metabolites that has reached the inside of the body. However, not all of an internalized dose is available to reach individual target cells. Techniques for the measurement of DNA damage in appropriate cells are helpful in estimating the *biologically effective dose of an agent*, or the amount that has reached the biological target thought to be relevant to carcinogenesis, DNA. The tissues that give rise to neoplasia are usually inaccessible for biomonitoring. In such cases, determinations of DNA damage are performed by using surrogate tissues such as peripheral lymphocytes or leukocytes in the blood.

The DNA damage sustained by biologically effective doses of a genotoxic

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carcinogen can be modulated substantially by DNA repair processes. Some damage will eventually become fixed as gene mutations or as chromosome mutations that manifest themselves as microscopically observable aberrations. All these phenomena are viewed as *early biological effects* in the process of carcinogenesis. The later stages of carcinogenesis, namely the processes that give rise to the proliferation and clonal expansion of dormant tumor cells, are at present difficult to monitor.

In chromium biomonitoring, analyses of internalized doses and determinations of DNA damage have all played prominent roles in the screening of occupationally exposed subjects.

6.2 Determinations of Chromium in Blood and Urine as Indicators of Internalized Doses

The advances in metal trace analysis in the 1970s and 1980s have made it possible to establish the relationships between airborne chromium levels at workplaces and the concentration of the metal in urine, plasma, and blood of exposed workers. Urinary chromium is a sensitive and reliable marker of internal exposure to chromium, suitable for the biological monitoring of exposures well below the occupational exposure limits in most industrialized countries. Today, the analytical limit of detection is 0.2 μg/L urine (or 0.13 mg Cr/mg creatinine), approximately a tenth of the upper background level in occupationally unexposed people (2 μg/L urine) (116).

Determinations of chromium in erythrocytes make it possible to distinguish whether exposure to chromium(VI) or chromium(III) has occurred. This is because the chromate anion is able to cross cell membranes readily via anion carrier systems. In contrast, chromium(III) complexes cannot reach the inside of erythrocytes (37). It is therefore not detected in red blood cells if the donor was exposed to chromium(III). Elevated levels of chromium in plasma or urine, however, are indicative of exposure to both chromium(III) and chromium(VI).

The work by Angerer and his colleagues was important in establishing that the diagnostic specificity that measurements of chromium in erythrocytes offer with respect to ascertaining chromium(VI) exposure can only be achieved at the expense of diagnostic sensitivity. In their study (117), elevated concentrations of chromium in erythrocytes were only seen at relatively high levels of airborne chromium(VI). In contrast, urinary and plasma chromium concentrations above background levels were observed at considerably lower exposure levels. Its superior sensitivity has made measurements of chromium in urine the method of choice for the monitoring of internal chromium exposure. It offers the additional advantage of being a more convenient, noninvasive sampling method than the collection of blood.

Variations in renal function have an influence on urinary chromium levels.

TABLE 2 Urinary Chromium Levels Among Occupationally Exposed and Nonexposed Individuals

Mean Cr(VI) in air (μg/m³)	Group	Mean urinary Cr (μg/g crea)	Ref.
13.8	SS, smokers	14.2	119
13.5	SS, nonsmokers	5.3	
1–55	Cr(VI) production	6	124
0	Nonexposed	0.76	
0	Diabetics	1.38	120
	Referents	0.58	
0	Beer drinkers	0.67	120
	Referents	0.49	

SS: stainless steel welders (MMA).

Such variations are easily corrected for by relating urinary chromium concentrations to the amount of creatinine present in the urine (118). Other factors that have to be taken into consideration during the design of monitoring programmes include the effect of smoking as a confounding factor. Kalliomäki et al. (6) and Strindsklev et al. (119) have found that urinary chromium levels in stainless steel welders who smoke can be two to three times higher than those of their nonsmoking colleagues (Table 2).

Strindsklev et al. (119) have carried out an extensive monitoring program among MMA stainless steel welders aimed at documenting daily variations of chromium concentrations in plasma, urine, and erythrocytes among welders. They found that during a working week in an atmosphere with chromium(VI) concentrations in the range of 10–34 μg/m³ the mean daily increase was 1.0 μg Cr/L in plasma and 5.6 μg Cr/g creatinine in urine. It is interesting to note that no such correlations between exposure and internal dose were observed for nickel.

Urinary chromium analyses are sufficiently sensitive to detect even differences in drinking habits and exercise. Bukowski and his colleagues (120) were able to demonstrate that drinking beer, lack of exercise, and diabetes all lead to elevated levels of chromium in urine. Thus, when monitoring weakly exposed subjects it becomes necessary to correct for these factors.

6.3 Determinations of DNA Damage in Lymphocytes as Measures of Biologically Effective Doses of Chromium(VI)

The assessment of DNA damage as a method of biomonitoring has not been widely used in the chromium field, with only a few studies published to date.

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Almost exclusively, peripheral lymphocytes are employed as a readily accessible tissue providing nucleated cells for the analysis of DNA damage.

One of the first studies of this kind was published by Popp and colleagues (121). As part of a cytogenetic survey among stainless steel workers (see below), DNA from blood lymphocytes was analyzed for cross-link-type DNA damage by using filter elution techniques. The samples obtained from welders showed reduced elution rates, which the authors interpreted as being indicative of DNA-protein cross-links (DPC). There were no indications of DNA strand breaks. However, in a more recent study (122), the same group of researchers found significantly elevated DNA strand breaks and additional DPC in the lymphocytes of welders. Elevated chromium levels in erythrocytes confirmed that exposure to chromium(VI) (and nickel) had occurred. The authors explained the different outcome of the two studies in terms of higher exposure to chromium(VI) among the more recent cohort.

Zhitkovich et al. (123) have examined Bulgarian platers for evidence of DNA damage in white blood cells. To monitor DPC they employed a potassium-SDS precipitation assay, which provides more direct evidence for the existence of this type of DNA damage than filter elution techniques. Although elevated concentrations of chromium in the red blood cells and urine of the platers confirmed that exposure to chromium(VI) had occurred, they failed to observe significant differences in DPC levels relative to controls not engaged in plating.

Gao et al. (124) chose to study a population of chromium(VI) production workers and assessed oxidative DNA damage by monitoring the number of single-strand breaks and modified DNA bases (8-hydroxydeoxyguanosine) in lymphocyte DNA. There was no evidence for increased levels of oxidative DNA damage, although the workers' exposure to chromium could be verified using markers of internal exposure.

In parallel with measurements of DNA damage, Gao et al. (124) and Zhitkovitch et al. (123) have determined chromium levels in blood lymphocytes. The striking observation in both cases was that the differences in lymphocyte chromium levels between exposed individuals and controls were not very pronounced, when at the same time all the other measures of internal exposure were markedly elevated in relation to controls. The lymphocyte chromium levels in exposed individuals were approximately twice as high relative to controls, while the concentrations in erythrocytes were 7–9 times higher. Expressed as amount of chromium per cell, the lymphocytes in both exposed and unexposed persons showed levels that were about 2 orders of magnitude higher than those found in erythrocytes (1.01 $\mu g/10^{10}$ lymphocytes vs. 0.01 $\mu g/10^{10}$ erythrocytes in exposed and 0.76 $\mu g/10^{10}$ lymphocytes vs. 0.002 $\mu g/10^{10}$ erythrocytes in unexposed), using the data of Gao et al. (124).

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6.4 Cytogenetic Surveillance Studies Among Exposed Subjects: The Monitoring of Early Biological Effects

The first studies looking for chromosome damage in the peripheral lymphocytes of chromium(VI)-exposed workers were carried out in the 1970s. Bigaliev et al. (125) have analyzed the number of chromosome aberrations in blood lymphocytes of 132 chromium(VI) production workers and found significant increases (3.6–9% aberrant metaphases) relative to 37 unexposed controls (1.88% ± 0.74%). In a surveillance study among chromium platers Stella et al. (126) observed increased SCE frequencies with levels of 8.08 ± 2.67 per cell versus 6.31 ± 1.56 in controls.

The focus of this early work was on cytogenetics and consequently no attempts were made to relate chromosome damage to internal chromium exposure or even concentrations of the metal compound in the workplace air. In contrast, the majority of subsequent studies in the 1980s and 1990s provide detailed information about measures of external and internal exposure to chromium, thus enabling us to evaluate the relative sensitivity of markers of early biological effects and of internal exposure in a variety of occupational settings.

The results of such a meta-analysis (127) of studies carried out among chromium platers and ferrochromium workers (128–132) were surprising. Although exposure to chromium could be readily ascertained (elevated urinary chromium levels) the numbers of chromosome aberrations or SCE's in exposed subjects did not differ from those in unexposed controls. Overall, the values of SCE's observed in these studies (5–9 SCEs per cell) were well within the range of SCEs reported for control populations (8.12 ± 1.82 SCEs per cell) (133). Age and smoking status had a strong influence on the level of SCEs.

The cytogenetic studies carried out among stainless steel welders using the manual metal arc method (which, unlike the other two major welding methods, generates welding fumes particularly high in chromium(VI) particulates) showed similar trends. Similar to the surveys among platers and ferrochromium workers, urinary chromium proved to be a reliable marker of internal chromium exposure even at levels of airborne chromium(VI) below 10 $\mu g/m^3$. In contrast, the frequency of SCEs appeared to be surprisingly insensitive to exposure to chromium(VI). All but one of the studies (134) failed to detect elevated numbers of SCEs in the exposed groups (121,135–138). In the surveys published after 1990 there was a marked tendency for SCEs to be slightly lower in manual metal arc welders than in unexposed controls.

A different picture emerges when chromosome aberrations are considered. Koshi et al. (134), Knudsen et al. (137), and Jelmert et al. (138) observed statistically significant increases of rare chromosome aberrations such as dicentric chromosomes, translocations, minutes, and rings. Importantly, these effects were only

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seen among nonsmoking manual metal arc welders. Knudsen et al. (137) have emphasized that such aberrations are severe genetic effects that require two independent damaging events to occur.

Overall, the results of these investigations clearly show that there is evidence for genotoxic effects among stainless steel welders using the manual metal arc method. It is, however, important to interpret these data in the context of other cytogenetic surveillance studies. The frequencies of aberrations detected among welders are similar to those observed in unexposed controls of many studies, which is about $1.42 \pm 0.96\%$ of aberrant cells (excluding gaps) (133).

In view of the strong genotoxicity of chromium(VI) compounds it is surprising that the effect markers in cytogenetic surveillance studies responded so weakly. One possible explanation would be that the study cohorts were exposed to relatively low levels of chromium(VI) and that the resultant genotoxic effects were small. Alternatively, are blood lymphocytes appropriate as a surrogate tissue for the monitoring of genetic effects of chromium(VI) in humans?

To clarify whether it makes sense to use blood lymphocytes for chromium(VI) effect monitoring it is necessary to reconsider the toxicokinetics of chromium(VI) after exposure by inhalation. In view of the fact that the bulk of inhaled chromium(VI) stays in the lungs, we can envisage two ways for lymphocytes to come into contact with chromium(VI) following exposure by inhalation: (1) lymphocytes, while traveling via the blood, take up chromium(VI) from the plasma that has leached from the lungs; (2) lymphocytes homing to the supporting tissues of the lungs are exposed to chromium before migrating back to the bloodstream.

6.5 Lymphocyte Traffic

Lymphocytes continuously enter and leave lymphoid and nonlymphoid tissues via the blood. Consequently, only about 2% of the total lymphocyte pool in the human body resides in the blood at any given time, with an estimated residence time of about 30 min (139). During 1 day approximately 500×10^9 lymphocytes travel through the blood, a number equivalent to the total lymphocyte population in the human body. However, not every single lymphocyte enters and leaves the blood during 1 day. There are subsets of the lymphocyte population that reside in certain tissues for long times without migrating through the blood. Lymphocytes in the blood are therefore hardly representative of the other lymphocytes distributed in the body.

Lymphocytes migrate through most of the organs of the body and have the ability to return to the blood. A well-established pathway of recirculation from organs to the blood is via the lymph nodes and the thoracic duct, accounting for approximately 5–10% of the lymphocyte population that returns to the blood

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each day. The spleen is by far the most important organ in lymphocyte recirculation, with 50% of all the recirculated lymphocytes being released by the spleen. In comparison, the daily recirculation of lymphocytes from the lungs back to the blood is negligible (139).

Thus, only a very small fraction of the lymphocytes residing in the blood will have had the opportunity to take up chromium(VI) while traveling through the lungs. The short blood transit time of lymphocytes is likely to be another complicating factor in the uptake of chromium(VI) by lymphocytes and might help explain the small differences in the lymphocyte chromium levels of exposed and unexposed observed by Gao et al. (124) and Zhitkovich et al. (123).

In conclusion, the vast majority of lymphocytes will have come into contact with chromium(VI) during their migration through the blood. They may take up chromium(VI) that has leached from the respiratory tract into the blood.

These features are important when considering whether chromium-induced genotoxic effects occurring in lymphocytes are in any way predictive of the processes leading to mutations and eventually cancer in the respiratory tract. In view of the toxicokinetics of chromium(VI) upon inhalation, it is likely that cells of the respiratory tract are exposed to much higher amounts of chromium(VI) than lymphocytes. It would appear that the likelihood that cancer-initiating events occur in lung cells in considerably higher. Therefore, the absence of any genotoxic effects in blood lymphocytes of exposed human subjects can by no means be taken as an indication of absence of lung cancer risks.

There is no doubt that urinary chromium is a useful marker of exposure to chromium in oxidation states (VI) and (III). At exposure levels likely to be encountered in occupational settings today it provides a sensitive measure of the internalized dose of chromium.

In contrast, the lack of success of cytogenetic surveillance studies using blood lymphocytes as surrogate tissue is surprising, particularly in view of the strong genotoxicity of chromium(VI). We consider it highly likely that the weak responses observed in these studies are due to the disproportionately small differences in lymphocyte chromium levels between exposed persons and controls. It would be of great importance to establish why the levels of chromium in lymphocytes are relatively high in unexposed controls.

The toxicokinetics of inhaled chromium(VI) and the dynamics of lymphocyte traffic probably mean that lymphocytes are exposed to lower concentrations of chromium(VI) than cells in the respiratory tract. Taken together, these considerations suggest that blood lymphocytes are not well suited for the monitoring of the biologically effective dose, and of early biological effects arising from exposure to chromium(VI) at levels below 50 $\mu g/m^3$, the current exposure limit in many industrialized countries. At higher exposure levels, however, effect monitoring using lymphocytes may well be useful.

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These rather sobering conclusions provoke the question whether there are alternative approaches to chromium biomonitoring that are more meaningful in relation to lung carcinogenesis.

6.6 Effect Monitoring in Tissues of the Upper Respiratory Tract?

The biggest challenge in chromium biomonitoring is to bridge the gap between the monitoring of internalized doses and effect monitoring. Given the problems associated with utilizing lymphocytes as a surrogate tissue, effect monitoring ideally should concentrate on cells of the airways. However, this is complicated by the difficulty of access to these tissues. A further problem lies in the small number of cells that can be obtained by biopsy or lavage.

Clearly, what is needed is a technique that allows the determination of DNA damage in single cells. The comet assay, or single-cell gel electrophoresis assay, is such a technique. It was first developed by Östling and Johanson in 1984 and later modified and refined by Singh and coworkers and by Olive and her colleagues (see ref. 140). A small number of cells are embedded in agarose on a microscope slide, lysed, electrophoresed, and then stained with a fluorescent DNA-binding dye. In the electrical field, DNA moves out of the nucleus, such that relaxed or broken regions of the genome migrate further. The objects thus created look strikingly like comets, hence the name of the assay.

Östling and Johanson worked with irradiated cells recovered from biopsy samples of patients receiving radiation therapy. They observed that the extent to which DNA could be liberated from the nucleus during electrophoresis depended on radiation dose. The more damage there was in DNA, the more DNA resided in the ''tail'' of the comet.

Virtually any eukaryotic cell can be examined using the comet assay and there are well-established methods for generating single-cell suspensions from biopsy samples. The most commonly used human cells are blood lymphocytes, but cells derived from other tissues including gastric and nasal mucosa have also been used (141). The comets are evaluated by using image analysis. A parameter often referred to as ''tail moment'' is most frequently determined. ''Tail moments'' are defined as the product of the length of the comet and the fraction of DNA residing in the tail.

The comet assay is sensitive to DNA damage such as single- and double-strand breaks and abasic sites. Strand breaks that are produced by the cell as it initiates DNA repair are also detected. Cross-link-type lesions, on the other hand, can only be analyzed indirectly. Under standardized conditions, X-rays introduce strand breaks to the DNA of cells, which subsequently will appear as comets with high tail moments. If, however, cross-links are present, the formation of comets will be reduced after pretreatment with radiation. In this way, cross-links

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formed by nitrogen mustard or cisplatin were readily detected using the comet assay (140).

It is an attractive proposition to use the comet assay for determinations of DNA damage in cells of the nasal epithelia obtained by using nasal lavage techniques. However, before commencing with such studies it would be necessary to establish in vitro whether the strand breaks and abasic sites induced by chromium(VI) are masked by concurrently formed cross-link-type lesions. Whether oxidative DNA damage or cross-links will dominate in cells exposed to chromium(VI) is likely to depend on factors such as the intracellular concentrations of glutathione and ascorbate anions, with high glutathione levels favoring cross-links (see above). Should cross-links be the major lesions, the comet assay protocols developed for cross-linking agents will have to be applied to nasal epithelial cells. The work of Pool-Zobel and her colleagues (141) and of Blasiak and colleagues (142) shows that chromium(VI) induces comets directly. It is important that human subjects with single-compound exposure, i.e., chrome platers, should be selected for initial studies.

7. EVALUATION AND REGULATORY STATUS

The Working Group on Chromium and Chromium Compounds of the International Agency for Research on Cancer has made an overall evaluation for chromium(VI) based on the concept that chromium(VI) ions generated at critical sites in target cells are responsible for the carcinogenicity observed and has classified chromium(VI) compounds in Group 1 (sufficient evidence of carcinogenicity in humans) (4).

The classifications of many national governmental bodies are in line with the evaluation of IARC. The American Conference of Governmental and Industrial Hygienists (ACGIH), U.S. EPA, and the German MAK Commission have classified chromium(VI) compounds as known human carcinogens. Poorly water-soluble chromates such as lead chromate are classed as agents justifiably suspected of having carcinogenic potential in humans.

7.1 Defining Threshold Limit Values (TLV) and Other Exposure Limits for Chromium(VI) Compounds

Although the basis for a classification of chromium(VI) compounds as carcinogens is sound, the published epidemiological and experimental data do not allow us to establish a quantitative relationship between the level of exposure and the risk of developing cancer. This is mainly due to the fact that records on exposure levels in the past are patchy or do not exist at all. Furthermore, both nature and level of exposure usually change as people move to different jobs in the same plant, making it very difficult to reconstruct past exposure profiles.

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It is therefore difficult, if not impossible, to define safe exposure limits (apart from zero) on an epidemiological basis. However, epidemiology can address the question whether existing exposure limits pose unacceptable risks. The recent survey among chromium platers published by Sorahan and colleagues (15) indicates that the current maximum exposure limit for chromium(VI) of 50 μg/m^3 in the United Kingdom is too high.

The occupational exposure limits for airborne chromium(VI) vary considerably from country to country. They range from 1 μg/m^3, the level recommended by the U.S. National Institute for Occupational Safety and Health (NIOSH) for insoluble chromium(VI) compounds as a time-weighted average, to 500 μg/m^3, which is the limit in Mexico. Most European countries have set exposure limits between 20 μg/m^3 (Denmark, Norway) and 50 μg/m^3 (United Kingdom, Finland, France) (4). These figures demonstrate that the criteria that have guided governmental bodies to decide on particular threshold limit values are varied. Toxicological considerations have certainly not always played a prominent role.

The question whether it is prudent to define threshold limits for carcinogenic agents such as chromium(VI) is an old bone of contention in regulatory toxicology. Based on the assumption that there cannot be a harmless level of exposure for carcinogenic agents, bodies such as the German MAK Commission do not define exposure limits for chromium(VI). Even though this position may not be helpful in guiding practice when it comes to negotiating improvements in occupational hygiene, it is at least on safe theoretical grounds. Whether or not there is a threshold exposure below which the risks associated with chromium(VI) are zero is almost impossible to decide. The argument that there may be a ''biological'' threshold for chromium(VI), because various tissues have the capacity to clear chromium(VI) by reduction (38), does not pay due regard to the special toxicokinetics of airborne chromium(VI). The formation of hot spots of deposition in the lung may quickly render any ''reductive barriers'' in the airways irrelevant. Furthermore, even if there were at least in principle a ''biological'' threshold for chromium(VI), toxicologists certainly lack the methodological means to determine, quantitatively and reliably, what such levels would be.

In conclusion, there is no alternative to minimizing exposure to airborne chromium(VI) to levels that approach the analytical detection limit as far as possible.

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10

Aluminum

John Savory, R. Bruce Martin, and Othman Ghribi
University of Virginia, Charlottesville, Virginia

Mary M. Herman
National Institutes of Health, Bethesda, Maryland

1. INTRODUCTION

In 1856 Charles Dickens expressed enthusiasm about the newly discovered metal aluminum (Al), but it was not until 1886 that large-scale production was introduced. Since that time the use of Al has increased enormously and has become the focus of a major industry. A few studies on Al toxicity were carried out as early as 1888, but over the years, exposure to Al has generally been considered to be a minor problem. In a report in 1957, Campbell et al. expressed few concerns about hazards to human health presented by Al (1). The extensive literature that formed the basis of this report was published prior to the development of reliable analytical methods for the measurement of Al.

Assessment of the hazard presented by certain forms of Al exposure to humans, animals, and plants has proved to be a difficult task. Aluminum is highly abundant in the environment and represents 8% of the earth's crust, with only oxygen and silicon exceeding it in quantity, and is the most abundant metal.

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However, Al is complexed in minerals that conceal its abundance and, surprisingly, the concentration in the ocean is less than 1 μg/L. Most natural waters also have low concentrations of Al; any free Al^{3+} is deposited in sediment as a hydroxide. It is with an increase in the acidity of fresh waters that Al can potentially pose a threat to living systems. Despite the abundance of Al in the environment, it is present in relatively small amounts in healthy living systems. Normally, the total body content of healthy humans is less than 30 mg. However, in certain human clinical conditions such as chronic renal failure, hyperaluminemia can occur, producing blood concentrations of Al that are as just as neurotoxic as equimolar blood lead levels that result from excessive lead exposure.

2. ALUMINUM IN BIOLOGICAL SYSTEMS

2.1 Chemistry

Appreciation of the toxicity of Al has been hindered by a general lack of understanding of the chemical properties of this complex element. Al^{3+} is a small ion with an effective ionic radius in sixfold coordination of only 54 pm. By way of comparison, other values are Fe^{3+}, 65; Mg^{2+}, 72; Zn^{2+}, 74; and Ca^{2+}, 100 pm (2). On the basis of ionic radii, Al^{3+} is closest in size to Fe^{3+} and Mg^{2+}. High concentrations of Al colocalize with iron in brain cells (3). Ca^{2+} is much larger, and in its favored eightfold coordination exhibits a radius of 112 pm, yielding a volume 9 times that of Al^{3+}. In the mixed crystal $Ca_3Al_2(OH)_{12}$, each hexacoordinate Al^{3+} is surrounded by six hydroxide ions and each cubic Ca^{2+} by eight hydroxide ions. Each metal ion adopts its own favored coordination number. The Al-O distances are 192 pm and the average of the Ca-O distances is 250 pm (4). The difference of 58 pm agrees exactly with the difference of ionic radii quoted above between six coordinate Al^{3+} and eight coordinate Ca^{2+}. Thus, the Al^{3+} and Ca^{2+} sites are distinctly different; one metal ion does not substitute for the other. For these reasons it is unlikely that Al^{3+} binds strongly to the Ca^{2+} sites of calmodulin (5,6). With one-quarter of its amino acid residues bearing carboxylate side chains, calmodulin is an acidic protein that should bind multiply charged ions as a polyelectrolyte. When it does so, physical changes upon addition of Al^{3+} are merely those of denaturation. It is, however, likely that Al^{3+} interacts with calmodulin-regulated proteins that involve phosphate groups. By this route calmodulin-dependent reactions may exhibit an Al^{3+} dependence (5,6).

Martin has argued that in biological systems Al^{3+} will be more competitive with Mg^{2+} than with Ca^{2+} (5,7). In both mineralogy and biology, comparable ionic radii frequently outweigh charge in determining behavior. More Al^{3+} is accumulated by central nervous system tissue when the Mg^{2+} concentration is low (8). Both Al^{3+} and Mg^{2+} favor oxygen donor ligands, especially phosphate groups (9). Al^{3+} is 10^7 times more effective than Mg^{2+} in promoting polymeriza-

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tion of tubulin to microtubules (10). In this study the free Al^{3+} concentration was controlled near 10^{12} M with nitrilotriacetate (NTA). Thus, wherever there is a process involving Mg^{2+}, there exists an opportunity for interference by Al^{3+}.

The most likely Al^{3+} binding sites are oxygen atoms, especially if they are negatively charged. Carboxylate, deprotonated hydroxy groups (as in catecholates, serine, and threonine), and phosphate groups are the strongest Al^{3+} binders. These binding characteristics differ sharply from those of the heavy metal ions that bind to sulfhydryl and amine groups. Even when part of a potential chelate ring, sulfhydryl groups do not bind Al^{3+}. Amines bind Al^{3+} strongly only as part of multidentate ligand systems, as in NTA and EDTA. Amino acids are weak binders, barely competing with metal ion hydrolysis (11). The nitrogenous bases of DNA and RNA do not bind Al^{3+} strongly (5,6). The weakly basic phosphate group of RNA and DNA also binds Al^{3+} weakly (12), while the basic and chelating phosphate groups of nucleoside di- and triphosphates bind Al^{3+} strongly (13). Within cells, Al^{3+} is likely bound to nucleoside di- and triphosphates (13).

In addition to stability of metal ion complexes, an important and often overlooked feature is the rate of ligand exchange out of and into the metal ion coordination sphere. Ligand exchange rates take on special importance for Al^{3+}, because they are slow and systems may not be at equilibrium. The rate for exchange of inner-sphere water with solvent water is known for many metal ions, and the order of increasing rate constants in acidic solutions for some biologically important metal ions is as follows: $Al^{3+} \ll Fe^{3+} \lll Mg^{2+} \ll Zn^{2+} < Ca^{2+}$. Each inequality symbol indicates an approximate 10-fold increase in rate constant from 1.3 s^{-1} for Al^{3+}, increasing through 8 powers of 10 to > 108 s^{-1} for Ca^{2+} at 25°C. Although these specific rate constants refer to water exchange in aquo metal ions, they also reflect relative rates of exchange of other ligands. Chelated ligands exchange more slowly, but the order remains. The slow ligand exchange rate for Al^{3+} makes it useless as a metal ion engaged in enzyme active site reactions. The 10^5 times faster rate for Mg^{2+} furnishes enough reason for Al^{3+} inhibition of enzymes with Mg^{2+} cofactors. Processes involving rapid Ca^{2+} exchange would be thwarted by substitution of the 10^8-fold slower Al^{3+} (4). Slow exchange of Al^{3+} may be an important factor affecting the efficacy of administered Al^{3+} compounds.

Regardless of the type of ligand present, it is necessary to consider the hydrolysis equilibria of Al(III). At pH < 5, Al(III) exists as an octahedral hexahydrate, $Al(H_2O)_6^{3+}$, usually abbreviated as Al^{3+}. As a solution becomes less acidic, $Al(H_2O)_6^{3+}$ undergoes successive deprotonations to yield $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ (5,14). Neutral solutions give an $Al(OH)_3$ precipitate that redissolves, because of the formation of tetrahedral aluminate, $Al(OH)_4^{-}$, the primary soluble Al(III) species at pH > 6.2. Only two species dominate over the entire pH range, the octahedral hexahydrate $Al(H_2O)_6^{3+}$ at pH < 5.5, and the tetrahedral $Al(OH)_4^{-}$ at pH > 6.2, while there is a mixture of hydrolyzed species and coordination

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numbers between $5.5 < pH < 6.2$ (distribution curves appear in the references) (11,14,15).

If in addition other ligands are incapable of holding Al(III) in solution, it becomes necessary to include the solubility equilibrium with $Al(OH)_3$ (5,11,14). Inorganic Al(III) salts should not be added to neutral solutions in the absence of a solubilizing ligand. At pH 7.5, the maximum concentration of total Al(III) is about 8 μM, most of which is present as $Al(OH)_4^-$; the free Al^{3+} concentration is only 3×10^{-12} M. Unless the remainder of added Al(III) has been sequestered by other ligands, it will form insoluble $Al(OH)_3$ (5,6).

2.2 Al Speciation in Cerebrospinal Fluid and Brain Tissue

Citrate is the main small-molecule binder of Al^{3+} in the plasma compartment; 10% of the Al^{3+} is bound to citrate and 90% to transferrin (6,12,16). Cerebrospinal fluid contains much less transferrin than plasma, and Al speciation studies (4) indicate that most of the Al is in the form of Al citrate. The pH of cerebrospinal fluid is 7.33, with concentrations of inorganic phosphate, transferrin, citrate, and amino acids of 0.49 mM, 0.25 μM, 0.18 mM, and 1.8 mM, respectively. Compared to plasma, cerebrospinal fluid has a higher citrate concentration (1.8 times), which favors Al citrate over Al transferrin, since the transferrin concentration is about 0.5% of that in the plasma. The citrate/transferrin ratio is 2.0 in the plasma and >720 in the cerebrospinal fluid. Thus in cerebrospinal fluid Al(III) exists mainly as a citrate complex, with the free Al^{3+} concentration comparable to that in plasma (4).

2.3 Where Is Al^{3+} Most Apt to Reside Within a Cell?

Typical intracellular fluids contain about 10 mM total inorganic phosphate at pH 6.6. Analysis indicates that as for plasma and cerebrospinal fluid, the insoluble $A1PO_4$ in the presence of ligands such as transferrin and citrate, will become soluble, giving rise to a greater free Al^{3+} concentration (4).

For the purposes of metal ion binding, soluble phosphate groups may usefully be divided into two classes: basic phosphates and weak or nonbasic phosphates (12). Basic phosphates with pKa = 6–7 are monosubstituted with a 2^- charge and occur as $HOPO_3^{2-}$, as the terminal phosphate in nucleoside mono-, di-, and tri-phosphates, and in many other compounds. Weakly or nonbasic phosphates with the only $pKa < 2$ are di (or tri)-substituted, bear a 1^- charge, and appear as the internal phosphates in nucleoside di- and tri-phosphates and in DNA and RNA. Metal ions bind strongly to the basic phosphates but only weakly to the nonbasic phosphates. The disubstituted phosphates of the nucleotide polymers bear one negative charge per nucleotide residue, and the polymers behave as polyelectrolytes, binding most metal ions weakly and nonspecifically.

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Al^{3+} binds strongly to basic phosphate groups. The strongest stability constants appear where chelation occurs: for ADP ($\log K_1 = 7.82$ and $\log K_2 = 4.34$) and for ATP ($\log K_1 = 7.92$ and $\log K_2 = 4.55$) (13). For comparison, the stability constant for Mg^{2+} binding to ATP and other nucleoside triphosphates is $\log K_1 = 4.3$ (17), 4000 times weaker than for Al^{3+}. Thus, 0.2 μM Al^{3+} competes with 1 mM Mg^{2+} for ATP. Within a cell, ATP competes effectively with solid $A1PO_4$ for Al^{3+}, and the ATP complex promises to be the predominant binder for small-molecule Al^{3+}.

It has often been supposed that Al^{3+} binds to DNA in the cell nucleus. However, Al^{3+} binding to DNA is so weak that a quantitative study was limited to a high pH = 5.5 owing to metal ion hydrolysis and precipitation. Therefore, DNA cannot compete with ATP and other ligands for Al^{3+}. We deduce that Al^{3+} binding to DNA is so weak under normal intracellular conditions that it fails by several orders of magnitude to compete with either metal ion hydrolysis or insolubility of even an amorphous $Al(OH)_3$. These chemical conclusions are supported by the lack of DNA or RNA phosphate-bound Al^{3+} in human neuroblastoma cells (18). Therefore, we conclude that the observation of Al binding with nuclear chromatin is due not to its coordination to DNA but to ligands containing basic phosphates.

2.4 What Ligands Might Bind Al^{3+} in the Cell, Especially in the Nuclear Chromatin Region?

ATP and ADP are comparably strong Al^{3+} binders (13). A crucial Al^{3+} binding site in chromatin promises to be phosphorylated proteins, perhaps phosphorylated histones. Phosphorylation and dephosphorylation reactions normally accompany cellular processes. The phosphate groups of any phosphorylated protein provide the requisite basicity, and in conjunction with juxtaposed carboxylate or other phosphate groups become strong Al^{3+} binding sites. Abnormally phosphorylated proteins have been found in brain tissue from Alzheimer's disease patients (19). Al(III) induces covalent incorporation of phosphate into human microtubule-associated tau (tau) protein (20). Al^{3+} aggregates highly phosphorylated brain cytoskeletal proteins (21) and induces conformational changes in phosphorylated neurofilament peptides that are irreversible to added citrate (22). More recent studies indicate that Al can induce conformational changes in tau peptides independent of phosphorylation, suggesting that there are binding sites that possess a high affinity for Al, and that phosphorylation, while decreasing the affinity of tau to microtubules, might have little effect on conformation (23). High Al(III) concentrations have been found associated with increased linker histones in the nuclear region of brain tissue obtained from patients with Alzheimer's disease (24). Al(III) induces neurofibrillary tangles in the perikaryon of neurons (25).

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Ternary Al^{3+} complexes have received little study, and Al(III) has been used as a tanning or cross-linking reagent. Al^{3+} seems capable of cross-linking proteins, and proteins and nucleic acids.

In fluids low in citrate, transferrin, and nucleotides, the catecholamines may well become important Al^{3+} binders. While DOPA and epinephrine fail to bind Mg^{2+} at pH 7.4, they bind Al^{3+} at picomolar levels. In neutral solutions the main species is a 3:1 complex, with the catechol moiety chelating the Al^{3+} and the ammonium group remaining protonated (26). The norepinephrine-Al^{3+} complex inhibits enzymatic *O*-methylation but not *N*-methylation by catechol-*O*-methyl transferase (27). This result conforms to that expected if Al^{3+} were to bind only to the catechol moiety of norepinephrine. When other metal ions are deficient, Al(III) decreases catecholamine levels in the rat brain (28). By binding to the catechol moiety of catecholamines, trace amounts of Al^{3+} may disrupt neurochemical processes.

Signal transduction pathways, particularly inositol phosphate and cAMP-mediated signaling, appear to be targets of Al both in vivo and in vitro. Al in drinking water decreases hippocampal inositol triphosphate levels, increases cAMP, and alters the distribution of protein kinase C (29,30). In vitro exposure to Al decreases agonist-stimulated inositol phosphate accumulation in rat brain slices (31,32). The potential mechanisms of inositol phosphate inhibition have been reviewed (33). Al can also interact with calcium and calcium-binding sites and probably disrupts calcium signaling and homeostasis, and can block calcium entry into the cell via voltage-sensitive channels (32). Several groups have shown that exposure to Al produces a decrease in choline acetyl transferase activity (34,35). There are regional reductions in glucose metabolism in Alzheimer's disease (36) and also following chronic Al chloride exposure to rats (31), which suggests that this effect may be important in human neurodegeneration. These mechanisms of Al neurotoxicity have been reviewed by Strong et al. (37).

3. HUMAN EXPOSURE

3.1 Aluminum Toxicity and Chronic Renal Failure

There is considerable controversy regarding the toxicity of Al in individuals with normal renal function. However, there is no doubt about the importance of Al toxicity in patients with chronic renal failure, on treatment with hemodialysis. This topic, reviewed by us (38–40), has been the subject of intensive investigation since the original report by Alfrey and his colleagues (41), which proposed that dialysis encephalopathy, a feature of patients on long-term treatment with intermittent hemodialysis for chronic renal failure, resulted from Al intoxication. Berlyne et al. were the first to recognize that hyperaluminemia occurred in these

patients, and that Al toxicity could be demonstrated in experimental animals (42,43). Aluminum in the dialysis solution is the major source of exposure to this metal ion in patients being treated long-term with either hemo- or peritoneal dialysis. The Al content is of course dependent on the water from which it is prepared, and it was this particular source of Al that caused major clinical problems when city water treated with alum was used to produce dialysis solutions, resulting in severe Al toxicity in many dialysis patients. This phenomenon has been largely eliminated by the use of deionized water, but the problem occasionally reoccurs (44). Adding to the problem of Al contamination of dialysis solutions has been the extensive use of Al salts in the therapeutic management of the hyperphosphatemia that arises in chronic renal failure. Intestinal absorption of Al from this treatment adds to the hyperaluminemia, and consequently to the clinical complications associated with this condition in patients with impaired renal function. There is no doubt that hyperaluminemia in patients with chronic renal failure has constituted one of the major clinical problems in modern times associated with metal poisoning, and few, if any, other occurrences of iatrogenic poisoning have been more serious. Dialysis encephalopathy was in fact a fatal complication of hemodialysis treatment until Alfrey et al. elucidated the problem (41). Guidelines developed in the early 1980s for Al monitoring of both dialysis patients and water supplies (45), together with refinement of analytical methods (46), played a major role in controlling this iatrogenic poisoning. Although this aspect of Al toxicity is well understood, there are still ongoing investigations, particularly in the mechanisms of the metabolic bone disease associated with the treatment (47–50). Complications of bladder irrigation with alum in patients with compromised renal function have also been reported (51).

3.2 Exposure to Al in Parenteral Nutrition Products

Most of the reported complications of contamination by Al of commercial intravenous-feeding solutions are related to its involvement as a key factor in the pathogenesis of metabolic bone disease. There is also a highly significant report of impairment of cognitive function in infants exposed in this manner to Al. The major clinical problem of this type of Al contamination is its occurrence in preterm infants. The U.S. Food and Drug Administration (FDA) has been investigating this problem since 1986, which has led to recommendations that were best summarized in a position paper of the North American Society for Pediatric Gastroenterology and Nutrition (52). This position statement supports the FDA's proposal to add certain labeling requirements for large- and small-volume parenterals used in total parenteral nutrition, to provide information on Al content, and to require validated analytical methods to be used for Al measurements. The FDA has taken this stance because of evidence linking the use of Al-containing

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materials being associated with morbidity and mortality among patients on total parenteral nutrition therapy, particularly premature infants and patients with impaired renal function

The first study of an increased body burden of Al being linked to total parenteral nutrition solutions as assessed by increased plasma, bone, and urine concentrations was that of Sedman et al. in 1985 (53). Bone disease in adult patients who were undergoing this treatment but without renal impairment was reported earlier (54,55). The source of the Al initially was casein hydrolysate, which was the protein source commonly in use at that time. Substitution of crystalline amino acids for casein hydrolysate, together with other conventional practices for reducing Al loading such as the use of low-Al dialysate solutions (prepared from deionized water) and the restriction of Al-containing phosphate binders, led to a resolution of bone pain in these patients (56). In the report of Sedman et al. (53) the Al souces were identified as being contaminated with calcium and phosphate salts, albumin, and heparin. The contamination of phosphate salts is not surprising in view of the high affinity of Al for phosphate. Infant formulas were also identified as being potentially contaminated by Al (53). Koo et al. also contributed to this aspect of Al toxicity, showing that Al accumulated at the mineralization front in the bones of premature infants (57), and later that preterm infants were able to increase Al excretion in the urine with increased Al load, but that this response could not prevent bone Al deposition and hyperaluminemia (58). Koo et al. (59) also demonstrated that infant formulas can contain high concentrations of Al. The highest levels (up to 2346 μg/L) are found in highly processed and modified formulas, including soy formula, preterm infant formula, and formulas for specific metabolic disorders. Human milk has the lowest concentration of Al, being less than 50 μg/L. Bishop et al. in 1997 (60) showed that preterm infants who received total parenteral nutrition containing 45 μg/L of Al, which is the usual solution used for these patients, had a lower score on the Bayley Mental Development Index at age 18 months than did age-matched infants who were given total parenteral nutrition solutions having much lower Al concentrations.

Thus, Al contamination of products used for preterm infants represents an important toxicity problem, which produces impairment of bone formation and neurological deficits. Preterm infants appear to be especially at risk. Intravenous administration circumvents the usual gastrointestinal barrier that keeps the majority of ingested Al out of the circulation. Once in the circulation Al becomes rapidly bound to transferrin (61) and cannot be readily excreted into the urine because of the relatively high molecular weight of this protein complex. The fact that renal function in preterm infants is developmentally impaired, taking up to 34 weeks to reach maturity, only adds to the problem.

Total parenteral nutrition appears to be less of a problem in adult patients, but still exists and has been well documented (62–64). Metabolic bone disease

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can develop in these patients, as characterized by patchy osteomalacia and reduced bone activity. There is also a reduction in serum levels of 1α,25-dihydroxyvitamin D, with normal levels of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D. Discontinuation of total parenteral nutrition containing Al-contaminated solutions has resolved the metabolic bone disease within 6 weeks (64).

Bone lesions have also been reported in adult patients with severe burn injury, and this complication has been related to Al toxicity resulting from contamination of human serum albumin and calcium gluconate (52,65). Contamination of blood products such as factors VIII and IX with Al have also caused concern (66,67).

3.3 Al-Containing Fumes and Dust

Several studies on occupational exposure to Al have been reported in which it has been observed that the mental status of exposed workers was impaired as compared to appropriate controls. This topic has been reviewed by McLachlan (68), Flaten et al. (69), and Sjogren et al. (70). Although there are a significant number of investigations reporting the possible hazards of occupational Al exposure, such reports are few in number when the vastness of the Al industry, thence the extent of worker exposure, is taken into consideration. There is certainly no clear evidence that this type of exposure leads to the development of Alzheimer's disease, although there is some indication that excessive exposure can lead to cognitive impairment. The handling of Al-containing minerals also exposes the worker to silica; hence pulmonary disease is also a major concern in this type of occupation (70).

Aluminum appears to be absorbed by all workers exposed to this metal in the course of their occupation, as demonstrated by increased urinary (71–76) and blood (74,75) Al levels. The urinary excretion of two workers who were exposed to welding fumes over several years was >10-fold higher than controls, and remained high for many years after cessation of exposure (75). Blood and bone Al levels were also increased, but not quite so dramatically as the urine level (75). A later study compared 38 welders exposed to Al fumes, but not manganese, to 39 unexposed controls (76). Assessment of these workers with a psychological examination showed that the workers exposed to Al achieved a significantly lower score in four of the tests than did the control group, and for two tests the effect was dose-related as assessed by urinary Al concentrations. An isolated case reported by these same workers described a man with aluminosis recognized in 1946 who developed a dementia with motor disturbances and elevated cerebrospinal fluid Al concentrations (77). This individual died in 1998 and his cerebrospinal fluid Al level was low, suggesting that the earlier measurement had been subjected to contamination. It was finally concluded that the patient had Alzheimer's disease, and that it was not related to Al exposure (78). There have been

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other reports of Al exposure from working in the potroom of an Al plant. A significant number of the workers revealed mild to moderate impairment of memory, as assessed by two separate memory tests (79). As in the other study discussed above (76), Al was identified as the probable cause of the syndrome, since exposures to other agents by these same workers had caused no problems in other workers exposed to the same agents but not to Al. In a separate study the psychomotor and intellectual abilities were assessed in workers in an Al foundry in Yugoslavia (80). Eighty-seven exposed and 60 unexposed workers were evaluated. These tests revealed slower psychomotor reaction and dissociation of oculomotor coordination in the exposed group. These workers also had memory impairment and emotional disturbances. Treatment with the Al chelator desferrioxamine resulted in mobilization of Al as detected by elevated concentrations in blood and urine (81). Salib and Hillier used the risk of developing Alzheimer's disease later in life as a monitor of occupational hazard for workers in the Al industry (82). Aluminum workers reported to have been directly exposed to Al dust and fumes did not appear to be more at risk for developing Alzheimer's disease than were unexposed workers in the same factory. This same conclusion was the result of a more recent study of occupational exposures to solvents and Al (83). An interesting exposure to Al powder occurred between 1944 and 1979 in mines in northern Ontario, when McIntyre powder (which consists of finely ground Al and Al oxide) was used as a prophylactic agent against silicosis (84). Exposed miners performed worse than unexposed controls on cognitive state examinations and this impairment increased with the duration of exposure.

3.4 Medications

3.4.1 Antacids

Al-containing antacids are used extensively for the treatment of dyspepsia. Quantities of this medication are consumed in gram amounts, contrasting markedly with the milligram quantities of Al consumed daily in food and drinking water. In a study of epidemiological aspects of Alzheimer's disease in 1984, Heyman et al. reported that the intake of Al-containing antacids was slightly higher in controls than in patients with Alzheimer's disease (85). House demonstrated that office workers who were not occupationally exposed to Al had significant elevations of their plasma Al concentrations if they were using antacids (86). In a surprising study, Graves et al. showed an association between antacid consumption and Alzheimer's disease, but demonstrated that this association was less obvious if Al-containing antacid users were removed from the analysis (87). Flaten et al. have performed perhaps the largest study of patients with an apparent high intake of Al-containing antacids for gastroduodenal ulcer disease (88). The results of this study provide no significant evidence that a large intake of Al in the form of antacids causes an increased incidence of Alzheimer's disease. The

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power of this investigation to detect an Al effect was diluted by the fact that not all patients took Al-containing antacids, and that non-Alzheimer's dementias were included. Plasma Al concentrations have been evaluated in a reference population and the effects of Al-containing antacids have been investigated (89). Both acute and medium-term Al-containing antacid consumption results in increased plasma Al concentrations, which occasionally reach the levels seen in patients with renal disease who are ingesting such medications. Although hyperaluminemia in patients undergoing long-term hemodialysis treatment for chronic renal failure can lead to development of metabolic bone disease (38,39), there has been no evidence of inhibition of bone mineralization in subjects consuming Al-containing antacids (89).

3.4.2 Antiperspirants

Aluminum compounds have been applied extensively for many years as antiperspirants, probably because of their antimicrobial properties (90). Graves et al. studied the same group of subjects as in their antacid study and were able to identify Alzheimer's disease patients and controls with no antiperspirant use, and others with low, moderate, and high use (87). Although the number of subjects was low, the results showed that the odds ratios associated with any antiperspirant/deodorant use at the various dose levels did not differ significantly from the null.

3.4.3 Food

Several reports of Al-induced gastrointestinal problems and effects on the central nervous system were reported in the late nineteenth and early twentieth centuries, and are summarized by Betts in a book published in 1928 (91). Concern has therefore existed for well over a century about excessive exposure to Al.

Only a few reports of the Al content of foodstuffs have been published, and the earlier ones, such as in the book by Betts (91), are of limited value because of the inaccuracy of the analytical methods available at the time. Most foods and beverages contain only low concentrations of Al (92,93), probably because they are mainly derived from living organisms to which Al is toxic. Cooking utensils add some Al, but the amount of this element consumed from foods, beverages, and utensils is small compared to the intake in some individuals derived from pharmaceutical products (94). The addition of Al during processing of foods increases its concentration appreciably. Herbs and tea contain more Al, but do not represent major contributors to the daily intake, since the Al in tea leaves does not dissolve in the liquid consumed (93). Probably the average individual in the more industrialized affluent nations consumes 20–30 mg of Al daily, but this might range from 2 to 100 mg. In a balance study of human subjects fed an Al test diet it was shown that minimal Al was retained in the body, with fecal excretion predominating (95). Several reports detailing the dietary intake

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of Al have been published (92,94,96,97). This topic has been revisited in a recent publication of a pilot study that evaluated dietary Al intake as a risk factor for Alzheimer's disease (98). Although there was some suggestion of a relationship, the authors acknowledge that more extensive investigation is warranted.

4. ADVERSE NEUROLOGICAL EFFECTS FOLLOWING ORAL ADMINISTRATION OF Al COMPOUNDS TO EXPERIMENTAL ANIMALS

4.1 Evidence for Transfer of Al from the Gastrointestinal Tract to the Brain

Only a small amount of the total ingested Al is absorbed via the gastrointestinal tract, and the majority of that is excreted into the urine (99). The form of the ingested Al is important, as stated in the first section of this review, which deals with speciation. Slanina et al. have shown in both humans and rats (100,101) that citrate enhances the gut absorption of Al. In humans this amounts to a four-fold increase in plasma concentrations (101). In rats there are significant elevations in bone and in the brain in cerebral cortex, hippocampus, and cerebellum (100); Al hydroxide alone did not produce these increases. However, study of the uptake and distribution of Al in tissues following exposure was made difficult by the lack of availability of a radioisotope, or even a stable isotope, which could complement studies of naturally occurring ^{27}Al. However, in the past few years, ^{26}Al, a by-product of the nuclear industry, has become available to researchers. This long-lived isotope of Al has a half-life of 7.1×10^5 years and can be measured with exquisite sensitivity by accelerator mass spectrometry. Using this method, Walton et al. have demonstrated that alum-treated water containing ^{26}Al, gavaged into the stomachs of rats, produced elevations of ^{26}Al in brain tissue (102). Only six animals were studied, but four of these had 10-20-fold increases of ^{26}Al in their brain and the other two had amounts that were 200–300 times greater. This study has been criticized because of the limited number of animals examined (103), but the author of this criticism failed to recognize the great complexity and expense entailed in the analytical measurement of ^{26}Al. ^{26}Al has been used in human volunteers to demonstrate gastrointestinal absorption, urinary excretion, and distribution of this metal in the circulation (104,105) and the analytical methods applied have been reported in detail (106). Thus it has been demonstrated, albeit with only a limited number of animals, that Al can be taken up by the brain following oral ingestion.

An important recent observation has been the identification of mechanisms whereby Al is transported out of the extracellular fluid in the brain. Allen et al. recognized the limited information available on the permeability of the brain-

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blood barrier to Al, and applied microdialysis to determine the distribution between frontal cortex and blood of unbound Al in extracellular fluid. Their results suggested the presence of an energy-dependent carrier that removes Al from extracellular fluid and transfers it into blood or into cells in the brain (107). Subsequent studies have shown that Al citrate is transported from brain to blood via the monocarboxylic transporter located at the blood-brain barrier (108,109).

4.2 Neurobehavioral Effects of Al in Experimental Animals

The key question that can be answered by experimental animal studies is whether oral intake of Al compounds can produce neurotoxicity. Rats have been used in the few studies performed, but with inconsistent results. Bowdler et al. gave rats a daily oral gavage of Al ($AlCl_3$) and correlated behavioral results with brain Al concentrations (110). It was found that this orally ingested Al was absorbed and deposited in brain. Conditioned avoidance response did not correlate with brain Al levels, but there was an increased sensitivity to flicker. Interestingly, behavioral tests were also given to elderly humans, and performance was correlated with serum Al concentrations. High serum Al levels were associated with poor long-term memory and increased sensitivity to flicker. In 1979 the techniques for measuring serum or plasma Al concentrations were poorly standardized, and the validity of these results could reasonably be questioned. Al chloride administered in the diet produced variable deficits on shuttle-box avoidance behavior, depending on rat strain and sex (111). Adult rats fed rat chow with no Al added, and others fed chow containing Al hydroxide, showed an inverse relationship between brain Al concentrations and open-field activity. Elevated brain levels correlated with relatively poor performance on a single-trial passive avoidance task and on a visual discrimination with reversal task. No behavioral problems were seen when Al was administered orally to rats at weaning, suggesting that developing animals are more resistant than adults to Al neurotoxicity (112). Connor et al. used a battery of behavioral tasks to evaluate the effect of chronic oral administration of Al sulfate to rats (113). No impairment of performance was observed on an active avoidance task, radial arm maze, or open field activity. Repeating the study demonstrated no Al effect on the passive conditioned avoidance response (114). A behavioral deficit induced by Al could be reversed by the Al chelator desferrioxamine (114).

Rabbits should be a more relevant species than rats for Al-related behavioral studies, since in rabbits the neuropathological and biochemical changes induced by Al bear a greater resemblance to those in human diseases associated with clinical dementia. However, to our knowledge, no behavioral studies on rabbits treated orally with Al have been performed. We have evaluated the long-term oral administration of Al maltolate to rabbits (115–117). Although de-

creased weight gain was noted, no significant histological changes were found in the central or peripheral nervous systems, nor were Al concentrations in brain found to be elevated by bulk analysis (116). There was some renal accumulation of Al and occasional hepatic changes (117), as well as decreases in hematocrit and hemoglobin levels and in red blood cell counts (115). We performed no specific behavioral studies. Investigations of this nature that have been carried out on rabbits have involved the direct administration of Al compounds into the brain, such as will be discussed in detail in the section on Alzheimer's disease (*see* p. 332, Al-induced neurodegeneration in animals), or the systemic administration of Al. Intracisternal administration of Al has been reported to produce deficits in water maze acquisition (118). Using this same route of Al administration, Pendlebury et al. (119,120) demonstrated learning and memory deficits in rabbits by using the acquisition or retention of the eyeblink reflex. The rabbit is not typically used for behavioral studies, but the classically conditioned-defensive eyeblink reflex is a useful tool and has been studied in this animal. This test appears to reflect the effects of Al on neural pathways and structures subserving this simple form of learning and memory; the hippocampus and cerebellum are structures that may be involved in these processes (121). Yokel, in a series of experiments, used the subcutaneous route of injection of Al lactate and was able to demonstrate learning and memory deficits, but only in adult and aged rabbits (122–126) suggesting, as we have also proposed (127), that aging increases susceptibility of the brain to Al toxicity, at least in rabbits. Yokel at al. related their Al-induced learning deficits to patients with Alzheimer's disease when they demonstrated that 4-aminopyridine, which has been reported to improve learning in Alzheimer's disease subjects, attenuates the Al-induced learning deficit in rabbits (128).

4.3 Phytotoxicity and Ecotoxicology of Al to Fish and Wildlife

Aluminum phytotoxicity is a major agricultural problem, since it limits crop productivity on acid soils, which represent approximately 30% of the world's land area. The lack of a suitable Al tracer has limited a detailed understanding of Al transport mechanisms. However, much is known about this important aspect of Al toxicity, and the topic has been reviewed in detail by Kochian and Jones (129).

The toxicity of Al has been studied extensively in fish and to a lesser extent in invertebrates, amphibians, and birds. There is essentially no information on its effect on reptiles and free-ranging mammals. A decrease in water acidity to pH 5.5–7.0 has a marked effect on life existing in this environment; for example, fish adsorb freed Al onto gill surfaces, which can subsequently lead to their asphyxiation. This important aspect of Al toxicity has been reviewed (130).

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5. THE POSSIBLE ROLE OF Al IN NEURODEGENERATIVE DISEASES

5.1 Alzheimer's Disease

This topic was reviewed in detail in a paper coauthored by one of the present authors (JS) (131). Few hypotheses concerning the pathogenesis of a common disease have caused so much controversy as the one linking Al to Alzheimer's disease, and it is fair to say that the majority of neurologists, neuropathologists, or neuroscientists in general do not consider Al to be a major player in the pathogenesis of this disease. The major factors that make this a contentious issue are of course the high incidence of sporadic Alzheimer's disease and the lack of well-accepted mechanisms for the cause(s) of this devastating neurological disorder. Three key arguments have persuaded most scientists to dismiss the Al hypothesis. First, patients with hyperaluminemia resulting from hemodialysis treatment do not consistently demonstrate neuritic pathology of Alzheimer's disease. The second has resulted from a review by the eminent epidemiologist Sir Richard Doll (132), who failed to draw any firm conclusions as to whether Al exposure might result in neurodegeneration. However, this review covered only the subject of human exposure to environmental Al and did not address the subject in its entirety; in particular, it failed to take into account the finding of deposition of Al in brain tissue, and furthermore did not consider the results of animal experiments and biochemical investigations. The third report casting doubt on the Al hypothesis came from Landsberg et al. (133), whose study reported a failure to detect Al in neuritic plaques in Alzheimer's disease patients; these workers concluded emphatically that therefore Al was not associated with Alzheimer's disease. Regrettably, the technique employed in this study was not particularly sensitive, thus limiting the value of the report. In the present review these important questions will be critically addressed.

Alzheimer's disease is characterized by the presence of (1) intraneuronal protein aggregates consisting primarily of abnormally phosphorylated tau, and (2) extracellular neuritic plaques containing the peptide Aβ as its chief constituent. There is also synaptic and neuronal loss. These characteristic neuropathological features (neurofibrillary tangles and neuritic plaques) are obviously important events and have been reviewed recently by Trojanowski et al. (134), but may represent later markers resulting from a more fundamental early process.

1. Is Al Present at Elevated Concentrations in the Neurofibrillary Tangles and/or Neuritic Plaques of Alzheimer's Disease?

Two approaches have been taken to determine whether in fact Al is elevated in brain tissue from individuals with Alzheimer's disease. The first studies of such

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Al measurements used the conventional approach of bulk analysis, whereas more recent investigations have employed several different microprobe techniques in addition to bulk assay. Crapper et al. (135) were the first to describe an elevation of Al concentrations in some regions of the brains of patients with Alzheimer's disease, and compared these results with brain analyses from Al chloride–treated cats. In the Alzheimer's disease patients, a wide range of Al concentrations was observed; in some regions these approached 12 μg/g (dry weight), whereas no control value was greater than 2.7 μg/g. The experimental animals treated with Al chloride yielded even higher tissue Al concentrations, although controls were similar to the non-Alzheimer's disease human controls. Subsequently it has been stressed by these investigators that the key to their findings was the selection of appropriate tissue for analysis, and also that they only included patients with well-defined disease. A later study failed to confirm the findings of Crapper et al. (135) and found no differences between Alzheimer's disease subjects and controls (136). Following these two initial and contradictory reports, three studies have described an elevation of Al in Alzheimer's disease patients (137–139), and two others have suggested no increases (140,141). Traub et al. (142) reported that four out of seven Alzheimer's disease patients showed no elevation of Al by bulk analysis; the other three patients did in fact demonstrate increases. A more recent study reevaluated this question of Al analysis and reported small but significant increases in Al concentrations in tissue from Alzheimer's disease patients (143). A more extensive study was reported in 1996 by Bjertness et al. (144). These workers examined 92 clinically and histopathologically diagnosed Alzheimer's disease patients along with normal elderly nursing home residents, and performed bulk tissue Al measurements on specimens of frontal cortex and temporal cortex, both of which regions are known to be vulnerable to the neuropathological changes associated with Alzheimer's disease. There were no significant differences between the severely affected Alzheimer's disease patients and normal controls, and there was no correlation between the density of neuritic plaques and neurofibrillary tangles with Al concentrations. This study would have been of greater significance had the hippocampus also been analyzed. However, because of the earlier contradictory bulk analysis results and this latest negative study, it seems unlikely that Al in bulk tissue is elevated to any significant extent in Alzheimer's disease. Even if Al is present in neurofibrillary tangles in levels capable of producing pathological changes, this amount might still be insufficient to elevate the bulk tissue concentration to any significant extent, which thus adds relevance to the ensuing discussion of microanalysis results. Perl et al. (145) have calculated the expected increase in bulk tissue Al concentration based on a normal concentration in cerebral cortex of 1.5 ppm, a density of 25 neurofibrillary tangle-bearing neurons/mm^2 in a 10-μm-thick section, and an Al concentration of 100 ppm within the neurofibrillary tangles. The expected increase in bulk Al concentration with these assumptions would be 0.0002%, which would be extremely

difficult to detect by bulk analysis methods; for this reason, Perl et al. recommended the microprobe analytical approach (145).

Controversy also has surrounded reports of microprobe analysis techniques for evaluating the Al content of neurofibrillary tangle-bearing neurons and neuritic plaques. In 1980, Perl and Brody (146) applied the technique of scanning electron microscopy combined with energy dispersive X-ray spectrometry to demonstrate the presence of Al in the nuclear region of neurons that contained neurofibrillary tangles. Two more recent reports using the same microanalytical technique, however, failed to detect a significant amount of Al in these lesions (141,147). Advances in this area of investigation were made by the application of a far more sensitive microanalysis technique, laser microprobe mass analysis (LAMMA). Using this technique, Good et al. (148) demonstrated the accumulation of Al within neurofibrillary tangle-bearing neurons within the hippocampus of all of the Alzheimer's disease patients they examined. In this report, Al was localized within the neurofibrillary tangles but not in the nuclear region, as had previously been reported by the same workers (146). Iron also was shown to be present in these lesions. The first studies were carried out on plastic-embedded semithin sections, and the question of contamination with exogenous Al during processing had to be addressed. Selected tissues were snap-frozen, dried, stained with cold toluidine blue, and analyzed. These workers state in this paper that the concentration of Al detected in the neurofibrillary tangles ranged from 15 to 80 ppm. The question of contamination is important, especially in view of the later work of Makjanic et al. reported below (149). Could the toluidine blue used by Good et al. (148) be contaminated as suggested by Makjanic et al. (149)? Dr. Perl (D. P. Perl, personal communication, 1999) has addressed this point and although his findings were not incorporated into the report of Good et al. (148), he analyzed the toluidine blue powder by LAMMA and saw no evidence of Al contamination. The brain sections these workers analyzed were very lightly counterstained with a 1% aqueous solution of toluidine blue. Since the native toluidine blue powder contained no demonstrable Al, a dilute (1%) solution prepared with deionized (Al-free) water should also not be contaminated. Additionally, in this work of Good et al., no other structures that stained with toluidine blue were found to be Al-positive. The sensitivity of the LAMMA technique is claimed by Good et al. (148) to be 1–2 ppm. Another study has been carried out by Lovell et al. (150) applying the same LAMMA technique, also on brain tissue from patients with Alzheimer's disease. These workers demonstrated intraneuronal elevations of Al in the Alzheimer's disease group when compared to controls, but relatively few cells were in fact positive. Also, the same percentages of elevations were seen in the neurofibrillary tangle-bearing as in the neurofibrillary tangle-free cells, thus suggesting that Al does not selectively accumulate in neurofibrillary tangle-containing neurons. The LAMMA instrument settings for the two studies were dramatically different. Good et al. (148) used a laser energy of 6–

8 μJ, whereas Lovell et al. (150) set their instrument at 90 μJ. Whether both instruments were operating optimally can be questioned; this aspect of the two studies has been the subject of some discussion in the literature by both groups of investigators (151), and is also discussed by Lovell et al. (152). As mentioned above, one question raised in the course of these microanalysis studies was possible contamination during tissue collection, processing, and analysis. It appears that Good et al. (148) have addressed this problem; the relatively small amount of Al detected in control tissues argues against significant contamination.

More recently the technique of nuclear microscopy has been applied to the microanalysis of brain tissue from patients with Alzheimer's disease. The initial report addressed the question of whether Al was present in neuritic plaques and this is discussed in more detail below (133). The technique has also been applied to the incorporation of Al into neurofibrillary tangles (149). In unstained and untreated sections there was no evidence (at a detection limit of 20 ppm) for the presence of Al in pyramidal neurons from the hippocampus of six patients with Alzheimer's disease; tissue from four age-matched controls was also analyzed. Although neurons with neurofibrillary tangles could not be visualized, an adjacent section when stained for tau and counterstained with cresyl violet revealed that 62% of the neurons contained tangles. The structure of the pyramidal neurons could be visualized with this method. Freeze-dried and toluidine blue–stained tissue gave a mean of 30 ppm of Al, and fixed, osmicated, and toluidine blue–stained tissue gave a mean of 90 ppm. If we directly compare these studies of Makjanic et al. (149) with those of Good et al. (148) we see that they both employed frozen sections stained with toluidine blue, and in both cases Al was detected. Makjanic et al. (149) had the advantage of using unstained tissue, and failed to detect Al within neurons in a region containing neurofibrillary tangles. However, in view of the careful evaluation of contamination by Good et al. (148), and the lack of detection of Al in the toluidine blue counterstain (D. P. Perl, personal communication, 1999), it is the opinion of the present reviewers that Al is indeed present in the neurofibrillary tangles of Alzheimer's disease. The limits of detection of the nuclear microscopy technque are hardly sufficient to detect toxic levels of Al. Investigations in the present reviewer's laboratory have shown that the in vivo injection of 65 μg of Al into the cisterna magnum of an adult rabbit brain with a brain weight of approximately 10 g (unpublished data) is sufficient to induce a lethal neurotoxic effect, with neurofibrillary degeneration accruing throughout the brain stem, midbrain, hippocampus, and cortical regions (127). The maximum concentration of Al in this rabbit brain, assuming a uniform distribution, would be 6 ppm; it is reasonable to expect a higher concentration close to the injection site, i.e., brain stem, and lower amounts in the hippocampus. There is the possibility that certain neurons might concentrate Al but it seems unlikely, even in this acute neurotoxicity experiment, that Al levels attaining

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the limit of sensitivity required for assessment by nuclear microscopy would be achieved.

This important although contradictory series of reports and data in the literature may recently have been clarified by an independent technique. Murayama et al. recently demonstrated, using rigorous chelation of tissue sections from Alzheimer's disease brain, that immunostaining with conventional tau monoclonal antibodies (mAbs) is markedly enhanced (153). These results strongly suggest that Al is an integral component of neurofibrillary tangles, and that it is sequestered in such a way that only by rigorous chelation is it released. In vitro aggregation of tau by Al was also observed. It is possible that contamination could have occurred in the tissue processing, since paraffin sections were used either from tissue fixed with 10% neutral buffered formalin or 70% ethanol/0.15 mol/L sodium chloride. It seems unlikely, however, that autoclaving with desferrioxamine should be required to remove exogenous Al contamination. Confirmation with contamination-free frozen sections would be a useful addendum to this highly relevant work.

Additional studies have unequivocally demonstrated the presence of far higher concentrations of Al in neurofibrillary tangle-bearing neurons of patients with the ALS/parkinsonism-dementia complex of Guam (154–156), and this will be discussed later.

Another contentious issue revolves around the possible presence of Al in the form of aluminosilicate in the core of senile plaques. Candy et al. (157) reported the colocalization of Al and silicon, although Landsberg et al. (133) failed to confirm this finding with the alternative microanalytical technique of particle-induced X-ray emission (PIXE). However, this nuclear microscopic technique is relatively insensitive below 15 μg/g of Al, making the significance of this report questionable. The authors (133) suggested that previous studies demonstrating the presence of Al in brain tissue should be repeated to rule out possible contamination, and this has been discussed in detail above. There appears to be a better case for endogenous Al having been detected in neurofibrillary tangles than in neuritic plaques, since the more sensitive techniques such as LAMMA have been used in the analysis of the former. One can also make a much better case for the presence of Al within neurons playing a more significant role in the neurodegenerative process than for its presence in the extracellular neuritic plaques. The presence of intraneuronal Al might not only perturb the cytoskeleton, but would be available to induce mitochondrial damage as well. It is of interest that in a later study, Landsberg et al. (158) reported that, at a sensitivity of 50 μg/g or greater, Al and silicon were detected in 20% of senile plaques, thereby contradicting their own widely cited 1992 study.

The lack of agreement on the question of whether the brain content of Al is increased in Alzheimer's disease simply attests to the complexity of the problem.

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Procurement of adequate numbers of brains from both correctly diagnosed Alzheimer's disease cases and appropriate controls is no trivial undertaking. Tissue must be uncontaminated, and dissection of suitable specimens for analysis also must be carried out in a clean air facility. The process requires the collaboration of an experienced neuropathologist with a highly skilled analyst. The actual analyses are challenging, particularly where microprobe techniques are involved. The more sensitive microanalysis instruments, particularly LAMMA, are only accessible to a handful of research groups worldwide. In the studies discussed above, only a relatively small number of brain specimens have been examined. The report of Good et al. (148) included 10 Alzheimer's disease patients and four controls. Lovell et al. (150) analyzed tissue from seven patients and five controls, and the bulk analysis study of Xu et al. (143) included 10 Alzheimer's disease patients and 10 controls. In the report of Landsberg et al. (133) only five Alzheimer's patients were studied, and six Alzheimer's disease patients were included in the report of Makjanic et al. (149). To answer the question of whether Al is present in Alzheimer's disease brains, more work needs to be carried out on many other brains using sensitive microprobe techniques. Elevations of Al may be small or nonexistent and a large number of patients will be required for statistically significant data. Assuming that there are detectable increases in Al, the question arises as to whether this necessarily implicates Al as a pathogenic factor as opposed to its presence representing a secondary phenomenon. Here the degree of elevation would have to be considered. Animal experiments, as discussed later, would also help to resolve this issue. The demonstration of Al deposition in animals at levels similar to those seen in Alzheimer's disease (at levels that consistently produce neurodegeneration in the experimental model) would strongly implicate Al as playing an active role.

2. Is Environmental Exposure to Al In Drinking Water or In the Workplace a Risk Factor for Alzheimer's Disease?

As with all other aspects of the potential Al–Alzheimer's disease relationship, all of the epidemiological studies focusing on Al in the environment are highly controversial, and the topic has been reviewed previously by us (131). Because data on Al in drinking water are relatively readily available, most epidemiological studies have focused on this particular exposure source, although drinking water represents only a fraction of the total amount of Al ingested orally. However, the argument can be made that the Al in drinking water may be more readily absorbed than Al present in other sources; i.e., it is more soluble and hence more bioavailable.

Most of the data linking Al exposure to Alzheimer's disease have been derived from several epidemiological studies of Al in drinking water. The most widely publicized investigation was that of Martyn et al. (159). In a study of 88 county districts in the United Kingdom, these investigators found a 50% increase

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in the risk of Alzheimer's disease in districts where the mean water Al concentration was greater than 111 μg/L, as compared to regions where it was less than 10 μg/L. Epidemiological data of this type are difficult to substantiate, since the disease may be underreported and the diagnosis may sometimes be wrong; however, supportive data have also been published (160,161). In a series of papers, Forbes et al. (162–166) reported a cohort of elderly men living in Ontario, in whom a statistically significant association was present between the risk of impaired cognitive function and elevated concentrations of Al in the finished drinking water supply. Although impaired cognitive function can be caused by conditions other than dementia, for example by depression, drug use, and cerebrovascular accidents, it represented a readily measurable end point, and the results obtained were consistent with those from other studies. Moreover, the results were analyzed in a multivariate manner; that is, the association of metal impairment with various water constituents could be examined after adjustment for other water quality variables. In this way, the presence of possible confounding variables was controlled, at least to some extent. The results demonstrated that, at neutral pH, relatively low Al concentrations and relatively high fluoride concentrations decreased the odds of exhibiting cognitive impairment by a factor of about five, as compared with other types of drinking water; also, a neutral pH alone was found to be associated with reduced odds.

More recently Forbes et al. (167) correlated death certificates from Ontario that mention Alzheimer's disease as the underlying cause of death with data for levels of Al in drinking water. These investigators concluded that the risk for dementia reached 3.2 (95% confidence interval 1.9–5.5) for communities having median concentrations of Al greater than 336 μg/L. This study employed a similar methodology as for the above-mentioned study that used impaired cognitive function as the outcome variable. Not unexpectedly, the relative risk estimates obtained were somewhat greater than in the earlier study since, as mentioned above, the former study would contain a number of individuals who were not demented. At the same time, the associations with Al concentrations were similar, as were the effects of fluoride and of pH. More specifically, the lowest risks were observed at a pH of about 7.9, both from the previously reported odds ratios, which were based on a mental status questionnaire, and from the death certificate data. In addition, although the risks are similar at silica concentrations of up to 5mg/L, silica concentrations above 6 mg/L may have reduced the risk, consistent with results obtained by Birchall and Chappell (168) and Edwardson et al. (169).

In another recent study, McLachlan et al. (170) investigated a possible relationship between Al in the drinking water and autopsy-verified Alzheimer's disease brains collected from 55 communities in Ontario. Aluminum exposure was estimated from the earliest available annual average Al concentrations, taken at monthly intervals, for municipalities participating in a Drinking Water Surveillance Program conducted by the Ontario Ministry of Environment and Energy

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since 1981. Alzheimer's disease was confirmed at autopsy, and controls were drawn from the records of the Canadian Brain Tissue Bank for donors from the Province of Ontario. Within the range of Al concentrations between 4 and 203 μg/L annual average exposure, the estimated relative-odds ratio, associated with Al concentrations greater than 100 μg/L, for Alzheimer's disease compared with all controls, was 1.7 (95% CI: 1.2–2.5) when only residence at the time of death was employed. However, estimating Al exposure from a 10-year-weighted residential exposure prior to death resulted in a relative risk of 2.5 (CI: 1.2–5.3) or greater. When cutoff values of 125, 150, or 175 μg/L were employed, the odds ratio for Alzheimer's disease, based on history of a 10-year residential exposure, was 3.6, 4.4, and 7.6, respectively. This study is of course not conclusive since the risk associated with the higher Al concentrations was not excessive. Also, only drinking water Al was investigated and other factors that might be associated with the disease, such as genetics, diet, and exercise, were not included.

Not all epidemiological studies report a relationship between impaired cognitive function and Al exposure in the drinking water, however. Perhaps the most significant negative study was that published in 1997 by Martyn et al. (171). Martyn and his colleagues were the first investigators to describe a positive relationship between drinking water Al concentrations and Alzheimer's disease in their report in 1989 (159). In this more recent report (171), which was carried out in eight regions of England and Wales, diagnoses were obtained by a review of case notes and included 106 men with Alzheimer's disease, 99 men with other dementing illnesses, 226 men with brain cancer, and 441 men with other diseases of the nervous system. The results indicated that any risk of Alzheimer's disease associated with Al concentrations in drinking water <200 μg/L was small. Two studies from Britain and Europe (172,173) failed to detect a relationship of Alzheimer's disease to levels of Al in the drinking water supply. The failure to detect such a relationship may represent geochemical differences in the drinking water supply. Also, Wettstein et al. (173) investigated only two drinking water sources, the water with high-Al levels having only about 100 μg/L of Al. There is also the possibility that there was a disproportionate removal of individuals from the population because of dementia and its associated comorbidities.

The indication from the studies discussed above, that high concentrations of Al in drinking water increase the risk for Alzheimer's disease, is surprising since this source represents only a small percentage of the total exposure. These results may mean that, at higher concentrations and in the presence of certain other water constituents, a more neurotoxic species of Al is formed. At the same time, quantification of the population at risk for Alzheimer's disease from other sources of Al, such as food additives, pharmaceuticals, adjuvants, cosmetics, deodorants, and repiratory dusts (84), is required before the total risk for Alzheimer's disease from the various sources of environmental Al can be fully evaluated. A problem with all studies of this type is that the exposure information is

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of poor quality. Nor is the duration of exposure (length of residence or patterns of migration) usually available, so accurate dose-response relationships cannot be adequately evaluated. In addition, potential confounders such as urban/rural residence, a family history of dementia, exploration of gene-environment interactions, level of education, and premorbid levels of cognitive functions are often not controlled. For example, if geographical regions with high levels of Al have more nursing homes, and people with Alzheimer's disease migrate to those regions to reside in nursing homes, a spurious relationship between Al and Alzheimer's disease may be observed (174). In addition, the earlier studies demonstrate weak dose relationships and do not support the hypothesis of a linear relationship.

Another weakness of many of the studies is reliance on death certificates for the diagnosis of the various dementias. Completeness and accuracy of death certificate data vary with access to medical care and with diagnostic and reporting practices associated with the type of medical facility or medical specialty of the physician completing the death certificate. It is also believed that death certificate codes that are used in these studies do not discriminate well between Alzheimer's disease and other causes of dementia, so that a test of the hypothesized association loses its specificity (175). Indeed, a definitive diagnosis of Alzheimer's disease requires autopsy confirmation, using silver stain (the Bielschowsky's method), or Congo Red stain, to exclude other causes of dementia, such as Pick's disease or multi-infarct dementia. These criticisms must be taken seriously, but it should be noted that the various investigators were well aware of these and, in some cases, appropriate adjustments have been made. For example, the studies on autopsy and special stain-verified Alzheimer's disease ensure that the diagnosis is correct, but the cases that reach a brain bank most probably represent a highly selected population. In the above reports, generally consistent results are obtained, and when the relationship is not found, reasonable explanations for this can be suggested. With respect to these criticisms, it might be noted that at least some of the various errors may reduce the estimates of the relative risks, and the actual risk of Alzheimer's disease may be higher in areas where the Al water concentrations are relatively high, compared with areas where the Al concentrations are relatively low; this in fact has been pointed out in two of the more recent studies, which estimate the relative risks to be above 5. The topic of Al ingestion and its relationship to Alzheimer's disease has been reviewed by McLachlan (68), who points out that epidemiological studies demonstrate association but do not establish cause and effect. The question of whether elevated concentrations of Al in drinking water is a risk factor for Alzheimer's disease has certainly not been answered conclusively, yet there is sufficient evidence to recommend that further investigations of this type should be carried out. If the association is confirmed, then evidence on what is a safe level of Al and what represents a toxic concentration may need to be reevaluated.

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3. Is Desferrioxamine an Effective Therapeutic Agent for Alzheimer's Disease and Do the Initial Results of Its Efficacy Implicate Al In the Pathogenesis of the Disease?

Adding to the list of studies linking Al neurotoxicity to Alzheimer's disease is the use of desferrioxamine chelation to treat affected patients. This work has been carried out by Crapper et al. and McLachlan et al. (176,177). Desferrioxamine is a trivalent ion-specific chelating agent with a high affinity for Fe^{3+} and Al^{3+}, and has been used extensively to treat Al toxicity associated with chronic renal failure (178). The studies described by Crapper et al. and McLachlan et al. (176,177) involved a 2-year trial to determine whether long-term treatment with desferrioxamine would slow down the progression of the dementia. A videotaped home behavior assessment tool was used to sample activities that are routine in daily living. This tool was selected over others such as the Mini-Mental State Exam, and proved to be effective for conducting unbiased analysis and at the same time providing a permanent record for future review. Forty-eight Alzheimer's disease patients were studied in a randomized single-blind manner in which either desferrioxamine (125 mg i.m.) or oral placebos were administered and the patients observed using the videotaped home behavior tool. Over a 2-year observation period the rate of decline of the no-treatment group was twice as rapid as that of the desferrioxamine-treated group. One drawback of the clinical study of Crapper et al. and McLachlan et al. (176,177) is that there was no true control group for ethical reasons. The controls were given oral placebos rather than intramuscular injections (the route of administration for the desferrioxamine). It is clear that these clinical trials need to be extended before any decision on the efficacy of chelation treatment can be made and before these data can be used to imply that Al is a risk factor for Alzheimer's disease. Desferrioxamine is also a chelating agent for other metals, particularly iron. Iron is critically important in oxidative stress-induced neuronal damage. Therefore, it could be proposed that iron removal might explain some of the beneficial effects of desferrioxamine treatment. However, to ascribe the change in the course of Alzheimer's disease following desferrioxamine chelation to the removal of iron and its resultant free radical damage is not supported by any evidence in the literature beyond the fact that desferrioxamine can chelate Fe^{3+}.

4. Does Al-induced Neurodegeneration In Experimental Animals Support the Hypothesis That Al Might Play a Role In the Pathogenesis of Alzheimer's Disease?

Several animal models have been proposed as an aid in understanding Alzheimer's disease neuropathology, including transgenic mice (182), rat monkey, and

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dog (179–181). Transgenic mice have been used mainly to examine the process of Aβ deposition (182), while individual events such as apoptosis, Aβ deposition, and neurofibrillary degeneration have been explored in other animals (179–181). Studies in the authors' laboratory have demonstrated that Al maltolate–treated rabbits (especially aged animals) exhibit widespread neurofibrillary degeneration and share significant immunochemical/antigenic characteristics with those found in the central nervous system of patients with Alzheimer's disease and in amyotrophic lateral sclerosis (ALS), a motoneuron disease. These characteristics include hyperphosphorylated tau, amyloid precursor protein, Aβ, α-1-antichymotrypsin, and ubiquitin (183,184). Interestingly, Woodruff-Pak and Trojanowski have recently reported similarities in eyeblink classical conditioning between aged New Zealand White rabbits and patients with Alzheimer's disease (185). Studies using rabbits may be particularly relevant to the investigation of human disease since, according to an impressive 88 protein sequences, they belong to the mammalian order Lagomorpha, a group that has been reported more closely to resemble primates than rodents (186).

The first experiment suggesting that Al-induced neuronal changes might have relevance to Alzheimer's disease was that of Klatzo et al. in 1965 (187), who reported that the intracisternal administration of Al phosphate to New Zealand white rabbits produced intraneuronal protein aggregates that with silver staining appeared remarkably similar to the neurofibrillary tangles of Alzheimer's disease. This was a serendipitous finding, since the experiment was designed to study the immune response of the central nervous system, and an antigen had been administered intracerebrally to rabbits in Holt's adjuvant (I. Klatzo, personal communication, 1997), which contained Al phosphate. Within 2 days, the rabbits developed severe neurological symptoms and had to be sacrificed. Examination of brain tissue from these animals revealed the characteristic Al-induced neurofibrillary degeneration: silver-impregnated (argyrophilic) fibrillary inclusions found predominantly in the neuronal cell bodies (perikarya) and proximal neurites (dendrites and axon hillock) (187). Al-induced neurofibrillary aggregates at the light microscopy level closely resemble neurofibrillary tangles, which, as discussed above, are one of the histological hallmarks of neuronal aging and of Alzheimer's disease. Other histopathological hallmarks of Alzheimer's disease, such as neuritic plaques, are not present in experimental Al-induced encephalopathy. Interestingly, an abundance of neurofibrillary tangles coupled with a relative paucity of neuritic plaques characterizes the ALS/parkinsonism-dementia complex of Guam, indicating that widespread neurofibrillary tangles (without plaques and/or significant Aβ deposition) may be the cellular correlate of neurological decline, as reviewed in Mawal-Dewan et al. (188). Often neuritic plaques show a perivascular predilection, suggesting that dystrophic neurites and attendant amyloidogenic accumulation may be pathogenetically linked to vascular (or microvascular) associated factor(s). Cerebrovascular pathology may be relevant in the

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pathogenesis of naturally occurring Alzheimer's disease, and may account for increased Al accumulation during central nervous system damage. Conversely, in experimental Al-induced neurofibrillary degeneration, the factor of cerebrovascular pathology is not present, at least with respect to the nonsenescent laboratory rabbit. Also, in experimental neurodegeneration, the mode of Al delivery in the central nervous system is often direct, as with intracisternal, intraventricular, or intracerebral modes of administration, thereby effectively bypassing the blood-brain barrier. Thus, it is not surprising that the type and distribution of neurofibrillary degeneration in rabbits is in some respects different from the more naturally occurring neurofibrillary tangles in Alzheimer's disease in humans. Furthermore, the formation of neuritic plaques appears to be species-dependent, being found in few mammalian species and then only in the aged (189,190). As is developed below, these differences are less compelling when compared to the broad array of immunochemical similarities between experimental Al-induced neurofibrillary degeneration in rabbits and the various neurofibrillary lesions of neurodegenerative disorders in humans.

The relationship of Al-induced neurofibrillary degeneration in rabbits to human neurodegenerative disorders has been viewed with skepticism, because of apparent ultrastructural differences between experimental lesions in rabbits and human neurofibrillary tangles. Compared to the tangles of Alzheimer's disease, which mostly contain paired helical filaments (191–193), those produced by Al are predominantly straight (intermediate-like) filaments (194). However, this line of argument may be of limited significance since tau can form Alzheimer's-like filaments in vitro (153,195).

Aluminum-induced neurofibrillary degeneration is characterized predominantly by immunohistochemical staining with antibodies specific for hyperphosphorylated epitopes of neurofilament protein (196,197). The primary constituent of paired helical filaments in human neurofibrillary tangles is tau, although other proteins are present, including abnormally phosphorylated neurofilament proteins, ubiquitin, α1-antichymotrypsin, amyloid precursor protein, and its derived peptide, Aβ. Studies in the authors' laboratory over the past 5 years, which now have been confirmed by others (198,199), demonstrate that immunochemical similarities between the composition of the Al-induced lesions and those found in Alzheimer's disease are far closer than was originally surmised.

Abnormally phosphorylated tau (184) is present in these neurofilamentous aggregates using a variety of mAbs that recognize both nonphosphorylated and phosphorylated tau (199,200). Among the mAbs applied for this immunostaining were Tau-1, Tau-2, AT8, PHF-1, and Alz-50, indicating that both phosphorylated and nonphosphorylated tau are present. The time course of aggregation of these cytoskeletal proteins has been evaluated in the authors' laboratory (183). The results indicate that the aggregates become detectable by silver staining within

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24 h following Al maltolate administration, and that neurofilament proteins predominate; nonphosphorylated phosphorylation-independent epitopes as detected by mAb SMI-33 (Sternberger Monoclonals Inc., Baltimore, MD) are found first, followed by phosphorylated forms, immunostained by mAb SMI 31, at approximately 72 h. Tau is also detectable by around 72 h, although the characteristic epitopes of Alzheimer's disease as recognized by mAbs AT8 and PHF-1 are most distinct at 6–7 days following Al injection. It has been proposed that phosphorylation of cytoskeletal proteins drives the formation of the neurofilamentous aggregates, particularly in human neurodegenerative disorders. Since these aggregates are hyperphosphorylated, phosphorylation alone would render these protein accumulations unstable, because of the preponderance of negative charges on the phosphate groups. Thus, it is reasonable to propose that some positively charged species constitute an inherent factor in the formation and stabilization of the neurofibrillary degeneration, both in Alzheimer's disease and in experimental Al-induced neurofibrillary degeneration; in the latter, Al^{3+} is an obvious candidate for this role.

We have proposed that aging is an important factor in the susceptibility of neurons to oxidative stress and to subsequent apoptosis; both processes are observed in the Alzheimer's disease brain (201,202). Protein changes controlling apoptosis such as those of the Bcl-2 family and caspases also are altered (203). We have shown similar responses in aged rabbits treated intracisternally with Al maltolate (127). Brain tissue from these aged animals exhibits intense intraneuronal silver positivity indicative of the formation of neurofilamentous aggregates, together with oxidative stress. These changes occur in the CA1 region of the hippocampus as well as in cerebral cortical areas. Apoptosis, assessed by the TUNEL in situ technique, colocalizes with oxidative stress. Young animals treated with Al show few of these alterations, while age-matched controls are essentially negative. Further studies on the time course of these and related changes demonstrate that oxidative stress and redox-active iron accumulation in hippocampal neurons occur very rapidly, within a period of 3 h, and increase in intensity at 72 h. Changes suggestive of apoptosis are apparent by 24 h and are pronounced at 72 h. In aged animals there is an initially intense immunopositivity at 3 h for Bcl-2, with negative staining for Bax. By 72 h, when apoptosis is strongly evident, Bcl-2 is negative and Bax strongly positive. In contrast to the aged rabbits, young animals treated similarly with Al exhibit much less oxidative stress with no apoptosis, and maintain Bcl-2 immunopositivity and negative Bax staining. Our findings strongly support the key role that oxidative damage plays in the process of neurodegeneration and in the increased vulnerability to Al-induced injury in the aged animal. These are novel observations that may have important implications for aiding in our understanding of the pathogenesis of neurodegeneration occurring in Alzheimer's disease. In addition, these findings

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demonstrate that Al can produce relevant neuropathological and biochemical changes in experimental animals, albeit in an artificial system quite different from Alzheimer's disease.

Another relevant finding in Al-induced neurodegeneration has been the observation of amyloid precursor protein and Aβ immunopositivity in neurons in Al-treated rabbits (184) and in rats (204). Neurofibrillary tangles in Azheimer's disease also exhibit this Aβ staining, but the most prevalent pattern is the presence of Aβ in the neuritic plaques. It is logical to hypothesize that in Alzheimer's disease, increased Aβ initially appears intracellularly, followed by extracellular deposition after cell death. Plassman and Breitner (205) have described an Al-induced secondary structural transition in the non-Aβ component of Alzheimer's disease amyloid (NACP), generating approximately a 33% α-helix, which renders NACP resistant to proteases. Based on this finding it is suggested that Al may influence NACP protein turnover and induce aggregation via structural modifications, thus leading to Aβ deposition.

Another recent study (3) describes the presence of high concentrations of Al and iron in the hippocampus and inferior temporal cortex in both Alzheimer's disease and dementia pugilistica. The predominant findings are those of the coaccumulation of Al and iron in both neurofibrillary tangles and in nuclei of tangle-bearing neurons. Also, concentrations of Al and iron in nuclei of tangle-free neurons and neuropil are similar in Alzheimer's disease and in dementia puglistica. These studies suggest the existence of an association between the deposition of Al and iron with neurofibrillary tangle formation, and support the possibility of a global dysregulation of Al and iron transport in Alzheimer's disease and dementia puglistica. Joshi (206) has hypothesized that deregulation of Al and iron homeostasis permits the existence of colocalization of these two metals, and contributes to the accumulation of metabolic errors leading to neuronal disorders, including the formation of neurofibrillary tangles and Aβ deposition. It has been shown that cells in the rat brain possess a specific high-affinity receptor for transferrin that is independent of the metal being transported. This system is postulated to be the route whereby the iron in the general circulation reaches the brain (207). Transferrin is predominantly considered an iron transporter protein, mainly because of the relatively high abundance of iron in the circulation and the high affinity this protein possesses for iron (61). However, as mentioned earlier, transferrin also has a high affinity for Al, although not to the same extent as iron (61), and this property should provide an avenue for the transportation and intracellular deposition of Al. Evidence for this pathway is provided in a report that Al accumulation was accompanied by an increase in iron uptake in primary cultures of rat cerebral cortex (208).

Shin et al. (209) have shown that Al selectively binds to the paired helical filament tau, induces aggregation, and retards the in vivo proteolysis of this protein aggregate. Their data suggest that Al may serve as a cofactor in the formation

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of neurofibrillary tangles by its interaction with tau. All of the above studies indicate that Al may contribute to the formation of neurofibrillary lesions in neurons and thus play a role in the pathology of Alzheimer's disease. More recently Tarbox and Goux have studied Al interactions with tau protein using circular dichroism spectroscopy, and have shown that Al induces marked conformational changes independent of tau phosphorylation (23).

The biochemical similarities of the Al animal model to the intraneuronal lesions of Alzheimer's disease make this system valuable for studying perturbations of the neuronal cytoskeleton, particularly since there are still no optimal animal models for Alzheimer's disease. In the context of the present review, the animal studies cited do not prove that Al is a risk factor for Alzheimer's disease, but they do convincingly demonstrate that this metal can produce certain features of the Alzheimer's disease brain.

5. Does Hyperaluminemia Associated with Longterm Hemodialysis Induce Neurofibrillary Degeneration?

Patients on long-term hemodialysis treatment for renal failure often have hyperaluminemia resulting in elevated brain Al concentrations (38,39). The lack of widespread neuropathological features of Alzheimer's disease in such patients has been used as an argument against Al playing a role in the neurodegenerative disease process. However, there are reports in some of these hemodialysis patients of protein changes specifically associated with neurofibrillary degeneration, together with the occurrence of neuritic plaques reminiscent of Alzheimer's disease (210–213). Candy et al. (211) reported a study of neuropathological and brain Al findings on 15 patients who had undergone hemodialysis for a prolonged time and compared the results to age-matched controls. One-third of the hemodialysis-treated patients with hyperaluminemia demonstrated β-positive plaques in the cerebral cortex compared with only 8% of the controls. Both serum and brain Al concentrations in the hemodialysis-treated individuals were increased. In a later study of dialysis patients by the same group (212) it was shown that insoluble Aβ could be detected in approximately 50% of patients, but there was no correlation between the quantity of Aβ and Al. There is still a question as to the occurrence and extent of Al-induced neurofibrillary degeneration in patients with hyperaluminemia associated with long-term intermittent hemodialysis treatment; more specifically, if more sensitive histopathological techniques were used would neurodegeneration become a more common finding?

5.2 Al and Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by distinctive neuropathological changes in the spinal cord. The occurrence of neurofilamentous aggregates in the somata and axons of spinal cord motor

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neurons represents a neuropathological hallmark of human ALS, although the composition of these aggregates is somewhat different from that of Alzheimer's disease. Neurofilament proteins are the predominant component of the intraneuronal lesions in ALS and are typically immunostained by mAbs that recognize phosphorylated epitopes (214). Ultrastructurally, these lesions largely consist of straight filaments (215), and are ubiquitinated (216).

5.2.1 Guam Disease

Aluminum has been implicated in the complex of neurodegenerative disorders of ALS/parkinsonism dementia complex seen in the Western Pacific regions, particularly in the Kii Peninsula of Japan, the West New Guinea islands, and Guam, which is one of the Marianas Islands. Environmental studies in these regions have shown the presence of high concentrations of Al, iron, and manganese in soil, drinking water, and vegetables (217). High concentrations of Al and iron have been detected in brain and spinal cord tissue of ALS- and ALS/parkinsonism-dementia-affected populations (154,155). More recent reports describe specific co-deposition of Al and iron in neurofibrillary tangle-bearing neurons, particularly in the perikaryon and dendritic process (156,218). This epidemiological, genetic, and environmental evidence, as well as experimental results from experimental animals (see above), increasingly supports the concept that in neurons of ALS and ALS/PD-affected patients, a basic defect in mineral metabolism and secondary hyperparathyroidism, provoked by chronic nutritional deficiences of calcium and magnesium, leads to the increased intestinal absorption of toxic metals and to the deposition of Al, calcium, and iron (and perhaps other metals such as silicon and manganese). This elemental deposition is thought to interfere with slow axonal transport by altering the normal production of neurofilaments, resulting in neurofibrillary tangle formation, a process probably occurring long before the onset of the clinical disease (154,156,218). Interestingly, ALS-parkinsonism dementia is characterized by an abundance of neurofibrillary tangles and a relative paucity of neuritic plaques.

Guamanian neurodegenerative disease was first noted in 1944 by Harry M. Zimmerman, a physician and neuropathologist who at that time was assigned to the U.S. Naval Medical Research Unit in Guam. Zimmerman was assigned to investigate diseases such as malaria, hepatitis, etc. that threatened American troops, but he also observed a form of ALS in the native population that was strikingly more prevalent than in most other parts of the world. The bulk of the evidence available suggests that Guam disease is environmental in its cause, since it occurred with equal frequency in the local population despite their heterogeneous heritage. As the population has become more westernized, particularly in diet, the incidence of the original ALS form of the disease has decreased, and there is now a higher incidence of Alzheimer's-like symptoms that appear at approximately 60 years of age. The nearest island to Guam in the Marianas is

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Rota, whose people also show this disease. Rota was once part of the same island as Guam, hence having a similar mineral composition of the rocks and soil, and presumably of the water. Other islands in the Marianas do not share these environmental similarities, and do not have the high incidence of neurodegenerative disease, although the populations are ethnically the same. Al probably plays a key role in the pathogenesis of this disease, since there is strong evidence for high Al levels in the neurofibrillary tangles of patients who have died from this devastating affliction. The presence of high soil concentrations of Al support this hypothesis, but as yet the actual route that Al follows from the environment into the brain has not been identified.

6. ANALYTICAL TECHNIQUES FOR MEASURING Al

The development of accurate and precise methods for the measurement of Al in biological materials and in the environment is an important component of assessing the impact of this metal on health and disease, and is a topic reviewed by us (219). Collection of specimens in a manner to ensure their arrival at the analytical laboratory free of contamination is vitally important, and is difficult since Al is ubiquitous in the environment. Acid-washed plasticware is preferred to glass, since the latter contains Al that can be leached out and can produce serious contamination problems. Elimination of contamination during analysis also is difficult and requires clean air facilities and Al-free reagents.

Aluminum can be measured using gravimetric, titrimetric, photometric, and fluorimetric methods, but these chemical and physiochemical techniques are subject to many interferences, requiring isolation of Al from interfering metals prior to the final measurement. Concentrations of Al in water can be measured by such assays, but generally the sensitivity requirements for analysis of biological materials precludes the application of physiochemical techniques for this purpose.

X-ray fluorescence is very specific but also lacks sufficient sensitivity for wide application as a general technique for determining Al. Neutron activation analysis has been used (140) but the short half-life of ^{28}Al, together with phosphorus interference, makes this technique impractical for most investigators. Atomic emission methods have also been applied; however, flame emission, even with nitrous oxide-acetylene, can only detect high levels of Al in plasma (220). Atomic emission with inductively coupled plasma excitation sources can provide adequate sensitivity (221), although relatively large sample requirements restrict its application for some research purposes.

Most investigators over the years have use electrothermal atomic absorption for the measurement of Al. This technique requires only 2–100 μL of sample and is an excellent technique, both for routine monitoring and for research purposes. Careful attention to specimen preparation, use of high-quality graphite cuvettes, a stabilized temperature platform, and matrix modifiers to reduce inter-

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ferences, all contribute to providing accurate and precise measurements (222). Nitric acid and $Mg(NO_3)_2$ are satisfactory matrix modifiers. Background correction using the Zeeman correction system enhances the specificity of the technique (223). We have developed a procedure that minimizes any potential matrix effects by protein precipitation prior to the final atomic spectroscopic analysis (224). Atomic absorption spectrometry with electrothermal atomization has been used extensively in clinical laboratories, where control of hyperaluminemia in patients with chronic renal failure undergoing hemodialysis treatment is an important clinical consideration. Urine analysis is straightforward using this techniqe, and a simple dilution with deionized water is the only sample preparation necessary. However, solid tissues must be homogenized, dried, and acid-digested to produce a liquid sample prior to analysis. Inductively coupled plasma excitation sources coupled with mass spectrometry (ICP-MS) for the detection of Al have recently attained popularity, particularly in reference laboratories where the cost of the equipment can be justified by the high-volume testing in such laboratories (225). A key advantage of ICP-MS over electrothermal atomic absorption spectrometry is the multielement analysis capability, hence the popularity of the technique in reference laboratories.

Localization of Al in tissue specimens has been accomplished by histochemical means, such as the use of ammonium aurintricarboxylate (226) or of solochrome azurine (227). More direct methods employing energy-dispersive X-ray microanalysis, LAMMA, nuclear microscopy, and SIMS are discussed above. Much valuable information has been obtained using such techniques, although standardization issues have stirred some controversy regarding interpretation of the results. Contamination also has been a major concern with these procedures.

7. CONCLUSION

Aluminum is highly prevalent in the earth's crust but is complexed so that the bioavailability of its toxic species is limited. The chemistry of Al is highly complicated but this metal in certain species can disrupt many biological processes, particularly in the central nervous system. Aluminum remains an important complication in patients with chronic renal failure, although efforts to reduce oral intake from Al-containing phosphate-binding agents and the use of deionized water to prepare dialysis solutions have minimized this problem. Exposure to Al in the workplace or from antacids and antiperspirants does not appear to be hazardous to health, although excessive exposure to Al-containing dust and fumes probably causes some cognitive impairment in workers exposed for many years. Ingestion of Al in food has not been shown to present a health problem. The use of parenteral nutrition products contaminated with Al, particularly for preterm infants, is a serious form of iatrogenic poisoning and needs to be recognized and controlled. The use of these products in adults also presents a problem, but this

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is not as serious as in infants. Few neurobehavioral studies involving Al ingestion or other routes of administration have been carried out on experimental animals. Those that have been performed suggest that Al can cause learning and memory deficits. There is circumstantial evidence for Al playing a role in the pathogenesis of Alzheimer's disease and in the amyotrophic lateral sclerosis/parkinsonism-dementia complex found in Guam and other Western Pacific regions. Evidence for the role of Al in these devastating neurodegenerative diseases, particularly Alzheimer's disease, is stronger than most of the scientific community realizes. This evidence is based on the detection of Al in Alzheimer's disease neurons and the epidemiological studies of Al levels in drinking water, the effect of Al in experimental animals, and the promising results from a very preliminary clinical trial using Al chelation to treat Alzheimer's disease patients.

The measurement of Al in a variety of specimens, such as air, drinking water, food, medications, intravenous solutions, blood, urine, and tissues, has contributed substantially to our knowledge of the toxicity of Al. Atomic absorption spectrometry with electrothermal atomization has been the most widely used technique, but many laboratories at the present time use ICP-MS. Localization of Al within cells using microprobe analytical approaches represent valuable techniques and wider application of these are needed, although access to the extremely expensive instrumentation required for their use is limited.

ACKNOWLEDGMENT

Preparation of this chapter was supported in part by a grant from the U.S. Department of the Army. The authors gratefully acknowledge the careful review of this manuscript by Dr. Michael S. Forbes.

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11

Nickel

Jessica E. Sutherland and Max Costa
New York University School of Medicine, Tuxedo, New York

1. CHEMISTRY

Nickel (Ni) is element 28 and, along with iron (Fe) and cobalt (Co), forms the first transition series group VIIIb of the periodic table. In aqueous solutions, nickel is most often divalent and exists primarily as the hexaquonickel $[Ni(H_2O)_6]^{2+}$ ion; other valences include -1, 0, $+1$, $+3$, and $+4$ (1). In solution, Ni^{2+} is 4- or 6-coordinated and most commonly occurs in square planar configuration and less often in tetrahedral or octahedral configurations (2). Ni^{2+} exhibits both ''hard'' and ''soft'' acid properties (3) and thus combines with nitrogen, oxygen, and sulfur-containing ligands in addition to donors from rows IV, V, VI, and VII of the periodic table. Nickel also can combine with carbon monoxide at atmospheric pressure to form highly toxic nickel carbonyl ($Ni(CO)_4$). The acetate, nitrate, sulfate, and halogen salts of nickel are all water soluble whereas the oxides, sulfides, carbonates, phosphate, and elemental forms of nickel are insoluble in water (4).

In biological systems, Ni^{2+} coordinates with water alone or with other soluble ligands. Nickel ions tend to be less ''soft'' than other toxic metal ions and hence are more likely to participate in ligand exchange reactions. Such reactions often govern the movement of nickel among different biological compartments.

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Important biological ligands for nickel are proteins containing the amino acids histidine and cysteine (5).

2. NICKEL IN THE ENVIRONMENT

2.1 Air

Atmospheric nickel arises primarily from anthropogenic sources such as the burning of residual and fuel oil, nickel metal refining, municipal waste incineration, steel production, nickel alloy production, and coal combustion (6). These activities release approximately 56 million kg of nickel into the atmosphere per year (7). Natural sources of atmospheric nickel are windblown dust, volcanoes, and wildfires, and approximately 8.5 million kg of nickel are released into the atmosphere from these sources each year (8).

Airborne nickel is primarily aerosolic with particles of many sizes. Schroeder et al. (9) reported particulate nickel atmospheric concentrations in the United States to be 0.01–60, 0.6–78, and 1–328 ng/m^3 for remote, rural, and urban areas, respectively. The species of nickel found in the aerosols vary with the source and include nickel oxides, nickel sulfate, metallic nickel, nickel silicate, nickel chloride, and nickel subsulfide (6).

2.2 Water

Nickel in surface water arises from runoff from soil and tailing piles, from landfill leachates, and from atmospheric deposition. Industrial and municipal wastewater is another important source of nickel in surface waters. Nriagu and Pacyna (7) estimated that anthropogenic contributions of nickel to water ranged between 33 and 194 million kg/year with a median value of 113 million kg Ni/year. Much of the nickel in surface water partitions into the sediments resulting in low surface water concentrations (6). Nickel concentrations in seawater range from 0.1 to 0.5 ppb whereas nickel levels in fresh surface waters are more variable and range from 0.5 to 600 ppb (6,10,11). Leaching of nickel from soil into groundwater accounts for much of the nickel found in groundwater and this process is accelerated in regions susceptible to acid precipitation. Groundwater nickel concentrations are generally lower than 10 ppb (12,13).

2.3 Soil

On average, nickel constitutes 0.0086% of the earth's crust (6). Soil concentrations of nickel vary with local geology and anthropogenic input with typical concentrations ranging from 4 to 80 ppm (8). Major emission sources of soil nickel include coal fly ash, waste from metal manufacturing, atmospheric deposition,

urban refuse, and sewage sludge (6). Hazardous waste sites frequently have elevated soil nickel concentrations (6).

3. HUMAN EXPOSURE

3.1 Ingestion

In the general population, ingestion of nickel-containing foodstuffs represents the primary route of nickel exposure. Estimates of average daily dietary intake of nickel range from 70 μg to 300 μg (14–17). Foods that typically contain fairly high concentrations of nickel (i.e., greater than 1 ppm) include oatmeal, dry legumes, hazelnuts, cocoa, soybeans, and soy products (10,15). Shellfish, depending upon the area from which they are harvested, can also contain high concentrations of nickel (6). Food preparation in stainless steel cookware can add up to 0.1 mg Ni to the diet per day (18). Drinking water nickel concentrations average 2 ppb and are usually less than 20 ppb (4,8). Consumption of 2 L of drinking water per day would therefore add 40 μg nickel to the daily amount of ingested nickel. In the United States, there is no Environmental Protection Agency (EPA)-mandated legal limit on the amount of nickel in drinking water but the agency has recommended a maximum contaminant level (MCL) of 0.1 mg Ni per liter of drinking water (19). Nickel levels in drinking water may be elevated due to corrosion of valves, pipes, or faucets made from nickel-containing alloys (6).

3.2 Inhalation

On average, individuals in the general population inhale 0.1–1.0 μg Ni/day (20). The highest reported general population intake of nickel from air is 18 μg Ni/day (8).

Exposure to nickel also occurs from tobacco smoking. Cigarettes, on average, contain 1–3 μg Ni (4,6) and mainstream smoke from one cigarette contains 0–0.51 μg Ni (21). Smoking a pack of cigarettes results in an inhalation exposure to 2–12 μg Ni (8).

Occupational exposure to nickel occurs via inhalation of nickel-containing aerosols, dusts, fumes, and mists. Nickel alloys and compounds have widespread industrial applications and each year, several million workers worldwide are occupationally exposed to nickel (22). Nickel mining and refining, nickel alloy production, nickel electroplating and thermal spraying, welding, production of nickel-cadmium batteries, manufacture of some types of enamel or glass, and the use of nickel compounds as chemical catalysts result in occupational nickel exposure (23–25). In these industrial settings, inhalation exposure varies in terms of amount and in terms of nickel speciation, depending on the activity. The American Conference of Governmental Industrial Hygienists (ACGIH) recently

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adopted threshold limit values (TLV) for an 8-h workday, 40-h workweek of 0.1 mg Ni/m^3 air for water-soluble nickel, 0.2 mg Ni/m^3 air for water-insoluble nickel, and 1.5 mg/m^3 for elemental/metallic nickel (26). The U.S. Occupational Safety and Health Administration (OSHA) has established permissible exposure limits (PEL) of 1 mg/m^3 as 8-h time-weighted averages for insoluble and soluble nickel compounds (27,28).

3.3 Dermal

Humans are also exposed to nickel via dermal contact with stainless steel, coins, fasteners, and jewelry and by occupational exposure to dusts, aerosols, and liquid solutions containing nickel (6). Sunderman (13) reported that soaps may also contain nickel if they were hydrogenated with nickel catalysts.

3.4 Iatrogenic

Nickel alloys used in surgical and dental prostheses, and clips, pins, and screws used for fractured bones release small amounts of nickel into the surrounding tissue and extracellular fluid (20,29). Nickel can also be absorbed from dialysis and intravenous solutions. Kidney dialysis solutions typically contain $\leq$1 μg Ni/L but have been reported to contain as much as 250 μg Ni/L (30). Intravenous solutions containing albumin have been reported to contain as much as 222 μg Ni/L (8).

4. ESSENTIALITY

4.1 Plants and Microorganisms

There are six known nickel metalloenzymes. In two of these enzymes, urease and bacterial glyoxalase I (GlxI), catalysis does not depend upon the redox chemistry of nickel at the active site. In the other enzymes [nickel superoxide dismutase (NiSOD), hydrogenase, carbon monoxide dehydrogenase (CodH), and methyl coenzyme M reductase (MCR)], the redox chemistry of nickel plays a key role.

Urease, found in plants and microorganisms, hydrolyzes urea to form ammonia and carbamate, which degrades further to form a second ammonia molecule and carbon dioxide (31). Two nickel atoms are present at each active site (32).

Glyoxalase I from *Escherichia coli* participates in the detoxification of α-keto aldehydes to 2-hydroxycarboxylic acids. *E. coli* Glx I is a homodimer with a single Ni^{2+} ion per dimer (33).

Bacterial nickel superoxide dismutase was isolated in 1996 (34). The gene for this enzyme, sodN, is upregulated by Ni^{2+}. Posttranslational modification of the enzyme is also regulated by Ni^{2+} (35). Like other cellular superoxide dismutases, NiSOD catalyzes the dismutation of superoxide to peroxide and molecular

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oxygen. In the reaction, Ni (III) is reduced to Ni (II) by superoxide and then reoxidized (33).

Two types of bacterial hydrogenases contain nickel in their catalytic sites. These enzymes catalyze the interconversion of dihydrogen to/from hydrogen ions (32).

Bacterial carbon monoxide dehydrogenase catalyzes the interconversion of carbon monoxide and carbon dioxide (33). In acetogenic and methanogenic bacteria, CodH also has acetyl-CoA synthase (ACS) activity (32). The site of CO binding and oxidation contains a nickel center with S-donor ligands linked to an iron/sulfur (Fe_4S_4) cluster (33).

Methyl-coM reductase catalyzes the final step of methanogenesis in bacteria (i.e., the reduction of methyl-coenzyme M by coenzyme B to methane) (36). Nickel porphinoid (coenzyme F430) is the prosthetic group of MCR.

Recently, Dai and colleagues (37) reported that E2 and E2[1] enzymes share the same protein component but catalyze two different oxidation products of the acireductone intermediate in the methionine salvage pathway in bacteria. E2 activity is gained after addition of Ni^{2+} or Co^{2+} to the apoenzyme whereas E2′ activity was detected after addition of Fe^{2+}. Production of each in intact *E. coli* was regulated by metal availability. Further work is needed to elucidate whether these metals constitute part of the active site or merely affect its structure, resulting in the two different reaction products.

Peptide deformylase (PDF) catalyzes the hydrolysis of *N*-formylmethionine from polypeptides in bacteria. When isolated in the presence of Ni^{2+}, PDF is bound to nickel and is highly active compared to its unbound state in which Zn is bound instead. It is not clear, however, whether nickel is the native metal used by PDF (33).

Organisms that employ nickel for enzymatic catalysis have evolved a number of nickel-binding proteins for acquisition, transport, storage, and enzyme assembly (33). It was recently shown that expression one of these transport systems (nickel-specific ABC transport system) in *E. coli* is repressed by nickel-responsive regulator when high extracellular concentrations of nickel exist. This prevents transport of potentially toxic amounts of nickel into the cell (38). In humans, *Heliobacter pylori*, the bacterium that causes peptic ulcer disease, relies upon urease to produce enough ammonia to neutralize gastric acid and hence allow bacterial colonization of the gastric mucosa. This bacterium needs to scavenge Ni^{2+} ions from gastric mucosal cells and has a specialized high-affinity nickel transporter (NixA) for this purpose (39).

4.2 Animals

Nickel is believed to be an essential element for rats (40,41), chicks (42), swine (43), goats, and sheep (44). The reported symptoms of nickel deficiency in these animals included depressed growth; depressed hematocrit; low plasma glucose;

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impaired reproductive performance; hepatic abnormalities including altered lipid metabolism; decreased ruminal urease activity; altered copper, iron, zinc, and calcium metabolism; and altered cobalamin (vitamin B_{12}) function (45). However most of these symptoms varied considerably among studies; therefore, reaching a consensus regarding the nutritive roles of nickel in animals is difficult. Moreover, interpretation of animal studies may be confounded by possible pharmacological actions of the high amounts of nickel added to control or ''nickel-adequate'' diets in some of the experiments (46,47). Nevertheless, Reeves (48) recommended addition of 500 mg Ni (as $NiCO_3$)/kg diet to purified laboratory animal diets.

To date, there is no evidence that nickel is essential in humans nor has a nickel-deficient state in humans been identified. There are no established nutritional standards for nickel; however, an ''acceptable daily dietary intake'' of 100–300 μg has been proposed (49).

5. METABOLISM

5.1 Cellular Uptake

Cellular uptake of nickel into cells is modulated by nickel's solubility. Experiments with cultured cells have indicated that insoluble nickel compounds are taken up by cells to a greater extent than are soluble nickel compounds (50). Uptake of soluble nickel from serum into tissues is believed to be governed by ligand exchange reactions. A proposed model suggests that L-histidine removes nickel from serum albumin and mediates its entry into cells. Active transport and diffusion probably function in movement of soluble nickel across plasma membranes but the actual mechanisms are not well understood. Soluble nickel and magnesium may share a common transport system (51). Uptake of ionic nickel may be low owing to competition with Mg^{2+} ions normally present in millimolar amounts (52). Some soluble nickel probably also enters cells via calcium channels (53,54). There is also evidence that nickel and iron may also share common cellular uptake mechanisms with nickel effectively competing with iron for low-affinity transport in cultured rabbit or rat reticulocytes (55,56). Iron-deficient rats, given intraperitoneal (i.p.) injections of 4 μg ^{63}Ni/kg body weight, accumulated more ^{63}Ni in tissues than did iron-sufficient rats (57). Nickel binds to the iron transport protein transferrin (58) and it is possible that some nickel enters cells on transferrin. Tandon et al. (59) reported that dietary iron deficiency had no effect on tissue disposition of nickel in rats following an intraperitoneal injection of 120 μmol $NiCl_2$-$6H_2O$/kg body weight. Tissue disposition following injection of such a high dose of nickel may not have accurately reflected physiological conditions.

Cellular uptake of soluble nickel is also temperature-dependent. Abbrachio

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et al. (60) reported that uptake of nickel following treatment of Chinese hamster ovary (CHO) cells with $NiCl_2$ at 4°C was decreased 50% compared to cells maintained at 37°C. Similarly, uptake of soluble nickel by cultured rat primary hepatocytes was decreased by 20% compared to uptake at 37°C (54), suggesting that nickel transport, at least in part, may be mediated by membrane carriers.

In contrast, insoluble nickel compounds enter the cell via phagocytosis (61–63). This process is influenced by crystalline structure, surface charge, and particle size (64–66). Although the mechanisms are unclear, cellular nickel accumulation following exposure to insoluble nickel is reduced in the presence of extracellular magnesium (50,67).

5.2 Absorption

5.2.1 Inhalation

In general, inhaled nickel-containing particles with diameters greater than 2 μm settle in the upper respiratory tract whereas particles smaller than 2 μm lodge in the lower respiratory tract and in lung tissue. In humans, absorption of respired nickel has been estimated by measuring urinary nickel levels following inhalation exposure. It has been estimated that approximately 35% of the nickel present in the respiratory tract of humans is absorbed into the bloodstream (6). It has been proposed that soluble nickel compounds (e.g., nickel sulfate, nickel chloride) are absorbed to a greater extent (as estimated from urinary nickel) than insoluble compounds (e.g., nickel subsulfide, nickel oxide) (68,69). However, greater elevations in urinary nickel following inhalation of soluble nickel may reflect more rapid clearance of this form rather than greater absorption per se. Accordingly, urinary nickel concentrations may not be reliable indicators of exposure to insoluble nickel via inhalation (70,71).

Uptake of inhaled nickel into the brain from the nasal epithelium via olfactory neurons may represent another route of exposure to inhaled nickel (72). In rats and pike, intranasal instillation of $^{63}Ni^{2+}$ resulted in migration along the olfactory neurons and entry into the cerebrum (72–74). The significance of this exposure route in terms of overall nickel uptake is unknown because of a lack of data regarding the proportion of inhaled nickel in the nasal epithelium that is taken up by the olfactory pathways. However, it is interesting to note that impairment of olfactory sensation has been observed in workers in nickel refineries and in rats exposed to soluble nickel (72).

5.2.2 Ingestion

Nickel absorption from the gastrointestinal tract is higher when the nickel is present in drinking water as opposed to food. Humans given 12, 18, and 50 μg/kg body weight absorbed 27 ± 17% of the nickel sulfate present in drinking water as compared to only 0.7 ± 0.4% when it was in food (75). Solomons et al. (76)

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and Nielsen et al. (77) reported a similar decrease in the bioavailability of nickel in food as compared to drinking water. These studies estimated absorption via balance studies where nickel concentrations in urine and feces were measured for up to 4 days following ingestion. Unfortunately, high doses of nickel were administered to produce detectable changes in nickel concentrations in urine and blood. Recently, nickel metabolism studies have been conducted in humans with stable isotope tracers (^{61}Ni and ^{62}Ni) (77–79). Nickel absorption in these studies ranged from 11 to 33%. In all of these tracer studies, the nickel isotope was administered in water; it is important to remember that nickel is much more bioavailable in water than when ingested in foodstuffs.

The mechanisms of intestinal nickel absorption have been studied using everted gut sacs (80), perfused rat jejunal and ileal segments (81–83), and Caco-2 cell monolayers (84). Absorption of nickel in the gut is believed to involve both active and passive transcellular processes; the role of paracellular transport in nickel absorption is not clearly defined (82,83).

Nickel and iron may share some absorptive mechanisms (57,84,85). However, from a nutritional standpoint, iron absorption is likely to be nonaffected by poorly bioavailable dietary nickel. Supplementation of diets with 3–100 mg Ni/kg diet did not affect iron status in rats (86). Cobalt may also compete with nickel and iron for uptake in the gut (87). Stangl et al. (88) reported that cattle deficient in vitamin B_{12} accumulated significantly more iron and nickel in liver than vitamin B_{12}-sufficient animals, which suggests increased absorption and/or increased hepatic uptake of nickel by the cobalt-deficient cattle.

There may be homeostatic regulation of nickel absorption from the gut. The rates of nickel uptake in everted jejunal sacs obtained from nickel-depleted rat pups were significantly greater than those in obtained from nickel-adequate pups (80). Homeostatic regulation of uptake is a hallmark of many essential trace metals (e.g., zinc, iron, copper, and manganese). Demonstration of this phenomenon in vivo for nickel is currently lacking but would do much to bolster arguments for nickel's essentiality.

5.2.3 Dermal

Soluble nickel salts are absorbed through the skin to a greater extent than insoluble compounds. Nickel chloride applied to excised human skin was absorbed approximately 50 times faster than nickel sulfate (89). However, dermal absorption was low; approximately 0.2% of the nickel chloride penetrated the skin sample in the 144 h immediately following application. Absorption of nickel chloride approximated 3.5% in occluded skin. Following dermal application, nickel is retained in the skin for extended periods (90). This is important toxicologically

because retention of nickel in the skin leads to nickel sensitivity and contact dermatitis.

5.3 Tissue Disposition

In the bloodstream, nickel binds to albumin, transferrin, L-histidine, and α-2-macroglobulin (also sometimes called nickeloplasmin) (91). The primary binding site of nickel to albumin is a histidine residue at the third position from the amino terminus of the protein (92). Neighboring residues (aspartate and alanine) are also involved in nickel complexation (93) forming a square planar N-terminal complex of nickel and albumin (94). Copper also binds to this site with an affinity one order of magnitude higher than nickel (93). Bal et al. (94) reported that human, bovine, and porcine albumins contained a second binding site for Ni(II), which also binds Cu(II), Zn(II), and Cd(II) with similar affinity but is not believed to be an important Cu(II) binding site under physiological conditions. In humans, approximately 76% of plasma nickel is bound to high-molecular-weight proteins (91). Nickel bound to α-2-macroglobulin is not readily exchangeable and hence this protein is not believed to be an important nickel transport protein (95).

In humans, serum and whole blood nickel concentrations in unexposed individuals range from 0.1 to 1 μg Ni/L (75,96–99). Plasma or serum concentrations of nickel in occupationally exposed workers range from 1 to 12 μg Ni/L (96,100–102). Average serum nickel concentrations of 6–7 μg Ni/L have been reported in hemodialysis patients (99,103). Workers who accidentally ingested 0.5–2.5 g of nickel in drinking water had serum nickel concentrations of 13–1340 μg Ni/L (104).

Numerous animal studies have indicated that the kidney and lung are the primary organs in which nickel accumulates following injection, intratracheal, or oral administration of soluble nickel compounds. Smaller amounts of nickel accumulate in liver, other soft tissues, and bone (2,105–107).

Nickel measurements made in human autopsy samples revealed that nickel was ubiquitously distributed in the body with highest concentrations present in lung (108) or bone (109). Whole-body nickel levels were found to be less than 600 mg Ni/kg dry tissue (91). In most studies, lung nickel concentrations increased with age (108,110–112) but Raithel et al. (113) and Fortoul et al. (114) found no such relationship. Lung nickel concentrations varied with topography within the lung and were generally highest in the upper lung regions (113,115,116).

5.4 Excretion

Animal studies reveal that most nickel absorbed from soluble forms, regardless of the route of exposure, is excreted in urine (Table 1). Smaller amounts of nickel

TABLE 1 Urinary and Fecal Excretion of Administered Nickel

Nickel compound	Animal species	Route of exposure	Period after dosing (h)	Percentage of Ni dose in urine	Percentage of Ni dose in feces	Ref.
$^{63}NiCl_2$	Rat	i.v.	72	61	5.9	541
$^{63}NiCl_2$	Rat	i.v.	72	78	15	119
^{63}Ni & ^{62}Ni	Rat	i.v.	80	60	5.4	79
$^{63}NiCl_2$	Rat	i.v.	96	100	0	542
$^{63}NiCl_2$	Rat	i.p.	144	80	6	543
$^{63}NiCl_2$	Rat	i.t.	72	75	NA	544
$^{63}NiCl_2$	Rat	i.t.	72	63	5	545
$^{63}NiCl_2$	Rat	i.t.	72	78.5	NA	546
$^{63}NiCl_2$	Rat	i.t.	96	54–82	13–31	127
^{63}NiO	Rat	i.t.	72	16	17	545
$^{63}Ni_3S_2$	Mouse	i.t.	72	33	57	130

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are also excreted in feces. Possible sources of the fecal isotopic nickel were biliary, pancreatic, and intestinal secretions (117,118). Rabbits excreted 9.2% of intravenously (i.v.) injected $^{63}NiCl_2$ in bile in the first 5 h following exposure (119); however, biliary excretion of i.v. ^{63}Ni in rats accounted for less than 0.5% of the administered dose (120).

When insoluble nickel compounds were intratracheally (i.t.) instilled, urine remained an important excretory route; however, fecal elimination also was significant (Table 1). In addition to biliary, intestinal, and pancreatic secretion of absorbed nickel, ingestion of nickel particles cleared from the lungs and trachea by mucociliary clearance is believed to contribute to fecal nickel content.

Humans who ingested tracer quantities of ^{62}Ni excreted 51–82% of the absorbed dose in urine in the 5 days following exposure (78). In nonexposed healthy humans, urinary nickel concentrations typically range from 0.1 to 13.3 μg Ni/L (71,121). Urinary nickel concentrations as high as 300 μg/L have been reported for occupationally exposed workers but are typically much less (3–50 μg/L) (122,123).

Renal excretion of nickel occurs via glomerular filtration of low-molecular-weight nickel complexes (e.g., histidine complexes) present in serum (124). Rates of nickel clearance in humans were found to be less than creatine clearance rates suggesting that up to 65% of the nickel present in the glomerular filtrate was reabsorbed by the kidney tubules (91).

The importance of biliary nickel excretion in humans is not well defined. At autopsy, bile from gallbladder specimens contained nickel concentrations of 2.3 μg Ni/L indicating that humans may secrete 2–5 μg of Ni/day in bile (108). This estimate is comparable to the amount of nickel excreted per day in urine by healthy individuals. However, biliary secretion of absorbed ^{62}Ni in humans following ingestion of a tracer dose of the isotope was believed to be negligible (78).

Relatively high nickel concentrations were reported to be present in human sweat (125). In some situations, substantial nickel excretion may occur via perspiration (96).

5.5 Toxicokinetics

5.5.1 Animals

Whole-body retention of nickel in mice equaled 0.02–0.36 percent and 1–6 percent 45–75 h after oral (p.o.) and i.p. administration of $^{57}NiCl_2$, respectively (126). Most often, pulmonary clearance rates have been measured following nickel inhalation. These estimates vary with dose and nickel compound (127–132). Soluble nickel compounds are cleared more rapidly than insoluble ones. Mathematical models of deposition, clearance, and retention kinetics of inhaled soluble and insoluble nickel compounds in rat lung have recently been published (133).

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5.5.2 Humans

In humans who had accidentally ingested nickel sulfate and nickel chloride, the mean biological half-time in serum was estimated to be 60 h (104). In human volunteers, the average elimination half-time following ingestion of nickel sulfate in drinking water or in food averaged 28 h (75). This estimate agrees with that of Tossavainen et al. (134), who reported half-times of nickel elimination of 17–39 h in electroplating workers who inhaled soluble nickel compounds.

Some nickel is apparently retained in long-term storage compartments within the body. Urinary nickel concentrations were elevated in nickel refinery workers following a 6-month plant closure (135), in nickel welders following 4 weeks of vacation (136), and in electrolytic nickel refinery workers and nickel platers after 1–5 weeks of vacation (102,123). Retired nickel workers had elevated plasma and nasal mucosal nickel concentrations (69). The half-life of nickel in the nasal mucosa was estimated to be 3.5 years. Biological half-lives of nickel in plasma following inhalation of insoluble nickel compounds have been estimated to range from 6 to 120 days and averaged 33 days in nickel workers (137).

6. SYSTEMIC TOXICOLOGY

Many nickel toxicology studies performed in laboratory animals have utilized high doses of nickel and routes of exposure that may not be relevant to the typical human situation. However, these studies often provided important mechanistic information regarding nickel's toxicity in various organ systems.

6.1 Respiratory Toxicity

6.1.1 Animals

Numerous animal studies have demonstrated significant respiratory toxicity following nickel exposure via inhalation. High inhalation exposures to Ni_3S_2 (3.6–7.3 mg Ni/m^3) have resulted in death, necrotizing pneumonia, emphysema, and chronic inflammation in lungs of rats (138,139). Exposure-related mortality (due to necrotizing pneumonia) was also observed in mice exposed to 7.3 mg Ni/m^3 as Ni_3S_2. Mice exposed to 3.6 mg/m^3 as Ni_3S_2 also developed fibrosis and had inflamed lung tissue (138,139). Inhalation of high concentrations of soluble nickel (13.3 mg Ni/m^3 and 1.6 mg Ni/m^3 as $NiSO_4{\cdot}6H_2O$) was lethal to rats and mice, respectively (139). Pulmonary inflammation was the cause of death in rats and necrotizing pneumonia was considered to be the cause of death in mice. The respiratory toxicity ranking in rats and mice was $NiSO_4{\cdot}6H_2O > Ni_3S_2 \gg NiO$ (139). Biochemical markers of lung inflammatory responses were elevated in bronchoalveolar fluid obtained from rats that were intratracheally instilled with 50 μg $NiSO_4$ 2–3 days previously; no evidence for increased lipid peroxidation in the lung was observed (129).

More relevant to humans are studies that employed nickel exposure concentrations similar to the current threshold limit values (TLV). Respiratory toxicity has been found to vary among animal species and is also dependent upon the length of the exposure period and the chemical composition of the nickel compound.

Rats exposed to 0.4 mg Ni/m^3 and mice exposed to 0.9 mg Ni/m^3 as Ni_3S_2 for 12 days developed respiratory and olfactory lesions (138). In a subsequent study, inflammatory lesions in lung, alveolar macrophage hyperplasia, alveolar proteinosis, and increases in β-glucuronidase, lactate dehydrogenase, and total protein content in bronchoalveolar lavage fluid were observed in rats 2–7 days following inhalation of 0.4 or 1.8 mg Ni/m^3 as Ni_3S_2 indicating that inhalation of insoluble nickel near the current TLV caused damage to the respiratory tract after only a few days of exposure (140).

Inflammation was present in lungs of rats and atrophy of the nasal epithelium occurred in rats and mice that inhaled 0.8 mg Ni/m^3 as $NiSO_4 \cdot 6H_2O$ for 12 days (141). This is the smallest soluble nickel dose that has been used in short-term toxicity tests to date.

In a longer exposure period, rabbits exposed to 0.13 mg/m^3 of metallic nickel dust for 4 or 8 months or 0.3 mg/m^3 as $NiCl_2$ for 1 month exhibited increases in alveolar type II cell numbers and cell volume and increased total lung phosopholipid content (especially disaturated phosphatidylcholines, which are a primary constituent of surfactant) (142,143). Rats exposed to 1 mg Ni_3S_2/m^3 for 78 weeks via inhalation had shortened life spans, reduced body weights, and increased inflammatory (pneumonitis, atelectasis, bronchitis, bronchiectasis, and emphysema) and hyperplastic lesions in lung compared to controls (144). Lung lesions developed at exposure levels of $NiSO_4 \cdot 6H_2O$ and Ni_3S_2 of 0.1 mg Ni/m^3 in rats and 0.2 mg Ni/m^3 in mice following 13 weeks of inhalation exposure (145). Rats and mice exposed for 13 weeks to Ni_3S_2, $NiSO_4$, and NiO at human occupational levels had elevated levels of lactate dehydrogenase, β-glucuronidase, total protein, total cells, and neutrophils in their bronchoalveolar lavage fluid indicating the occurrence of cytotoxic and inflammatory responses in the lung (146). Nickel sulfate was more toxic than Ni_3S_2, which was more toxic than NiO. Dunnick et al. (145) also found that soluble nickel was more toxic to the respiratory system than insoluble nickel and that rats were more sensitive than mice to effects of inhaled nickel. Similarly, Tanaka et al. (147) reported that green NiO, though cleared slowly from the lung, was relatively nontoxic to rats following inhalation exposure to 0.2 or 0.9 mg Ni/m^3 for up to 12 months.

In a chronic exposure study, rats inhaled 0.03–0.11 mg Ni/m^3 as $NiSO_4 \cdot 6H_2O$, 0.11–0.73 mg Ni/m^3 as Ni_3S_2, or 0.5–2.0 mg Ni/m^3 as NiO for 2 years. Mice were exposed to the same compounds for 2 years at exposure concentrations of 0.06–0.22, 0–0.9, or 1–3.9 mg Ni/m^3 as $NiSO_4 \cdot 6H_2O$, Ni_3S_2, or NiO, respec-

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tively. Both species developed exposure-related nonneoplastic respiratory lesions including focal alveolar/bronchiolar hyperplasia, inflammation, and/or fibrosis of the lung (148).

Other investigators have reported respiratory toxicity following intratracheal instillation or i.m. or i.p. injections of nickel compounds. Lavage fluid obtained from rats instilled i.t. with 1 μmol Ni as Ni_3S_2, $NiSO_4$, or $NiCl_2$ contained significantly elevated levels of lactate dehydrogenase, β-glucuronidase, total protein, glutathione reductase, and sialic acid indicating increased cytotoxicity, phagocytic activity, and inflammatory response (149). Moreover, instillation of 0.1 or 1 μmol of nickel as $NiCl_2$ or $NiSO_4$ and instillation of 1 μmol of nickel as Ni_3S_2 resulted in significant increases in neutrophils and macrophages in the lavage fluid, which also indicated the presence of an inflammatory response in nickel-exposed lungs (149). The lungs of rats receiving a lethal dose of $NiSO_4$ (14 i.m. injections of 125 μmol/kg) exhibited proliferation of cells in the alveolar lining, thickening of the alveolar wall, and proteinaceous alveolar exudate (150). Increased lipid peroxidation, lactate dehydrogenase activity, total protein, phospholipid and Ca, Fe, and Zn and decreased glutathione and alkaline phosphatase activity were observed in the lungs of mice following i.p. injection of 5 mg $NiCl_2$/kg (151). Administration of the nickel chelators meso-2,3-dimercaptosuccinic acid (DMSA) and *N*-benzyl-D-glucaminedithiocarbamate (BGD) decreased pulmonary nickel concentrations and effectively protected against the nickel-induced pulmonary damage.

6.1.2 Humans

The lungs and nasal cavity are the primary targets for nickel-induced cancers. While these are the most hazardous respiratory effects of nickel exposure, other respiratory system effects in humans have been reported. Death from adult respiratory distress syndrome occurred in one worker exposed to very high concentrations (382 mg/m^3) of metallic nickel (152). Epithelial dysplasia and hyperplastic/polyploid nasal mucosa were observed in active and retired nickel workers (153–159). Some workers have developed occupational asthma as a result of nickel exposure (160–165) either as a hypersensitivity reaction or as a response to primary irritation (6). A dose-response model using noncancer end points for inhalation exposure to nickel compounds has recently been published (166).

6.2 Immunotoxicity

Nickel's effects on the immune system are twofold. It is a powerful sensitizing agent and, as such, elicits hypersensitivity reactions manifested as contact dermatitis and asthma. In addition, nickel is an immunosuppressant and decreases macrophage and natural killer (NK) cell activity. In terms of public health, nickel

hypersensitivity constitutes a far greater concern than nickel-induced immunosuppression.

6.2.1 Hypersensitivity

Animals. Mechanistic studies of nickel hypersensitivity have been hampered by lack of suitable animal models. It has been difficult to consistently induce nickel contact allergy in mice. Recent work has demonstrated sensitization in mice raised in metal-free cages for at least two generations and intradermally injected with $NiSO_4$ or $NiCl_2$ in Freund's complete adjuvant (FCA) (167,168) or in combination with an irritant or interleukin-2 (IL-2) (168). In addition, enhanced sensitization was achieved in mice following subcutaneous (s.c.) injection with Ni(III) or Ni(IV) (168). Ishii et al. (169) also demonstrated that mice could become nickel-sensitized following chronic epicutaneous administration of $NiSO_4$, which is a route of exposure most analogous to the human situation. Work with guinea pigs has yielded inconsistent results (20) although Wahlberg and Boman (170) have demonstrated consistent sensitization of guinea pigs to nickel, using intradermal injections of FCA and $NiSO_4$.

Humans. Hypersensitivity arises in the general population and in occupationally exposed individuals and there is also growing concern that nickel in air pollution particulate matter may constitute a risk to sensitive individuals. Such particles typically contain a mixture of toxic metals and hence nickel's role in air-pollution-induced asthma has not been clearly defined (171–173).

Type IV cell–mediated delayed-type hypersensitivity (DTH) reactions, presenting as contact dermatitis, are the most prevalent form of nickel-induced hypersensitivity in the general population (20). Dermal exposure to nickel-containing alloys in jewelry and coins is the primary cause of nickel contact dermatitis. Nickel sensitivity is fairly common; it was diagnosed in approximately 30% of women and 5% of men in two Norwegian study populations (174). Women are believed to be more at risk because of more frequent skin contact with jewelry. Ear piercing, also more common among females, is another activity strongly associated with nickel sensitivity (174–176). Occupationally, nickel contact dermatitis is fairly common among hairdressers, bank clerks, retail clerks, caterers, domestic cleaners, and metalworkers (177,178). Clinically, nickel sensitivity may arise in patients with dental prostheses (179–181) or metallic orthopedic implants (182,183). In some patients, nickel contact dermatitis is exacerbated by ingestion of dietary nickel (98,184,185).

In nickel contact dermatitis, nickel cations penetrate the epidermis and bind as haptens to serum or cellular proteins and interact with epidermal dendritic cells (i.e., Langerhans cells), which then migrate to the lymph nodes and act as antigen-presenting cells (APC) (186,187). T lymphocytes recognize the antigen

complexed to class II major histocompatibility complex (MHC) molecules on the cell surface of the APC and become activated and differentiate into nickel-specific memory T lymphocytes (188). These cells secrete cytokines that induce local inflammation and dermatitis (189–193).

Keratinocytes are also directly involved in the pathogenesis of nickel-induced contact dermatitis. Upon exposure to nickel, cultured normal human keratinocytes and transformed human keratinocytes expressed higher amounts of intercellular adhesion molecule 1 (ICAM-1) (194–196) and exhibited enhanced T-cell binding (196). Enhanced ICAM-1 expression on keratinocytes obtained from nickel-sensitive subjects had previously been reported (197). In addition, Garioch et al. (197) observed increased numbers of lymphocytes expressing leukocyte-function-associated antigen (LFA-1), a ligand for ICAM-1, in the skin of nickel-sensitive individuals. Nickel also caused increased expression of the inflammatory cytokines, IL-1, and tumor necrosis factor-alpha in cultured keratinocytes (194,195,198).

Expression of adhesion molecules in vascular endothelium, important for leukocyte recruitment during inflammation, is also upregulated by nickel. Expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin is upregulated following nickel exposure (199,200). On a molecular level, transcription of these adhesion molecules is regulated, at least in part, by the transcription factor NF-κB, which is upregulated by nickel (201).

As mentioned above, occupational asthma has occurred in some nickel workers. Nickel-induced asthma is believed to constitute a type I hypersensitivity reaction, mediated by nickel-specific IgE antibodies (162,164).

6.2.2 Immunosuppression

Animals. Studies in laboratory animals have also demonstrated that nickel is toxic to elements of the immune system and hence can cause immunosuppression. Immunosuppression was observed in mice exposed via inhalation to 0.25 mg Ni/m^3 as $NiCl_2$ for 2 h but not in those exposed to 0.1 mg Ni/m^3 (202). After inhalation of 0.46 mg Ni/m^3 as $NiSO_4$ or 0.50 mg Ni/m^3 as $NiCl_2$, mice were more susceptible than controls to challenge with *Streptococcus* (203). Mice that were challenged with a sublethal dose of murine cytomegalovirus (MCMV) and then given an i.m. injection of 20 mg $NiCl_2$/kg 3 days later had higher MCMV-induced mortality rates than challenged mice that had not been injected with $NiCl_2$. In contrast, MCMV-challenged mice exposed to 500 or 1000 μg Ni/m^3 as $NiCl_2$ via inhalation for 2 h on post-MCMV-injection days 0, 1, 2, and 3 did not have significantly increased MCMV-induced mortality rates compared to controls (204). This illustrates that route of nickel administration may influence immune parameters.

Nickel impacts both cellular and humoral immunity in laboratory animals. Humoral immunity, as gauged by decreased antibody production against injected

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antigens, decreased following nickel exposure in several animal studies (172,202,205–207).

Pulmonary macrophages represent one of the first lines of host defense against inhaled particles. Many animal studies have indicated that macrophages are susceptible to nickel toxicity as gauged by decreased phagocytic capacity (203,208–210), impaired trypan blue exclusion (140,211), decreased oxidative burst formation (212), reduced lysozyme production (208), and decreased antibacterial activity. Alveolar macrophages from rabbits exposed for 1 month to an aerosol of 0.43 mg Ni/m^3 as $NiCl_2$ had decreased bactericidal activity against *Staphylococcus* compared to those from nonexposed animals (213).

Natural killer (NK) cells participate in tumor surveillance and host defense against viral infection (20) and are detrimentally affected by nickel exposure (206,209,214,215). The increased susceptibility of mice to MCMV following nickel exposure (as described above) may have resulted, at least in part, from depressed NK-cell activity (204).

Experimentally, nickel's effects on NK cells are highly dependent upon route of administration. Animal studies have consistently demonstrated that parenteral administration of nickel depresses splenic NK cell activity; however, results from inhalation studies are ambiguous. Splenic NK activity in mice was not altered following a 12-day inhalation exposure to Ni_3S_2 (138). However, longer exposure (i.e., 13 weeks) to Ni_3S_2 or $NiSO_4$ either decreased splenic NK cell activity or increased susceptibility to challenge with NK-sensitive B16F10 melanoma cells (209).

The only studies examining NK activity in lungs following i.t. nickel exposure were conducted in cynomolgus monkeys and demonstrated that NK cell activity in all the lungs examined was increased regardless of nickel exposure or prior injection with sheep red blood cells (216).

Humans. Currently, there is no evidence for nickel-induced immune suppression in humans. Because of the relevance of human inhalation exposure to nickel compounds and the existing evidence for NK-cell tumor surveillance, more research effort should be devoted to examining lung-associated NK-cell activity following TLV exposures to soluble and insoluble nickel compounds.

6.3 Nephrotoxicity

6.3.1 Animals

In experimental animals, administration of high soluble nickel doses has resulted in nephrotoxic symptoms such as increased urinary protein and amino acid content (217–220,221), renal tubule lesions (222–225), binding of nickel to anionic glycosaminoglycan sites of glomerular basement membranes (124,226), and polyurea (227). Rats exposed to nickel carbonyl by inhalation of an LD_{50} dosage excreted elevated amounts of protein, amino acids, and ammonia in urine (228).

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Studies of animals receiving intrarenal injections of nickel revealed increased erythropoietin production resulting in polycythemia (229,230). Rats developed enhanced lipid peroxidation in kidney following s.c. injection of $NiCl_2$ (231) or i.p. injection of nickel acetate (232).

6.3.2 Humans

In humans, nickel exposure results in minimal changes in renal function. Workers who consumed 0.5–2.5 g of Ni in contaminated drinking water had elevated urinary albumin levels on day 2 postexposure, which returned to normal levels by day 5 (104). Sunderman and Horak (219) reported significant increases in urinary β_2-microglobulin among nickel workers whose urinary nickel concentrations exceeded 100 μg Ni/L. At lower concentrations of nickel, no elevations in urinary β_2-microglobulin levels were observed (233). Renal lesions have been reported in workers exposed to nickel carbonyl (234). No changes were noted in biochemical markers of kidney function in stainless steel welders exposed to nickel and chromium (235). In contrast, the urine of male chemical plant workers who were exposed to 0.2–1.3 mg soluble Ni/m^3 had elevated levels of lysozyme and *N*-acetyl-β-D-glucosaminidase (NAG) indicating damage to the proximal renal tubules; women in the same study excreted elevated amounts of NAG, total proteins, β_2-microglobulin, and retinol-binding proteins in their urine compared to nonexposed controls (236).

6.4 Hepatotoxicity

6.4.1 Animals

Nickel's hepatotoxicity has been demonstrated in laboratory animals. Enhanced lipid peroxidation in liver was observed in rats following s.c. or i.p. injection of a high dose of $NiCl_2$ (231,232,237–240). In most (232,241,242), but not all (238), nickel administration depleted hepatic glutathione in experimental animals. Intraperitoneal injection of 100–750 μmol $NiCl_2$/kg to rats resulted in dose-dependent increases in serum alanine transaminase (ALT) and aspartate (AST) activities indicating that hepatic toxicity had occurred (243).

Histological examinations revealed microvesicular fatty metamorphosis, mild hydropic degeneration, and foci of inflammation (237). Dose-responsive increases in serum AST and ALT activity were observed 24 h after s.c. injection of 125–750 μmol $NiCl_2$/kg body weight. Serum alkaline phosphatase activity was reduced compared to controls. Knight et al. (150) reported microvesicular steatosis and the presence of necrotic hepatocytes in rats following i.m. injections of 125 μmol $NiSO_4$/kg. Hepatocytes of mice receiving s.c. injections of metallic nickel solutions were swollen with clear cytoplasm (244).

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6.4.2 Humans

There is much less evidence that nickel is a significant hepatotoxin in humans. In acutely nickel-intoxicated workers, serum bilirubin levels were transiently elevated (104). In typical situations of environmental and occupational exposure, liver nickel concentrations would probably not reach hepatotoxic levels (6)

6.5 Cardiovascular Toxicity

6.5.1 Animals

Animal studies have indicated that exogenous $NiCl_2$ is a potent coronary vasoconstrictor in in situ dog hearts and isolated perfused rat hearts (245,246). Widespread arterioscelerotic lesions were observed in rats following intrarenal injection of 2.5 or 5 mg Ni_3S_2/rat (247,248).

6.5.2 Humans

Hypernickelemia has been reported in patients with myocardial infarction or unstable angina pectoris (249) and release of endogenous nickel has been postulated to be a possible cause of myocardial injury in burn victims (250). A clinical concern is that nickel in contaminated intravenous solutions may pose a risk to cardiac patients (251). In occupational settings, there is no evidence of increased cardiovascular disease in nickel workers (8).

6.6 Reproductive and Developmental Toxicity

6.6.1 Animals

Most studies examining reproductive toxicity of nickel compounds have focused on effects in males. Damage to the seminiferous tubules, edema, hemorrhage, lipid peroxidation, and epithelial degeneration in testes have been observed in rats following nickel exposure (138,141,252–255). Alterations in testicular metabolism and decreased testosterone production have also been reported in animals after nickel exposure (256,257).

No abnormalities in sperm number, morphology, or motility were observed in rats or mice exposed to 13-week inhalation exposure to 0.4–7.9 mg Ni/m^3 as NiO, 0.02–0.4 mg Ni/m^3 as $NiSO_4 \cdot 6H_2O$, or 0.11–1.8 mg Ni/m^3 as Ni_3S_2 (145). After oral exposure to 30 ppm $NiCl_2$ in drinking water for 28 days, rats had fewer basal spermatogonia and reduced fertility rates compared to control rats (255). Others investigators have reported reduced fertility rates in mice and rats after a high i.p. dose of soluble nickel (254,258).

In female rats, s.c. injection of 10–40 mg $NiSO_4$/kg disturbed ovarian cycles; ovulation was blocked following the 40 mg $NiSO_4$/kg dose (259). Nickel

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treatment did not alter the number of corpora lutea in the ovaries but the 40 mg $NiSO_4$/kg dose did abolish ovarian progesterone release following stimulation with hCG.

Nickel in high dosages is embryotoxic and fetotoxic to experimental animals. Ingestion of 1000 ppm $NiCl_2$ in drinking water by gestating mice resulted in reduced maternal weight gain, reduced fetal weight, and increased incidence of spontaneous abortions. None of these effects were observed in mice exposed to 500 ppm $NiCl_2$ in drinking water (260).

Intramuscular administration of 16 mg Ni/kg as $NiCl_2$ and 80 mg Ni/kg as Ni_3S_2 to female rats early in gestation resulted in increased mortality of embryos and impaired fetal growth but no teratogenicity (261). Intraperitoneal administration of sublethal doses of $NiCl_2$ to pregnant mice resulted in increased fetal resorption rates, decreased fetal weight, delayed skeletal ossification, and a high incidence of fetal malformation (262). Mas et al. (263) observed that i.p. injections of 1–4 mg/kg to pregnant mice on gestation days 8 and 12 resulted increased incidence of hydrocephalus, hydronephrosis, heart defects, and hemorrhage. Injections on day 16 were neither teratogenic nor highly fetotoxic indicating that nickel exposure during the period of active organogenesis is the most harmful (263). Exencephaly, everted viscera, skeletal abnormalities, hemorrhage, and reduced body size were observed in chicken embryos after eggs had been injected with 0.02–0.7 mg Ni/egg (264). Reduced fetal weights were observed after pregnant rats inhaled 1.6 or 3.2 mg Ni/m^3 as NiO (265).

In Syrian hamsters, inhalation of $Ni(CO)_4$ on gestation days 4–5 resulted in 24–33% incidence of malformed fetuses (i.e., exencephaly, cleft palate, and hemorrhage) (266). Inhalation exposure on days 7–8 of gestation by pregnant hamsters produced pups with ophthalamic defects (267).

Nickel may also indirectly affect fetal development by altering maternal endocrine status by inducing hyperglycemia (268–270). Intraperitoneal nickel administration (4 mg $NiCl_2$/kg) to pregnant rats increased maternal plasma and fetal glucose concentrations and this occurrence has been postulated to contribute to teratogenicity (263).

Experiments on embryos cultured in vitro have also revealed abnormalities caused by nickel exposure (271–274). Recently, the teratogenicity of nickel has been assessed using frog embryo teratogenesis assay (FETAX) with *Xenopus laevis*. Malformations were observed in frog embryos treated in vitro with $NiCl_2$ and were especially prevalent when embryos were treated during the period of most active organogenesis. Malformations included ocular, skeletal, intestinal, facial, cardiac, and integumentary deformities along with retarded growth, dermal hypopigmentation, and hemorrhages (275,276) and were significantly reduced in incidence and severity when the culture media was supplemented with magnesium (Mg^{+2}) (277). Frog embryos that were exposed for 4 days to the EC_{50} concentration of $NiCl_2$ and then allowed to metamorphose into juvenile frogs in

Ni-free water maintained malformations including ocular depigmentation, sacropelvic abnormalities, spina bifida, and scoliosis (278).

Mechanistically, nickel's embryotoxic effects may be related to nickel's binding to the serpin pNiXa (279). This protein is a protease inhibitor and it has been hypothesized that nickel binding may interfere with proteolysis during embryonic development (279,280).

Nickel is excreted into milk by lactating animals and ingestion may have toxic effects on the offspring. Milk from lactating rat dams injected s.c. with 100 μmol/kg $NiCl_2$ had altered biochemical composition (i.e., increased milk solids and lipid and decreased protein and lactose) (281). Pup mortality was significantly increased when female rats drank 10–250 ppm $NiCl_2$ in water during lactation (255,282).

6.6.2 Humans

In humans, placental transfer of nickel also occurs (283) but there is only one published report of possible reproductive and developmental effects caused by nickel exposure in humans. Increased risk of pregnancy complications and cardiovascular and musculoskeletal birth defects have been reported in women exposed to high concentrations of soluble nickel in industrial settings (284).

Soy-based infant formulae have very high nickel concentrations compared to human breast milk, cow's milk, and cow's-milk-based formulae (285,286) but developmental abnormalities in infants consuming soy-based formulae have not been reported.

6.7 Neurotoxicity

6.7.1 Animals

With the exception of the pituitary gland, the brain is not a major site of nickel accumulation following administration of soluble nickel salts to laboratory animals. However, accumulation of nickel in peripheral nerves and spinal cord in mice after oral administration of 0.58 mg Ni/kg body weight as $NiCl_2$ in the absence of significant nickel accumulation in the brain has been reported (287). Distribution of nickel to the brain was increased after coadministration of lipophilic chelators (107,287–289). As previously described, direct entry into the brain via the olfactory neurons has recently been reported in rats and fish (73,74).

Nickel-mediated neuroendocrine effects have been reported in laboratory animals and in vitro. Nickel affected the rate of release of growth hormone, thyrotropin, luteinizing hormone, follicle-stimulating hormone, adrenocoticotropin, and prolactin from the pituitary in vitro (290–292). Subcutaneous injection of 10 and 20 mg $NiCl_2$/kg to male rats resulted in significant increases in circulating plasma prolactin levels (291). In addition, nickel administration caused deregulation of hypothalamus-mediated thermoregulation in rats (293).

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6.7.2 Humans

Human autopsy samples from nonexposed subjects revealed modest nickel accumulation in the brain (108,109) and the extent to which nickel accumulates in brain in exposed workers has not been evaluated. The brain is a major target of nickel carbonyl poisoning (294). Symptoms of acute nickel carbonyl poisoning include headache, dizziness, vertigo, cerebral edema, cerebral hemorrhage, convulsions, delirium, and coma (10,234,295). Neurological symptoms (giddiness, lassitude, and headache) were reported in workers who accidentally ingested nickel in drinking water (104).

7. MOLECULAR TOXICOLOGY

7.1 Genotoxicity

7.1.1 Nickel-DNA Interactions

In the nucleus, nickel ions bind to nucleic acids and to chromatin (296,297). Nickel ions bind to the phosphate backbone of nucleic acids and N positions of guanine and adenine (2,298) but the exact nature of these interactions has been difficult to characterize because they are unstable to isolation procedures (66,299). Within chromatin, much more nickel is bound to proteins than to DNA because the functional groups of some amino acids (e.g., histidine and cysteine) have higher affinity for nickel than do the DNA phosphate groups (300). Experiments in vitro indicated that Ni-DNA-protein complexes were more stable than Ni-DNA complexes (298). Moreover, chromatin-associated proteins may limit the accessibility of nickel to potential binding sites on the DNA molecule itself.

Nickel binds selectively to heterochromatin. Treatment of CHO cells with $NiCl_2$ resulted in preferential damage to the heterochromatic centromeric regions of chromosomes. In addition, treatment with crystalline NiS particles also resulted in selective fragmentation of the heterochromatic long arms of the X chromosomes (301). The increased efficacy of NiS as compared to $NiCl_2$ was later found to be related to the increased delivery of nickel ions (from dissolution of phagocytized NiS) to the nucleus (302). Patierno and Costa (303) treated CHO cells with $NiCl_2$, biochemically fractionated chromatin into heterochromatin and euchromatin, and found that nickel binding and DNA protein cross-links occurred almost selectively in heterochromatin.

The preference of nickel ions for heterochromatin probably resulted from several factors. Heterochromatin is believed to form the inside lining of the interphase nucleus (304) and hence may be the first molecule that nickel encounters upon entering the nucleus. Heterochromatin also has a higher protein/DNA ratio than euchromatin and therefore has a higher number of potential binding sites for nickel ions (302). Magnesium is important for maintaining condensed hetero-

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chromatin. Nickel can substitute for magnesium and alter heterochromatin structure (52,305).

Within chromatin, nickel has been shown to bind to histones and nonhistone proteins. Patierno and Costa (306) demonstrated that most of the heterochromatic proteins to which nickel was tightly bound were nonhistone chromosomal proteins. However, they tentatively identified histone H1 as a nickel-binding protein within heterochromatin. Similarly, most of the nickel bound to whole liver chromatin obtained from rats injected with 40 mg/kg nickel carbonate 3 or 20 h previously was bound to nonhistone proteins (296). In contrast, a greater proportion of nickel was bound to DNA and histone proteins in whole kidney chromatin obtained from these rats. The authors proposed that this was due to the 40% greater nonhistone protein mass ratio found in liver. Nickel associated with histone and nonhistone proteins when incubated in vitro with whole liver and whole kidney chromatin or with intact nuclei obtained from rats (297).

Recently, interactions between nickel and histones have garnered much research attention. Bal and colleagues (307–309) have demonstrated nickel binding to model peptides corresponding to amino acid sequences from histones H2A and H3. Nickel binding to a model peptide corresponding to the N-terminal tail of histone H4 has also been demonstrated in vitro (M. Zoroddu, personal communication, 1999). Binding of nickel to histone H3 in core histone tetramers isolated from chicken erythrocytes has been characterized (309). The extent to which any of these interactions between nickel and histones occur in vivo is not unknown.

7.1.2 DNA-Protein Cross-Links

Persistent DNA-protein cross-links have been consistently observed in cultured cells that have been treated with nickel (300,303,305) and in tissues from animals that have been exposed to nickel in vivo (310,311). These lesions are potentially genotoxic because they are not easily repaired and possess the ability to interfere with DNA replication (305). Formation of these lesions was enhanced when cells were treated in late S phase of the cell cycle as compared to those treated at other cell cycle stages. Of interest is the fact that heterochromatic DNA is also replicated in late S phase (66).

Further biochemical characterization of these cross-links revealed that they were stable to high salt and nonionic detergents but disrupted by sodium dodecyl sulfate (SDS) suggesting that nickel mediated DNA-protein complexes were kinetically labile (306). Further investigations with cultured cells demonstrated that cross-linking between the amino acids cysteine and histidine and DNA in the presence of nickel was greatly enhanced by the addition of hydrogen peroxide (H_2O_2) (312). In addition, nickel bound to the DNA–amino acid complexes was readily removed by EDTA washing whereas 40–50% of the histidine or cysteine remained complexed with the DNA (312). Moreover, in this study, the amino acid-DNA complexes were stable in the presence of SDS. This suggests that

nickel did not directly participate in formation of the amino acid–DNA complexes but rather catalyzed the covalent cross-linking via oxidative means.

Mechanistically, the interactions between nickel ions and proteins or amino acids are very important in terms of causing oxidative damage to cellular constituents. At physiological pH, uncoordinated nickel ions are redox inactive but upon binding to certain intracellular ligands (e.g., histidine) become redox active via lowering of their redox potential (313,314). When this occurs, strong oxidants such as hydrogen peroxide or monoperoxysulfate can oxidize Ni(II) to Ni(III) and generate oxygen radicals (315,316). Increased production of oxygen radicals and hydrogen peroxide has been demonstrated in nickel-treated cells (317–319). Current hypotheses propose that protein or amino acid–nickel complexes bind to DNA and react with molecular oxygen to produce hydroxyl radicals at the site of DNA binding (320). Numerous studies have reported that nickel generates oxygen radicals oxidizing both DNA and protein in vitro and in vivo (321,322).

Owing to their abundance in chromatin, histones are likely ligands for nickel and hence may promote oxidative reactions. Bal and colleagues (307) demonstrated that a model peptide based upon a metal-binding amino acid sequence of histone H3 enhanced the formation of 8-oxo-2′-deoxyguanosine in the presence of Ni(II) especially if submillimolar concentrations of H_2O_2 were also present. In sperm cells, protamines are abundant and have been suspected to be an important intracellular ligand for nickel (314). Increased oxidative damage to DNA in vitro following incubation with a model peptide representing the N-terminal sequence of human protamine P2, Ni(II), and H_2O_2 has been demonstrated (314,323)

From a practical standpoint, new protein cross-linking strategies have been designed using nickel- and histidine-tagged proteins to cross-link proteins of interest for analysis of multiprotein complexes (324). Levine et al. (316) demonstrated that in the presence of Ni(II), sulfite, and ambient oxygen, spontaneous N-terminal oxidation occurred, producing a free carbonyl on the N-terminal α-carbon and suggested that this method may prove useful for artificially producing site-specific carbonyls on peptides and proteins.

In addition, DNA-protein cross-links may serve as a biomarker for assessing previous nickel exposure. Welders exposed to chromium and nickel had higher amounts of DNA-protein cross-links in peripheral lymphocytes than unexposed controls (325). Costa et al. (326,327) reported increased levels of DNA-protein cross-links in the peripheral white blood cells of welders exposed to nickel and chromium in welding fumes. Both metals are potent cross-linking agents, so the contribution of nickel alone to this event is not known.

7.1.3 DNA Strand Breakage

DNA single-strand breaks occurred in kidneys, liver, and lungs of rats after i.p. or s.c. nickel injection (310,328–330). Mice exposed to 13 mg/kg Ni_3S_2 for 2 h

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via inhalation exhibited an increased frequency of DNA strand breaks in nasal mucosa but not in lung cells (331).

Nickel chloride, crystalline NiS, and Ni_3S_2 caused dose- and/or time-dependent DNA single-strand breakage in cultured CHO, HOS, and cultured human lung fibroblasts (332–334). The frequency of DNA strand breaks increased in a concentration-dependent manner in freshly isolated mouse nasal mucosa and lung cells following a 2-h treatment with Ni_3S_2 (331).

DNA strand scission is likely to be mediated by oxidative events within the nucleus. Vicinal-thiol-containing molecules [i.e., meso-2,3-dimercaptosuccinic acid (DMSA), 2,3-dimercaptopropane-1-sulfonate, and 2,3 dimercaptopropanol] greatly enhanced $NiCl_2$-induced DNA strand breaks in a human leukemia cell line (335). Conversely, mono-thiol-containing molecules (i.e., D-penicillamide, glutathione, β-mercaptoethanol, and diethyl dithiocarbomate) reduced $NiCl_2$-induced DNA breaks. Vicininal thiol-containing molecules generated H_2O_2 in solution whereas mono-thiol-containing molecules did not suggesting that the DNA strand breaks induced by vicinal thiols and Ni were mediated by H_2O_2 molecules. This result could have important implications in occupational health settings because many of these vicininal thiol-containing molecules are used as chelating agents in metal-intoxicated individuals.

Supplemental catalase ameliorated nickel-induced DNA strand breakage in freshly isolated mouse nasal mucosa and lung cells (331). This provides further evidence for the involvement of H_2O_2 in nickel-mediated DNA strand breakage.

Nickel sulfate (25 μM–1 mM) in combination with 50 mM H_2O_2 did not cause increased DNA strand breakage in phenol-extracted salmon sperm DNA (336). However, this same combination in a subsequent experiment (337) caused a high number of single-strand breaks in double-stranded plasmid DNA. The difference might be explained by the increased sensitivity of detection in the latter assay.

Nickel-peptide-catalyzed DNA strand scission has been used as a research tool. Footer et al. (338) used a peptide nucleic acid (PNA) featuring a tripeptide consisting of glycine-glycine-histidine to induce nickel-mediated site-specific DNA cleavage in a target DNA molecule.

DNA strand breakage has also been evaluated as a potential biomaker of nickel exposure. Welders exposed to nickel and chromium had a significantly higher rate of DNA single-strand breakage in peripheral lymphocytes than unexposed controls (101). Hexavalent chromium induces DNA strand breakage (339); therefore, the exact role of nickel in this setting cannot be adequately addressed.

7.1.4 Oxidative Base Damage

Nickel also catalyzes oxidative DNA base damage. Rats injected i.p. with soluble nickel or a $Ni(II)(His)_2$ complex had detectable levels of several oxidized base products in renal and hepatic DNA (340–342). Nickel (II) in the presence of H_2O_2

caused oxidative DNA base modification in chromatin from cultured human cells (322). Interestingly, Ni(II) produced greater damage to DNA in chromatin than to isolated DNA. Formation of 8-hydroxy-2′-deoxyguanosine in calf thymus DNA exposed in vitro to $NiCl_2$ and H_2O_2 has been demonstrated (343). Addition of L-histidine greatly enhanced the reaction. In contrast, Lloyd et al. (337) reported that treatment of salmon sperm DNA with 25 μM–1 mM nickel sulfate in combination with 50 mM H_2O_2 failed to increase 8-oxoguanine formation. Nickel treatment increased 8-hydroxyguanine levels in HeLa cells but only at cytotoxic concentrations (i.e., 750 μM) (344).

One possible source of 8-oxoguanine in DNA is via insertion of 8-oxo-dGTP from the nucleotide pool. 8-Oxo-dGTPases eliminate this base from the nucleotide pool. Nickel (II) at fairly high concentrations (800–1461 μM) inhibited 8-oxo-dGTPases in vitro (345).

The ability of nickel to catalyze oxidation of N7 guanine has been exploited to study RNA structure. Zheng and colleagues (346) used a nickel-dependent reaction to oxidize N7s of guanine residues present in ribosomal 5S RNA, which helped them to identify key structural features of the RNA molecule. In a similar manner, Hickerson et al. (347) used a nickel (II) complex to derive structural information about *E. coli* tmRNA.

7.1.5 Altered DNA Structure

Nickel induced conformational changes in a poly[d(G-C)] oligodeoxynucleotide from a normal right-handed B helix to a left-handed Z helix (348,349). It is believed that interaction between Ni^{2+} ions and the N7 of guanine residues favors the transition (350,351). Abrescia et al. (352) cystallized an oligonucleotide in the presence of Ni^{2+} ions and demonstrated an association between Ni^{2+} and the N7 atoms of all the guanines. However, the presence of Ni^{2+} ions did not introduce any significant distortion in the B-helical oligonucleotide structure. At present, it is unclear as to how prevalent Ni-induced DNA conformational changes are in native DNA and whether or not these changes have genotoxic ramifications.

7.1.6 Intrastrand DNA Cross-Links

A recent study demonstrated that 25 μM–1 mM $NiSO_4$ caused a dose-dependent increase in bulky DNA lesions in salmon sperm DNA as detected by ^{32}P postlabeling (336). These lesions identified as putative intrastrand cross-links and were postulated to arise by a different reaction mechanism than DNA strand scission.

7.1.7 Chromosomal Aberrations

Preferential damage to heterochromatic regions of chromosomes by nickel has been already described. This preference was also observed in cell lines derived from crystalline NiS-induced mouse tumors (353). Nishimura and Umeda (354)

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reported that a variety of nickel compounds (i.e., $NiCl_2$, Ni acetate, potassium cyanonickelate, and NiS) induced chromosomal aberrations in a mouse mammary carcinoma cell line. On the other hand, Au et al. (355) exposed human lymphocytes to 0–1000 μM nickel acetate and reported no increases in chromosomal aberrations even though the highest dose caused mitotic inhibition.

Studies of nickel's clastogenicity in laboratory animals have yielded inconsistent results. The frequency of chromosomal aberrations in bone marrow and spermatogonia of rats did not increase 7 or 14 days following i.p. injection of 3 or 6 mg Ni/kg as $NiSO_4$ (356). However, bone marrow cells from mice injected with 6, 12, or 24 mg $NiCl_2$/kg had an increased frequency of chromosomal aberrations (357).

Increased levels of chromosomal gaps and breaks have been observed in peripheral lymphocytes obtained from active or retired nickel refinery workers (358,359). Deng et al. (360) reported that the frequency of chromosomal aberrations (gaps, breaks, and fragments) in lymphocytes from nickel-exposed electroplating workers was higher than in those from unexposed controls. Nickel exposure was reported to be correlated with increased frequency of chromosomal aberrations in chemical plant workers exposed to NiO and $NiSO_4$ (361). When interpreting these studies, it is important to bear in mind that the workers may have been exposed to other clastogenic agents; therefore, nickel can not be considered the sole causative agent.

Sister-chromatid exchange (SCE) is usually observed at doses below those that cause chromosomal aberrations (23). Low doses of $NiCl_2$ increased the freqency of SCE in CHO (362), macrophage (363), and Syrian hamster embryo (364,365) cell lines. Dose-dependent increases in SCE have been observed in human peripheral lymphocytes following treatment with soluble nickel (364,366,367).

Micronuclei are another cellular marker of chromosomal damage. Sobti and Gill (368) reported that mice exposed to $NiCl_2$, $NiNO_3$, or $NiSO_4$ in drinking water had a significantly higher incidence of micronuclei in bone marrow.

7.1.8 Effects on DNA Replication and Repair

Nickel also affects DNA replication and repair processes. The latter process is of utmost importance because the potentially promutagenic lesions described in this section represent genotoxic hazards only if they escape DNA repair processes and disrupt expression of vital genes.

Nickel is capable of stimulating DNA polymerases presumably by substituting for Mg^{2+} ions during catalysis. However, activation of polymerases by nickel is inefficient and appears to have a narrow concentration range; low concentrations are stimulatory whereas larger concentrations tend to be inhibitory (369). At low doses (0.125–0.25 mM), $NiCl_2$ stimulated DNA replication in cul-

tured HeLa cells and in *E. coli* (370). In the absence of Mg^{2+}, 0.25 mM Ni^{2+} activated DNA polymerase alpha in vitro (371). Above 0.25 mM, Ni^{2+} inhibited this enzyme. Moreover, when Mg^{2+} was present, Ni^{2+} also inhibited the DNA polymerase. Subsequent experiments revealed that the nickel-ion-induced activation of the polymerase was mediated by a single-stranded DNA binding protein (372). Snow et al. (373) examined nickel's effects on several DNA polymerases and found variable effects. T4 DNA polymerase was relatively insensitive to nickel inhibition as compared to the Klenow fragment of *E. coli* DNA polymerase I, T7 polymerase, and DNA polymerase α.

A high concentration of nickel (8 mM) decreased the fidelity of DNA synthesis in an in vitro assay (374). In a subsequent study, Ni^{2+} did not affect the fidelity of DNA replication by DNA polymerase from avian myeloblastoma virus when added in the absence of Mg^{2+}. However, addition of Ni^{2+} in the presence of Mg^{2+} decreased the fidelity of DNA synthesis (369). Snow et al. (373) investigated the effects of nickel on a variety of DNA polymerases and reported that the degree of alteration in replication fidelity was quite variable depending upon the identity of the polymerase.

Nickel has also been shown to inhibit DNA repair in several experimental systems. $NiCl_2$ was comutagenic to ultraviolet (UV) radiation in Chinese hamster V79 cells and enhanced the cytotoxicity of *cis*-diamminedichloroplatinum and these effects were ascribed to inhibition of DNA repair (365). Addition of nickel to UV-treated CHO cells inhibited ligation of DNA single-strand breaks and increased cytotoxicity but did not inhibit repair of MMS-induced single-strand breaks or influence cytotoxicity (375).

Nickel blocked removal of UV-induced cyclobutane pyrimidine dimers in irradiated HeLa cells and increased the repair time of DNA strand breaks suggesting that nickel interfered with the incision step in nucleotide excision repair (376). Repair of visible-light oxidative base damage and DNA single-strand breaks in HeLa cells was reduced at 50 μM and 100 μM Ni (II), respectively (344). These Ni(II) concentrations were not capable of inducing these lesions; therefore, the increased presence of oxidized bases and single-strand breaks was attributed to defective repair. Nickel (II) decreased the repair of *N*-methyl-*N*-nitrosurea (MNU)-induced O^6-methylguanine in a dose-dependent manner (starting at 50 μM) in Chinese hamster ovary cells stably transfected with human O^6-methylguanine-DNA methyltransferase (MGMT) cDNA (377). Activity of MGMT was diminished in cell extracts from nickel-treated cells compared to untreated cells. Repair inhibition was accompanied by increased MNU-induced cytotoxicity.

Krueger et al. (378) reported that $\geq$50 μM Ni(II) inhibited the repair of cisplatin- and transplatin-induced DNA lesions and proposed that the DNA damage recognition/incision step during nucleotide excision repair was affected. Nickel decreased DNA damage recognition in UV-irradiated HeLa cells (379).

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However, other investigators reported that nickel antagonized UV induction of micronuclei and SCE formation (380,381).

7.1.9 Mutagenicity

Prokaryotic Assays. Despite the evidence described above that nickel compounds are capable of causing several types of promutagenic lesions and interfering with DNA replication and/or DNA repair processes, most bacterial mutagenicity assays have yielded negative results for nickel (382–384). Whereas nickel has been generally negative in prokaryotic reversion assays, Rossman and colleagues (385) observed increased nickel induction of λ prophage in *E. coli* using a forward assay. This assay, however, primarily detects DNA damage rather than mutations (66). Another factor to consider when evaluating bacterial assay results is that bacteria and mammalian cells are likely to have very different nickel uptake systems. For example, bacterial assays are most likely inappropriate for evaluating the mutagenicity of insoluble nickel compounds because uptake requires phagocytosis (386).

Eukaryotic Assays. Nickel compounds have been shown to be nonmutagenic or weakly mutagenic in most eukaryotic assays (387). The stronger mutagenic response in eukaryotic cells than in prokaryotes may be a reflection of the greater amount of DNA-associated proteins, which are molecular targets for nickel, in eukaryotic cells (66).

The mutagenic response in eukaryotic cells is weak relative to that observed for classical mutagens. The weak response to nickel in traditional mutagenicity assays may reflect the fact that such tests require use of actively expressed target genes located in euchromatin, which would not be a primary target for nickel given its propensity to primarily attack heterochromatic regions (52). Kociok and colleagues (388) used a DNA fingerprint analysis with a synthetic minisatellite probe to examine mutation frequencies in nickel-induced peritoneal tumors in rats. They reported that the mutation frequency was 40.9%. This example suggests that assays not based upon differences in gene expression may be more sensitive for detecting nickel-induced mutations.

The importance of studying nickel's effects on expression of genes located in or near heterochromatin was demonstrated by mutagenesis experiments conducted with the Chinese hamster G10 and G12 cell lines (389–391). These cell lines are derived from nonrevertible *hprt*$^-$ V79 Chinese hamster cells and have been transformed with the pSV2gpt plasmid containing the complete xanthine-guanine phosphoribosyl transferase (*gpt*) gene from *E. coli* (389). In G12 cells, the *gpt* insert resides adjacent to a heterochromatic region on chromosome 1 whereas in G10 cells, the *gpt* insert is located in another autosome in a euchromatic region. Location of the *gpt* insert proved to be a very important determinant of its response to nickel. The G12 cell line responded strongly to insoluble nickel;

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gpt mutagenesis was at least 20 times higher than the spontaneous rate (390). In contrast, *gpt* mutagenesis by insoluble nickel in the G10 cells was only 2–3 times higher than in unexposed controls. In fact, the mutagenic response of the G12 cells to nickel is the highest reported for mammalian assays. Clearly, proximity of the reporter gene to heterochromatin influenced mutagenesis by nickel compounds.

Further characterization of the G12 *gpt*$^-$ mutants revealed that few contained detectable deletions or sequence mutations in the *gpt* gene (391,392). It was subsequently demonstrated that nickel primarily affected *gpt* gene expression via nonmutational epigenetic mechanisms involving hypermethylation of the gene's promoter and chromatin condensation (393). Previously, Klein et al. (394) demonstrated that nickel induced hypermethylation of a senescence gene on the X chromosome. These experiments support the hypothesis that nickel is capable of epigenetically inactivating genes located near heterochromatin by inducing increases in DNA methylation and chromatin condensation (395,396).

Further evidence for nickel-mediated epigenetic regulation of gene expression was reported by Mayer et al. (331), who assessed the mutagenic potential of Ni_3S_2 in respiratory tract tissues of Big Blue rats and Muta Mouse mice after inhalation exposure to Ni_3S_2. These transgenic animals carry the bacterial *lacZ* and *lacI* genes, respectively, as the target genes for mutagenicity assessment. Two-hour inhalation of 17.6–258 mg Ni/m^3 as Ni_3S_2 did not result in an increase of mutation frequencies in nasal mucosa or lung cells of the *lacZ* mouse or the *lacI* rat. However, Ni_3S_2 treatment in vitro resulted in increased *lacI* mutation frequency in the Big Blue Rat 2 embryo cell line. In one-third of these mutants, no sequence change in the *lacI* gene was detected prompting the investigators to suggest that loss of the *lacI* phenotype in these cells was perhaps due to nonmutational epigenetic regulatory processes.

Another way in which nickel can influence gene expression via nonmutational mechanisms is by influencing histone acetylation. Acetylation of lysine residues in histones is believed to increase transcription of certain genes by relaxing nucleosomal structure and weakening DNA-histone interactions (397). Recently, Broday et al. (398) demonstrated that nickel increased telomeric silencing in the yeast *Saccharomyces cerevisiae*. Moreover, the degree of nickel-induced silencing was dependent upon the distance between the reporter gene and the heterochromatic telomere. Yeast DNA is unmethylated; therefore, the effects of nickel upon histone acetylation were explored. Using antibodies against lysine residues in histone H4, Broday and colleagues (399) demonstrated that nickel decreased the degree of histone acetylation in yeast.

Although nickel by itself may be nonmutagenic in classical assays, it has been found to be cooperative or synergistic with other mutagenic agents (400,401). This may reflect nickel's detrimental effect on DNA repair processes (23,402). In contrast, Ni_3S_2 antagonized benzo[a]pyrene-induced HPRT mutations in human fibroblasts (403).

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7.2 Cytotoxicity and Other Cellular Effects

7.2.1 Altered Cellular Redox Status

As already described, nickel's oxidation potential is lowered upon binding to certain cellular ligands thereby increasing its reactivity toward cellular oxidants such as molecular oxygen, H_2O_2, and lipid hydroperoxides. Oxidation of Ni^{2+} to Ni^{3+} is accompanied by formation of reactive oxygen species, which, in addition to causing DNA damage, can cause cytotoxic damage to cellular membranes via lipid peroxidation (232) and to cellular proteins via oxidation.

Nickel also may decrease cellular antioxidant capacity. Inhibition of catalase and glutathione peroxidase by nickel has been demonstrated in vitro (404) and in vivo (232). Existing data regarding nickel's effects on the cellular glutathione (GSH) are equivocal. Athar et al. (238) reported that $NiCl_2$ enhanced hepatic GSH content in rats. In another rat study, nickel treatment caused an initial decrease in hepatic and renal levels of GSH followed by a rebound of hepatic but not renal GSH (405). Intraperitoneal administration of $NiCl_2$ depleted hepatic GSH in 8–12-week but not in 3- or 6-week-old mice (242). Rats had decreased GSH levels in kidney and liver 3 h after i.p. injection of 107 μmol nickel acetate/kg (232). Cellular GSH content declined significantly in rat renal cortical slices incubated in vitro with 1 mM $NiCl_2$ (406) and in mouse 3T3 cells incubated with 0.5–2 mM $NiCl_2$ (407).

Mouse 3T3 cells that acquired resistance to 200 μM $NiCl_2$ exhibited increased resistance to H_2O_2 and menadione (408) and had 1.8 times higher basal GSH levels than wild-type cells. These data suggest that increased antioxidant capacity contributed to the development of cellular resistance to nickel.

Other important cellular antioxidants are cytoplasmic Cu-Zn superoxide dismutase and mitochondrial Mn superoxide dismutase. Nickel's effects on these enzymes have not been well studied. Misra et al. (232) reported that i.p. administration of 107 μmol nickel acetate/kg to rats had no effect on total SOD activity in kidney and liver but increased muscle SOD activity by 30% 3 h after injection.

As previously described, increased hepatic and renal lipid peroxidation has been consistently demonstrated in laboratory animals injected with nickel salts (231,232,237–240,242,329,409). The relative potencies of nickel compounds to induce lipid peroxidation in CHO cells were estimated to be NiS, Ni_3S_2 > NiO black > NiO green > $NiCl_2$ (410). Lipid peroxidation negatively impacts cellular membranes via loss of fluidity, decreased membrane potential, altered permeability, and altered ionic transport (411,412). Moreover, lipid peroxidation products (e.g., MDA) may be mutagenic and/or carcinogenic (413,414).

Another potentially cytotoxic event induced by nickel is cross-linking of intracellular proteins. Gill et al. (415) reported that nickel, in the presence of monopersulfate, induced monomeric bovine pancreas ribonuclease A to form dimers, trimers, tetramers, and higher oligomers via nickel-catalyzed tyrosine-tyro-

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sine cross-links. Temporally, other types of oxidative cellular damage may precede that caused by lipid peroxidation. In rat renal cortical slices incubated with 1 mM $NiCl_2$, significant release of lactate dehydrogenase occurred within 1 h whereas markers of lipid peroxidation only became elevated after 3 h (406).

7.2.2 Hypoxia

It is interesting, in light of the evidence discussed above, that nickel exposure increases cellular oxidant levels and that nickel induces genes that are known to be induced by hypoxia. Transcriptional upregulation of erythropoetin (EPO) (416), vascular endothelial growth factor (VEGF) (417), and glyceraldehyde 3-phosphate dehydrogenase (418) occurs after nickel exposure in certain cultured cell lines. A similar response is observed following cobalt exposure. In addition, erythrocytosis in laboratory animals follows intrarenal injections of nickel (229,419,420).

Zhou and colleagues (421) recently identified and cloned a novel human gene, *Cap43*, based upon its transcriptional upregulation by nickel. Further experimentation revealed that *Cap43* transcription was also upregulated by hypoxia and by cobalt (422).

Nickel is believed to mimic hypoxia by disturbing cellular oxygen sensing. Current hypotheses propose that the cellular oxygen sensor is a heme protein that can be functionally compromised when nickel or cobalt substitutes for iron in the heme center (416,423). These putative oxygen-sensing molecules have not been positively identified but tentative identifications as a b-cytochrome (perhaps an NAD(P)H oxidase) in mammalian cells (424–427) and cytochrome c oxidase in yeast (428) have been proposed.

It has been further hypothesized that reactive oxygen species (ROS) produced by NAD(P)H oxidase when molecular oxygen is abundant (normoxia) destabilize the HIF-1α subunit of the transcription factor HIF-1 (429) responsible for the induction of hypoxia-responsive genes. During hypoxia, intracellular ROS levels are lowered and HIF-1 is stabilized leading to induction of hypoxia-responsive genes (430).

It is difficult to reconcile the above model with the abundant evidence that nickel exposure results in increased ROS levels in cells, induces HIF-1α protein (431), and induces HIF-1-regulated genes. Even if the presumptive nickel-substituted heme-containing oxidase is dysfunctional and does not produce ROS, other nickel-induced oxygen radicals present in the cell would destabilize HIF-1 and hypoxia-regulated genes would not be induced. This apparent paradox merits further experimental investigation.

The cellular consequences of induction of hypoxia-associated genes by nickel are at present unknown. However, based upon other studies of cellular hypoxia, it is likely that many cellular processes may be altered including glucose metabolism, vascularization, p53 status, iron homeostasis, tumorigenesis, and possibly apoptosis (432–435).

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7.2.3 Alteration of Cell Cycle and Apoptosis

Nickel has been shown to perturb cell cycle progression. Growth arrest has been demonstrated in cells following exposure to many DNA-damaging agents (436) and is believed to facilitate DNA repair processes. Cell cycle perturbation by nickel also is likely to be a response to DNA damage.

Treatment of CHO cells with crystalline NiS or $NiCl_2$ produced an S-phase-specific cell cycle block and a corresponding decrease in cell proliferation with NiS having higher potency (299,437). In contrast, others have reported that nickel acetate decreased the proportion of CHO cells in S-phase and arrested cells in the G2/M phase of the cell cycle (438,439). Blockage in S phase reflects perturbation of DNA synthesis whereas G2/M arrest is postulated to represent an attempt by the cell to allow for DNA repair following damage (440). Nickel-induced DNA-strand breakage occurred to the greatest extent during late S phase in CHO cells (305), which may correspond with replication of heterochromatin (66).

The nickel-induced G2/M arrest was associated with increased apoptosis; however, the mechanisms driving these phenomena have not been elucidated. Upregulation of p53 protein is a common response to DNA damage (441), is associated with G2/M arrest (442), and is believed to facilitate reentry into mitosis after DNA repair (443). Although they did not specify the status of the p53 gene in their cells, Shiao et al. (438) reported that p53 protein did not accumulate in CHO cells following treatment with nickel acetate. Accordingly, they concluded that p53 protein (whether wild-type or mutant) was not associated with G2/M arrest or apoptosis. However, they reported that the highest nickel acetate dose (640 μM) evoked increased production of an extra protein fraction in their p53 western blot, which remains to be characterized.

It is also possible that nickel may indirectly elicit apoptosis via induction of hypoxia or by raising intracellular calcium levels. Both events have been found to induce apoptosis (432,444); however, pharmacological calcium channel blockers have been found to have complex and contradictory effects on cellular apoptosis (445).

Nickel-induced apoptosis may be a cell-type-specific occurrence. Nickel-induced apoptosis has also been reported in myeloid precursor HL-60 cells but not in mouse L-929 fibroblasts or human gingival fibroblasts (446).

7.2.4 Cytoskeletal Abnormalities

In eukaryotic cells, the cytoskeleton consists of microfilaments, intermediate filaments, and microtubules and is an important regulator of cell morphology, motility, cell division, and mobility of cell surface receptors (447). Elements of the cytoskeleton are susceptible to damage by heavy metals including nickel.

Following exposure to $NiCl_2$, microtubules in mouse 3T3 cells aggregated in the perinuclear region (407,448) and were more numerous and shorter than

those in control cells (449). Soluble nickel promoted the rate of tubulin polymerization in vitro (449).

Mechanistically, oxidation of protein sulfhydryls and formation of disulfide bonds between individual microtubule polymers may contribute to nickel-induced microtubule aggregation (407,450). Acetylation of α-tubulin is a key process during microtubule stabilization (451). Treatment with 1–2 mM $NiCl_2$ increased α-tubulin acetylation in a dose- and time-dependent manner in cultured 3T3 cells (452). This result suggests that nickel-induced microtubule aggregation may also arise, at least in part, from their increased stabilization.

Nickel may also exert deleterious effects on microtubule formation by disturbing actin polymerization. Nickel enhanced the rate of actin assembly in vitro primarily by enhancing the elongation rate (453). Actin filaments polymerized in the presence of 2 mM Ni^{2+} were shorter than controls, frequently aggregated, and also exhibited faster rates of depolymerization.

8. CARCINOGENESIS

8.1 Epidemiology

Exposure to nickel oxides and nickel subsulfide has been consistently associated with lung and nasopharyngeal cancer among nickel refinery workers in Wales (451,454,455), Canada (456–458), Norway (459,460), and the United States (461). The International Committee on Nickel Carcinogenesis in Man (ICNCM) concluded that the respiratory cancer risk in nickel refinery workers could be attributed to high exposure to a mixture of oxidic and sulfidic nickel or to high concentrations of oxidic nickel alone (462). In addition, soluble, but not metallic, nickel was found to increase the risk of these cancers and enhance the risks associated with exposure to the insoluble forms of nickel. The International Agency for Research into Cancer (386) concluded that there is sufficient evidence for the carcinogenicity of nickel sulfate and of the combinations of nickel sulfides and oxides encountered in the nickel-refinining industries whereas there is inadequate evidence in humans for carcinogenicity of metalic nickel and alloys. Accordingly, both insoluble and soluble forms of nickel were categorized as Group 1 human carcinogens.

Although the debate regarding the carcinogenic risks associated with soluble nickel exposure continues (463), more evidence has been compiled suggesting that exposure to soluble nickel imposes an increased risk of respiratory carcinogenesis in occupational settings (464,465). Recent epidemological studies have also found slightly increased risks of stomach cancer (22,465) in workers exposed to soluble nickel.

In addition to nickel exposure in occupational settings, soluble and insoluble nickel inhaled in particulate matter from air pollution may pose a carcinogenic

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risk. Some oil-fired electric utility plants in the United States were estimated to pose inhalation cancer risks (i.e., maximum individual risks) above 10^{-6} with Ni, As, radionuclides, Cr, and Cd serving as primary contributors to these risks (466).

8.2 Animal Studies

The carcinogenic effects of nickel compounds have been studied in a variety of animal models using various routes of exposure (386). Results varied depending upon the form of nickel administered, the animal species, and the route of administration. In terms of relevance to human tumorigenesis, inhalation or tracheal instillation studies are the most appropriate. However, studies employing other exposure routes demonstrated that nickel was a complete carcinogen and, as such, was capable of inducing local tumors at the site of intraperitoneal, intramuscular, intrarenal, intratesticular, intraocular, or subcutaneous administration (386). These studies also demonstrated that insoluble nickel compounds were more tumorigenic than soluble forms.

Inhalation studies demonstrated that chronic exposure to Ni_3S_2 at or below the current human threshold limit value induced lung tumors in rats (144,148). Susceptibility to respiratory tumors was species-species; no nickel-exposure-related neoplastic responses occurred in mice exposed to Ni_3S_2 via inhalation (148) or in mice or hamsters exposed to Ni_3S_2 via intratracheal instillation (467,468).

Results from inhalation and intratracheal instillation studies with nickel oxides have varied with animal species and with the physicochemical characteristics of the nickel oxide used (463) and hence are difficult to interpret. Dunnick et al. (148) reported results from a National Toxicology Program (NTP) chronic nickel oxide inhalation study. Although a 2-year exposure to green nickel oxide caused increased lung and adrenal tumors in rats, no increase in nickel-induced tumor frequency was observed in male mice. Moreover, female mice exhibited more lung tumors than controls after exposure but the results were equivocal with respect to the amount of nickel exposure.

Only one chronic inhalation exposure study using soluble nickel has been conducted. Lung tumor incidence in rats and mice did not increase following a 2-year inhalation exposure to 0.03–0.11 mg Ni/m^3 as $NiSO_4$ or 0.06–0.22 mg Ni/m^3 as $NiSO_4$, respectively.

The species-specific differences in susceptibility to tumor induction by insoluble nickel may be partly explained by differences in nickel pulmonary clearance rates (463). Pulmonary clearance of inhaled green nickel oxide occurred more rapidly in mice than in rats (128). In addition, rats are susceptible to lung tumors secondary to particle overload (469,470) whereas mice are not (463), which may also explain the increased susceptibility in rats to nickel oxide.

It is likely that genetic/cellular differences between rats and mice also influence susceptibility to nickel carcinogenicity. For example, it has been reported

that mice have higher baseline levels of glutathione and glutathione reductase in bronchial and lung tissue than rats (463,471), which would confer mice with more resistance to nickel-generated ROS.

There is also empirical evidence of differences within rat strains in susceptibility to nickel tumorigenesis. An inbred colony of Wistar rats had a significantly lower rate of nickel-induced muscle sarcomas after i.m. injection of Ni_2S_3 than a common Wistar colony (472). Further genetic studies are needed to gain more insight regarding this difference.

8.3 Morphological Cell Transformation

Both soluble and insoluble nickel compounds promote morphological cell transformation in vitro (61,64,473–477). Because of their greater tendency to be phagocytized and delivered to the nucleus, insoluble nickel compounds are more potent inducers of transformation (473,474). Most neoplastic transformation studies with nickel have utilized rodent cell lines such as Syrian hamster embryo (SHE) cells and mouse C3H/10T1/2 cells. In rodent cells, insoluble nickel displayed the properties of a complete carcinogen. Exposure of SHE cells to Ni_3S_2 resulted in morphological transformation, increased proliferation, and acquisition of the ability to induce tumors in nude mice (478). Although the reasons are not completely understood, cultured human cells are generally more resistant than rodent cells to morphological transformation by nickel (479). Human osteoblastic cells have been transformed from anchorage-dependent to anchorage-independent growth by nickel in vitro (479,480). In addition, normal human kidney epithelial cells have been immortalized by nickel treatment (476,481).

8.4 Mechanisms of Nickel Carcinogenesis

The etiology of nickel carcinogenesis is not well understood. Many of the nickel-induced insults described earlier have been postulated to be involved in this process.

8.4.1 Chromosomal Aberrations

Given nickel's ability to increase oxidative damage to DNA and to chromatin, it is not surprising that chromosomal aberrations have been described in nickel-transformed cells (482) and in nickel-induced mouse rhabdomyosarcomas (353,483) and renal cancers induced in rats by Ni_3S_2 (484). Chromosomal aberrations can cause alterations in gene expression via translocation of a quiescent gene into an actively transcribed site, loss of a normally expressed gene by deletion or rearrangement, or gene amplification (484).

8.4.2 Epigenetic Regulation of Tumor Suppressor Genes

Nickel's carcinogenetic effects may be related to its selective damage to heterochromatic regions. Nickel-induced transformation of CHO cells was antagonized

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by extracellular magnesium. Interestingly, magnesium selectively suppressed nickel-induced heterochromatic but not euchromatic damage suggesting that the heterochromatic damage and morphological transformation were associated (333). In a subsequent study, it was observed that over half of male nickel-transformed CHO cells had a complete deletion of the heterochromatic long arm of the X chromosome (482). Restoration of the X chromosome caused a high proportion of the previously transformed cells to enter senescence, which suggests that during nickel-induced transformation, inactivation or deletion of a cellular senescence gene had occurred (394). However, senescence was also induced when a normal X chromosome was introduced to nickel-transformed cells not harboring the X-chromosome deletion. This result suggested that the nickel-inactivated senescence gene on the X chromosome was not located within the deleted portion of the heterochromatic long arm. The senescence activity could be restored following treatment with the DNA methylation inhibitor 5-azacytidine suggesting that nickel-induced inactivation of the senescence gene had occurred via DNA hypermethylation (394,396).

This occurrence and the previously described observations of nickel-induced hypermethylation of the *gpt* transgene inserted near heterochromatin led Costa and colleagues to hypothesize that nickel's carcinogenic effects may arise from epigenetic silencing of tumor suppressor genes via de novo DNA methylation of their promoters (313,396,485). The recent demonstration of nickel's reductive effect on histone H4 acetylation in yeast (399) suggests an additional possible mechanism of epigenetic gene regulation by nickel. Further evidence implicating nickel in epigenetic gene regulation via its effects upon chromatin is the reported downregulation of the homolog of the human chromatin remodeling factor hSNF2H in nickel-treated CHO cells (486).

8.4.3 Activation of Cellular Oncogenes

Elevated expression of oncogenic *c-myc* mRNA was reported in nickel-transformed mouse 10T1/2 cells (487). Sunderman et al. (484) reported that the *N-myc*, *H-ras*, and *c-fos* oncogenes were amplified in renal neoplasms induced in rats by intrarenal injections of Ni_3S_2 but no consistent pattern was observed. To date, there is a paucity of data linking oncogene activation with functional roles in nickel carcinogenesis.

8.4.4 Altered Expression of Other Cellular Genes

Expression of a number of cellular genes is altered upon exposure to nickel. The induction of HIF1-α and hypoxia-related genes by nickel (431) may be important in nickel-tumorigenesis. HIF-1 activity was significantly higher in nickel-transformed human or rodent cells than in parental cells (431). Upregulation of glycolytic enzymes by nickel in the presence of molecular oxygen resembles the ''Warburg effect'' described for cancer cells (488,489). This event may favor cell proliferation by causing intracellular accumulation of 5-phosphoribosyl-1-pyro-

phosphate, which is needed for nucleotide synthesis (490). Tumors frequently contain poorly oxygenated regions (491,492) and exhibit upregulation of HIF1-α (493) and it has been suggested that hypoxia promotes tumor aggressiveness and metastases (494,495). On the other hand, thrombospondin I, an antiangiogenic protein, is transcriptionally downregulated in nickel-transformed cells (496,497). This event would most likely promote increased tumor oxygenation but it may not be enough to fully oxygenate rapidly growing solid tumors.

Differential display analysis has revealed altered gene and/or protein expression of H-ferritin, vimentin (an intermediate filament protein), and *Cap43* following nickel treatment of cultured cells (421,486). The roles of these genes, if any, in nickel carcinogenesis are unknown. With the advent of DNA microarray technology (498,499), it is likely that more genes will be identified that have altered expression following exposure to nickel compounds. Analysis of these alterations in gene expression should do much to elucidate the genetic components of nickel carcinogenesis.

8.4.5 Alterations in Cell Cycle Checkpoints and Growth Regulation

Mutations in some tumor suppressor genes that regulate cell cycle progression have been suspected to participate in nickel carcinogenesis. Lin et al. (500) reported that NiS-transformed human osteoblastic clones had reduced phosphorylation of retinoblastoma (Rb) protein arising from a mutation in the *Rb* gene. Hypophosphorylated Rb protein binds the transcription factor E2F and delays entry of cells into S phase (501). A number of other transcription factors (e.g., Elf-1, DRTF-1, NF-IL-6) are also inactive when bound to hypophosphorylated Rb protein and are released and activated when Rb is phosphorylated (502). Moreover, Rb protein regulates expression of *c-fos*, *c-myc*, and Sp-1 (503). Hence, alteration of Rb protein status by nickel may have widespread effects upon gene expression.

Mutations in the tumor suppressor gene *p53* have also been associated with nickel carcinogenesis, although this is controversial. Maehle et al. (504) reported *p53* mutations in nickel-transformed human kidney epithelial cells. In addition, *p53* mutations in lung tumor samples were marginally associated with occupational exposure to nickel (505). However, no *p53* mutations were identified in nickel-induced rat renal tumors (506).

Mutations in p53 protein would increase the likelihood of cells with damaged DNA progressing through the cell cycle unchecked. However, Salnikow et al. (431) reported that wild-type p53 protein accumulated in MCF-7 and A549 human cancer cells following nickel treatment, which would be consistent with the induction of p53 in response to DNA damage. In addition, human HOS cells have mutant p53 protein but nickel treatment results in their further transformation indicating that this event is not solely dependent upon p53 status (431).

Nickel may impact p53 activity by nonmutational means. It was recently

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demonstrated that nickel, cobalt, and zinc inhibited binding of p53 protein to supercoiled DNA or to the p53 consensus sequence in linear DNA fragments (507). It is possible that nickel interferes with p53's ability to act as a transcription factor by interfering with its ability to bind to DNA. The net result would be similar to that caused by p53 mutations (i.e., altered cell cycle control).

Recently, a gene (*DAN*) encoding a nickel-binding protein was cloned from neuroblastoma cells (508). Expression of *DAN* is reduced in transformed cells and *DAN* has also been shown to have tumor-suppressive activity in vitro (509). Overexpression of *DAN* resulted in delayed entry into S phase (510). An intriguing hypothesis is that nickel binding to the DAN protein may perturb cell cycle regulation (489).

Nickel-immortalized human kidney epithelial cells were nonresponsive to transforming growth factor-β (TGF-β) resulting in increased cell proliferation (511). In addition, these cells exhibited increased expression of epidermal growth factor (EGF) receptor and increased EGF binding. These data were highly suggestive that altered responses to the growth regulatory factors EGF and TGF-β were involved in nickel-induced immortalization.

8.4.6 Inflammation-Induced Cell Proliferation and Reduced Immune Surveillance

Nickel's effects on the immune system may contribute to its carcinogenicity. Cellular inflammatory responses to nickel are likely to involve the release of growth factors and cytokines that may increase cell proliferation (463). Nickel may also enhance tumorigenesis by inhibiting natural killer (NK) cell activity (512–514).

9. INTERACTIONS WITH ESSENTIAL METAL IONS

9.1 Magnesium

Magnesium and nickel ions share many physiochemical properties. Ionic radii are 0.69 and 0.66 Å for nickel and magnesium, respectively, and both metals have similar affinities for biological ligands (333). Accordingly, magnesium antagonizes many of the toxic and carcinogenic effects of nickel (50,67,277,333,376,379,452,512,515–520).

9.2 Calcium

As discussed previously, soluble nickel may be taken up by cells via certain calcium channels, although this remains controversial (521). Nickel has also been shown to block R- and T-type calcium channels (522–527). Nickel is also a competitive inhibitor of a mitochondrial calcium carrier (528). In addition, nickel ions may be transported into cells via sodium/calcium exchangers, which nor-

mally function to extrude calcium from the cell (529). These exchangers also participate in calcium uptake and nickel has been shown to be a competitive inhibitor of this process (530). Nickel may also influence calcium influx via effects on receptor-regulated nonselective cation channels (531).

Given the dichotomous effects of nickel on calcium channels, it is not surprising that nickel's effects upon cellular calcium homeostasis are highly variable and may, in fact, be cell-type specific (532–535).

In A549 human lung epithelium-derived cells, extracellular $NiCl_2$ caused an increase in intracellular calcium as measured by fluorescence of Fluo-3 AM dye (536). Induction of the *Cap43* gene by nickel was blocked by BAPTA-AM, a specific chelator of intracellular calcium, demonstrating calcium's role in gene induction by nickel.

In general, the role that calcium homeostasis has in carcinogenesis is not well understood. In mice, coadministration of calcium abolished the enhancement of pulmonary adenomas caused by nickel (520). However, supplemental calcium had no effect on development of Ni_3S_2-induced muscle sarcomas in rats (518).

9.3 Iron

The evidence that nickel and iron may share a low-affinity cell transport pathway has been described earlier. Metallic iron was found to be a very effective local antagonist of Ni_3S_2 carcinogenesis in rat muscle (519). Coadministration of ferric iron (Fe III) with Ni_3S_2, though not as effective as metallic iron, also reduced the frequency of Ni_3S_2-induced muscle sarcomas in rats. Neither form of iron affected nickel retention in the injected muscle.

9.4 Zinc

Intracellular zinc concentrations in rat hepatocytes (as measured by fluorescence of zinquin) were not affected by incubation in <80 μM $NiCl_2$ (537). The significance of this finding is questionable because such low concentrations of soluble nickel were used. Coadministration of zinc oxide or zinc acetate diminished sarcoma occurrence rates in rats following i.m. injection with Ni_3S_2 although neither form of zinc affected muscular retention of nickel (519). Interestingly, zinc oxide also somewhat protected rats from metastatic kidney and lung tumors. This suggested that zinc conferred both local and systemic protection. It has been proposed that nickel can substitute for zinc in the zinc fingers of DNA binding proteins and potentially interfere with gene expression (538).

9.5 Manganese

Manganese dust protected against Ni_3S_2 muscle tumor development when it was coinjected with the Ni_3S_2 (539,540) and the effect was strictly local. Moreover,

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manganese dust caused changes in the subcellular compartmentalization of nickel at the injection site.

10. FUTURE RESEARCH DIRECTIONS

The increasing availability of DNA microarray technology should enable researchers to explore and elucidate the genetic components regulating nickel's carcinogenic effects. In addition, such analyses may further define the exact roles of nickel, if any, in animal and human nutrition.

The factors responsible for species differences in susceptibility to nickel carcinogenesis should be explored further. Such research may identify key cellular and genetic differences between susceptible and nonsusceptible species and would identify the best animal model for human nickel carcinogenesis. In addition, such work may help researchers determine whether certain human populations are at increased risk of contracting nickel-induced cancers. In future animal studies, more attention should be devoted to delivering biologically relevant nickel doses by appropriate routes of exposure to best mimic the human condition.

Additional exploration of nickel's selective effects upon heterochromatin and chromatin remodeling is needed. The biochemical mechanisms of nickel-induced chromatin condensation and DNA hypermethylation are not presently understood and additional work should also focus on identifying tumor suppressor genes potentially impacted by these processes. The functional consequences of nickel's perturbation of histone acetylation also need to be defined.

The association between nickel carcinogenesis and induction of hypoxia is intriguing. More work is needed to determine whether increased expression of hypoxia-related genes contributes to the etiology of nickel-induced tumorigenesis.

Another important research area with important applications with regard to occupational health and air pollution is elucidating the role of nickel compounds as components of chemical mixtures. Certain chemicals may synergistically enhance nickel's detrimental effects or vice versa. On the other hand, other chemicals may suppress nickel's toxicity and/or carcinogenicity. Evaluation of these interactions would enhance regulatory decision-making processes.

In conclusion, both basic and applied research approaches are needed to unravel the complex and multifactorial nature of nickel's toxic and carcinogenic effects. An understanding of the combination of cellular events that confer a selective growth advantage to nickel-induced cancer cells remains the focus of current nickel research. Such knowledge will enable better human risk assessment and may elucidate events important in other types of environmentally induced cancers.

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12

Lead

Emily F. Madden
U.S. Food and Drug Administration, Rockville, Maryland

Mary J. Sexton
University of Maryland, Baltimore, Maryland

Donald R. Smith
University of California, Santa Cruz, California

Bruce A. Fowler
University of Maryland, Baltimore, Maryland

1. INTRODUCTION

1.1 Release of Lead into the Environment

Lead (Pb) may be released into the general environment as a result of automobiles using leaded gasoline as an antiknock agent (1), lead-containing paint (2), water due to lead solder in water pipe systems (2), and atmospheric emissions of lead for industrial sources such as smelters (1). In recent years, the atmospheric releases of lead have been reduced in many countries owing to the removal of lead from gasoline and restrictions on the release of lead from point sources. Lead-based paint is still a problem in some countries, such as the United States, as a result of its use in older housing stocks. The decreased use of lead solder in water systems has greatly reduced exposure to lead from this source.

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1.2 Human Body Burdens

Once absorbed into the system, the largest stores of lead in the body are found in the bone (2) followed by the kidney and liver (2). The blood is a short-term storage compartment that is useful as a measure of lead exposure during the last 30 days (2). There is a dynamic relationship between the blood and bone compartments (2) and the other target organ systems, such as the brain and kidney (2). Once the skeleton has achieved an elevated concentration it will continue to release a portion of its stores over a long time period even following cessation of exposure.

1.3 Populations at Risk

The toxic properties of lead have been known for centuries (2). As noted above, this element is found in food, air, and water (1,2) and humans are exposed to it in varying quantities. In recent years, concern has been focused on populations of special risk as a result of increased exposure, as in the case of workers (2), age or physiological status (2) for both the young and senior sectors of the population, genetic susceptibility (2), and nutritional status (2). In the case of the young, such as the fetus that may be exposed in utero to lead as a result of its transference across the placenta, or infants owing to exposure from lead in breast milk (2) or dust from leaded paint (2), exposure may result in deleterious biological effects during sensitive growing periods. For the aged, release of bone lead stores during demineralization of the skeleton as a result of osteoporosis or simple demineralization may result in increased exposure of target organ systems, such as the brain and kidney, which may exacerbate declining functionality at that stage of life.

It has been recognized for a number of years that individuals vary greatly in their susceptibility to lead toxicity for equivalent exposure or blood lead concentrations (2). The identification of target-organ-specific lead-binding proteins (PbBPs) in fish (3), rodents (4–6), nonhuman primates (7), and humans (8,9), as discussed below, provides one possible explanation for these differences in susceptibility. Individuals may vary in the expression of these molecules, which appear to be particularly important in regulating the intracellular bioavailability of lead in target tissues at low concentrations (2,6).

Nutritional factors, such as calcium (2) and iron (2) content of the diet, have been shown in rats to influence the absorption of lead from the gastrointestinal (GI) tract. In addition, dietary protein content and starvation have been also suggested to influence the absorption of lead from the GI tract (5). Zinc status (10,11) with regard to the activity of lead-sensitive zinc-dependent enzymes is also an important consideration with regard to the use of this enzyme activity in blood as a biomarker, and iron status with regard to the use of erythrocyte

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protoporphyrin as a biomarker for increased lead exposure is a potentially important confounding factor.

2. ANALYTICAL METHODS FOR THE ASSESSMENT OF LEAD EXPOSURE AND METABOLISM

The ability to perform accurate, precise, and meaningful measurements of lead in environmental and biological systems has provided the foundation for understanding the introduction, transport, and fate of lead in the environment, as well as the routes and magnitudes of exposure and mechanisms of toxicity in organisms (2,12–15). Furthermore, accurate and meaningful risk assessments depend upon the availability of accurate and precise lead measurements. However, while the ability to perform high-quality lead measurements is now nearly commonplace, instances still occur where improper procedures and/or lead analyses are conducted, leading to data that may confound study outcomes at best or completely invalidate the study at worst. Therefore, the overall importance of high-quality lead measurements in lead toxicity studies cannot be overstated.

Since there exists no single biological marker of lead exposure and toxicity, multiple complementary measurements of lead exposure and effect are needed to understand magnitudes and pathways of exposure, metabolism and mechanism(s) of toxicity, and the efficacy of interventions (e.g., lead abatement, chelation treatment) to alleviate exposure and toxicity. Measurements of blood lead levels are still most commonly utilized in evaluating exposure and toxicity (2,12,16), although the limitations of this biological marker are becoming increasingly evident (17–19). Thus, many of the analytical methods described below have been developed or utilized to derive more meaningful measures of human lead exposure and effect.

The discussions here on analytical methodologies for lead measurements update summaries of methodologies presented in several previous reviews (12,20). Since those reviews, there have been several important advancements in analytical methodologies or their applications in investigations of lead exposure, metabolism, and toxicity. These include advancements in in vivo K-XRF measurements of bone lead levels as a marker of lead exposure and toxicity, and the development of inductively coupled plasma–mass spectrometry methods for routine use in stable lead isotope methods. An additional advancement that has proven useful in clinical settings includes the development of a portable electrochemical method for the rapid screening of blood lead levels (e.g., LeadCare system). One future benefit of these improvements in lead measurement sensitivity and the common use of trace metal clean techniques may be in studies at ultralow lead concentrations in biological systems that may be important in better understanding lead toxicodynamics (e.g., plasma lead) (21–24), as well as in

future investigations seeking to better define low-level lead effects in experimental systems (12,25,26).

2.1 Importance of Trace Metal Clean Techniques

Numerous studies have demonstrated the importance of trace metal clean techniques, which have become nearly essential in performing meaningful measurements of low lead levels in biological (e.g., blood plasma) and environmental (e.g., natural waters) samples (22,27,28). However, while the potential confounding effects of sample lead contamination are now recognized, they still may not be sufficiently appreciated in measurements of low lead samples (e.g., $<$10 ng/ml). Investigators still encounter substantial problems with the inadvertent introduction of contaminant lead during the collection, storage, processing, and analysis of samples. Contaminated analytical reagents and inadequately cleaned labware, as well as contaminated sample collection containers, continue to be significant sources of contamination for low lead samples (17,29). However, this is avoidable, since most reagents can now be purchased in a ''trace metal analyses'' grade, and laboratory environments and laboratory-ware can be selected and prepared to substantially reduce lead contamination. The procedures that compose trace metal clean techniques for lead analyses have been detailed elsewhere and will not be repeated here (26,29,30).

2.2 Analytical Methods

While many analytical methods exist for the measurement of lead concentrations in biological and environmental samples, the discussion here will focus only on the more commonly utilized methods. These include atomic absorption spectrometry (AAS), inductively coupled plasma–mass spectrometry (ICP-MS), and X-ray fluorescence (XRF). Intercomparisons of analytical methods generally involve assessments based on measurement sensitivity (e.g., detection limits), accuracy, and reproducibility (precision). There also are other considerations that become important, such as the degree of sample pretreatment needed prior to analyses, and other unique strengths and limitations of a particular analytical method that in part determines its overall utility. In general, AAS is probably the most widely utilized methodology, whereas ICP-MS may possess greater measurement sensitivity and fewer sample matrix problems. XRF methodologies are valuable for the measurement of in vivo lead levels, which are most commonly restricted to bone tissues due to measurement sensitivity.

2.2.1 Atomic Absorption Spectrometry

Atomic absorption spectrometry measures lead concentrations from the absorbance of lead spectra emitted from a source lamp by lead atoms vaporized into the light path of the source lamp (31). Flame AAS techniques are appropriate

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for measurements of relatively high (μg/ml) analyte concentrations of lead, whereas flameless AAS (also known as graphite furnace AAS) techniques are required for low (e.g., low ng/ml or below) level measurements. These include graphite furnace (GFAAS) measurements of low lead concentrations in complex matrices, such as blood, that commonly utilize advanced furnace designs and matrix background correction (e.g., Zeeman background correction) (2,32). Atomic absorption spectrometry, including GFAAS, is a relatively straightforward and inexpensive analytical method. Preparation of samples for measurement by AAS generally requires strong acid digestions to axidize organic matter, although several relatively simple sample preparation methods are now widely used for the analyses of whole-blood lead levels that require only sample mixing with surfactants (e.g., Triton X) or weak acids. Continued advances in AAS instrumentation have resulted in instruments that are now capable of measuring multiple analyte metals simultaneously (e.g., Perkin-Elmer SIMAA instruments) (33).

2.2.2 Mass Spectrometry

Mass spectrometry is a powerful class of analytical instrumentation capable of separating and detecting single charged ions. These include ICP-MS (both quadrupole and magnetic sector) and thermal ionization magnetic sector mass spectrometry (TIMS). It is noteworthy that while TIMS is still the definitive means for measuring lead isotopic abundances (28,34–36), the sensitivity and mass resolution of recently available ICP-MS, particularly the newer generation of magnetic sector ICP-MS, is sufficient for quantitative isotope abundance analyses of lead (37–39). The following sections briefly summarize some aspects of current and projected applications of mass spectrometry for analyses of lead concentrations and isotopic compositions in environmental toxicology.

Thermal ionization mass spectrometry is considered the definitive method for accurate and sensitive lead analyses when isotope dilution mass spectrometry (IDMS) is used (40). These benefits are due in part to the very high vacuum (e.g., 10^{-9} torr), precise electronic focussing optics, and strong magnetic field surrounding the flight tube that are characteristic of sector TIMS instruments, which combine to maximize the delivery of ionized lead atoms to the Daly or Faraday detector(s) (40,41). However, sample analyses for lead isotope abundances and concentrations are limited by a number of factors, including (a) the relatively lengthy sample preparation procedures required for analyses, especially for biological samples. These often involve strong acid digestion and anion-exchange column chromatography to purify sample lead from the surrounding sample matrix. And (b) the relatively limited availability and sample throughput capabilities of TIMS instruments.

ICP-MS, which occur now as quadrupole or magnetic sector instruments, have become a viable alternative to TIMS for isotopic measurements and highly accurate IDMS concentration measurements (magnetic sector ICP-MS), and to

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other established methods (AAS, ASV, ICP-AES) for more routine lead concentration measurements (18,21,39,42–44). With ICP-MS the sample is introduced into a plasma with excitation temperatures >5000°K that efficiently atomize and ionize lead into a mass spectrometer, effectively reducing potential sample matrix interferences that might otherwise confound the analyses (42). An additional benefit of ICP-MS is the capability to measure multiple elements simultaneously, thereby dramatically reducing analysis effort compared to other methods when multiple elements are measured (42,43). Most applications of ICP-MS utilize external standards to quantify sample lead concentrations in combination with internal standards (usually of a different element, such as bismuth) to correct for sample matrix interferences and changes in instrument sensitivity over the course of analyses.

There are limitations of ICP-MS that may compromise its utility in stable lead isotope measurements. In particular, typical ICP-MS (quadrupole or single-detector magnetic-sector) has not yet achieved the level of isotope measurement accuracy and precision achieved with TIMS (45–47). The recently available multidetector ICP-MS instruments, which are capable of producing accurate and precise lead isotope data on par with TIMS analyses, are an exception to this, although those instruments are very costly and not commonly available. Nonetheless, recent studies utilizing more commonly available single-detector ICP-MS, particularly magnetic-sector ICP-MS, now have the capacity to produce highly precise ($\pm$<0.1% RSD) measurements of lead isotopic ratios in biological matrices (38,39) that are approaching the data produced by TIMS (35,36,48) (Figs. 1 and 2; see below). Other improvements in ICP-MS methodologies that have been realized include the development of ultrasonic nebulizers and special spray chambers that help reduce interferences from the sample matrix, as well as plasma torch guard electrodes that dramatically (e.g., 10-fold or more) increase the analyte signal/noise ratio and have increased measurement sensitivity.

For example, studies from our (DRS) lab intercalibrating ICP-MS with TIMS analyses of blood and environmental samples have shown that the external measurement error or between-sample reproducibility of magnetic sector ICP-MS is close to that obtained with TIMS (38,39) (Figs. 1 and 2). Those studies also pointed out that the 'long-term external measurement reproducibility' is the most relevant expression of measurement error for isotope ratio analyses of environmental and biological samples. As such, this should be the measure of analysis error used for comparing different analytical methods for measuring lead isotopes. This is justified, since (a) the external error typically encompasses the internal sample measurement error, and (b) the external reproducibility is the indication of the probability that the measured value agrees with the 'true' value. The relevance of this point is based on ongoing disputes over the lead isotope ratio measurement precision capabilities of ICP-MS compared to TIMS. Typically, reports of TIMS measurement precision for lead isotopes are based on

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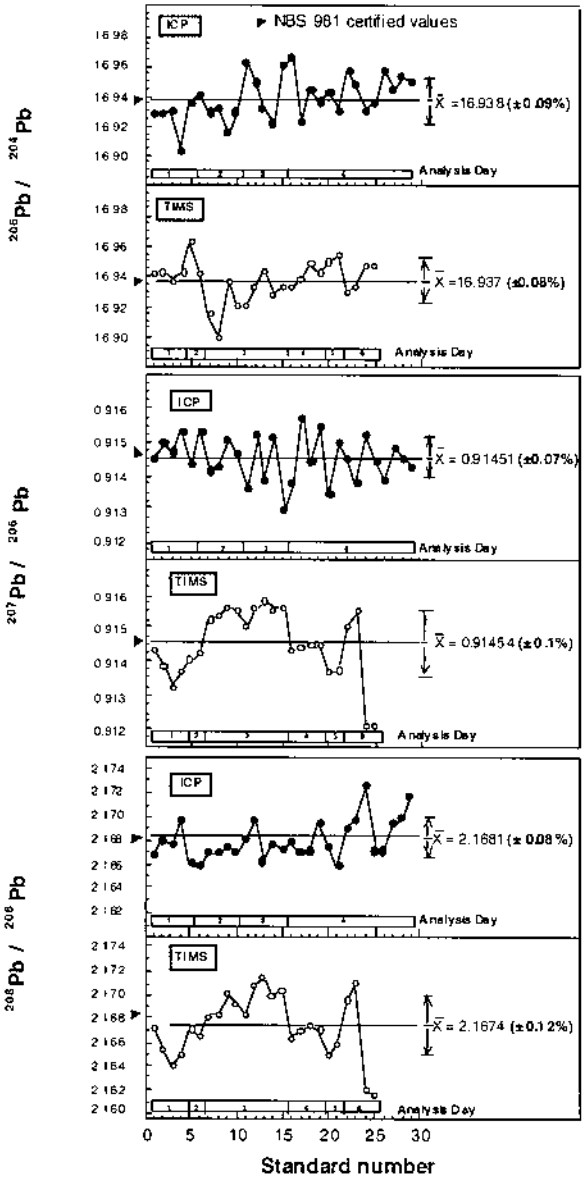

FIGURE 1 Comparison of the long-term external reproducibility of $^{206}Pb/^{204}Pb$, $^{207}Pb/^{206}Pb$, and $^{208}Pb/^{206}Pb$ ratios in NBS 981 standards analyzed by magnetic sector ICP-MS and by TIMS on four to six different analysis days over the course of one year. The certified NBS 981 value is indicated on the ordinate (▶). The grand average isotope ratio (± %RSD) is shown on the right in each figure. ICP-MS analyses were run according to the protocol described in Gwiazda et al. (38). TIMS measurements were corrected for instrument mass fractionation (0.92‰ amu $^{-1}$). (Note: Subsequent to this study replacement of the Faraday cups in the TIMS instrument led to a three-fold increase in the reproducibility of isotope ratios). (Figure reproduced from Gwiazda et al., Ref. 38, with permission).

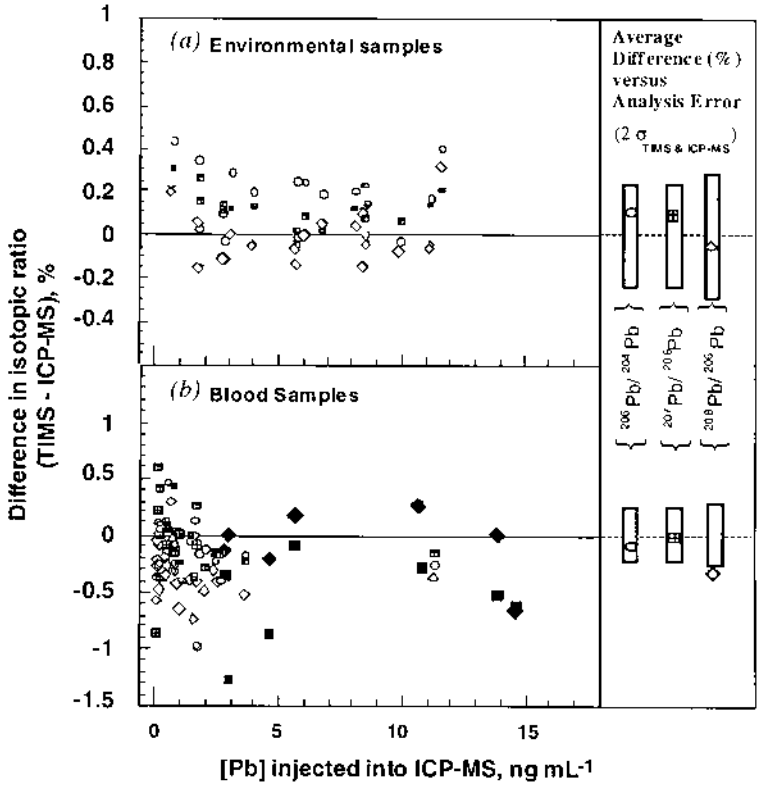

FIGURE 2 Percent (%) difference in the $^{206}Pb/^{204}Pb$ (○), $^{207}Pb/^{206}Pb$ (⊞), and $^{208}Pb/^{206}Pb$ (◇) isotope ratios of (a) environmental (dust), and (b) blood samples measured by magnetic sector ICP-MS versus TIMS as a function of the lead concentration of the sample solution injected into the ICP-MS instrument. The average percent difference of all isotope ratios (○, ⊞, and ◇ symbols, right side of each panel) was within the combined analyses error of both methods ($2 \times \sqrt{\sigma^2_{TIMS} + \sigma^2_{ICP\text{-}MS}}$), with the exception of the blood $^{208}Pb/^{206}Pb$. Also included in (b) are data from Delves and Campbell, 1988: $^{207}Pb/^{206}Pb$ (■), and $^{208}Pb/^{206}Pb$ (◆). Figure reproduced from Gwiazda et al. (Ref. 38), with permission.

within-sample measures, rather than on sample measurement reproducibility (i.e., between samples). In our work, the ICP-MS method yielded long-term reproducibility imprecision in isotope ratio measurements of $<0.1\%$ RSD, with an inaccuracy of $<0.11\%$ and $<0.36\%$ RSD for environmental and blood samples, respectively (Figs. 1 and 2). These values compare very well with the TIMS

measurements of Inskip et al. (48), and they demonstrate that ICP-MS is a viable alternative to the more costly and time-consuming TIMS analyses for determining lead isotope ratios in biological and environmental samples.

2.2.3 Isotope Dilution Mass Spectrometry for Lead Concentration Measurements

The most accurate lead concentration measurements are made with IDMS, using either TIMS or ICP-MS (40). Isotope dilution mass spectrometry is considered the definitive method because it is a yield-independent method of analysis, extremely sensitive, and precise (49). Isotope dilution measurements rely on the addition of a known amount of isotopically characterized stable lead isotope spike (e.g., a spike highly enriched in ^{208}Pb or the manmade ^{205}Pb) to the sample preparation. Assuming the stable lead isotope spike completely equilibrates with the lead inherent to the sample, measurement of the lead isotope abundances within the spiked sample can yield very accurate measurements of the lead content in the sample. Isotope dilution mass spectrometry methods using TIMS or ICP-MS are superior to other lead concentration measurement methodologies, such as GFAAS and ICP–atomic emission spectrometry (AES), since the latter are secondary measurement techniques that rely on calibration with secondary standards and standard reference materials.

2.3 Stable Lead Isotope Methods in Studies of Lead Exposure and Metabolism

Different sources of industrial lead in the environment, as well as different sources of lead exposure to humans, may be characterized and distinguished by their stable lead isotopic compositions (12,39,50–52). This is because lead exists naturally as four stable isotopes, ^{204}Pb, ^{206}Pb, ^{207}Pb, and ^{208}Pb, and because the relative abundance of these four isotopes varies naturally throughout the environment. These natural variations in stable lead isotopic abundances are due to the geological ages of different lead-bearing ores, and the amounts of stable ^{204}Pb and the radioactive progenitor isotopes ^{238}U ($t_{1/2} = 4.5 \times 9^{10}$ years), ^{235}U ($t_{1/2} = 0.70 \times 10^9$ years), and ^{232}Th ($t_{1/2} = 1.4 \times 10^{10}$ years) that form ^{206}Pb, ^{207}Pb, and ^{208}Pb, respectively (53). Although stable lead isotopic compositions in the environment may vary naturally by as much as 10–15%, they generally vary less than that (e.g., 1–5%) (52,54).

Stable lead isotope methods have proven useful in evaluating lead metabolism in humans using inherent differences in lead isotope abundances between different tissues (e.g., skeletal versus soft tissues (35,36), as well as in the identification of anthropogenic sources of lead exposure to lead-poisoned children (39,55,56).

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2.3.1 Identification of Environmental Lead Hazards

Stable lead isotopes may be the most accurate tool available for the lead hazard assessor to identify sources of lead exposure, because the isotopic fingerprint of a biological sample (e.g., blood) may be traced to the household source(s) of the lead exposure. For this approach to work, different sources of lead must possess distinct isotopic compositions that are distinguishable within the error of the measurement. However, while the lead isotope methodology using TIMS has been used to identify sources of exposure to humans (50,55–59) these approaches have been limited owing to the relatively costly and time-consuming sample preparation methods required for TIMS analyses, and the fact that TIMS instruments are not readily available. Magnetic-sector ICP-MS measurements of environmental samples (soils, dusts, paints) are less precise than those by TIMS (based on external sample measurement reproducibility), but are typically five times more economical. Thus, ICP-MS is far better suited for routine use in lead hazard assessments (38,39).

The utility of the stable lead isotope method for assessing sources of household lead exposure to lead-poisoned children can be illustrated with data from a household case study reported by Gwiazda and Smith (39). In the case provided here, environmental (soil, household dust, and paint) samples and a blood sample from the lead-poisoned child (blood lead = 16 μg/dl) were collected and analyzed for lead concentrations and stable lead isotopic compositions by magnetic sector–ICP-MS (38,39). These data show how lead isotope data can augment lead hazard evaluation performed by a certified lead hazard assessor.

Lead Hazards Evaluation Based on Lead Concentrations and Loadings. Two paint samples (Exterior window frame, #3, and Child's bedroom window sill, #8, Fig. 3a) had high lead content and were potential contributors to the child's elevated blood lead level. In particular, #8 was more accessible to the child and covered a friction surface (window jamb). The dust loading of the bedroom carpet (#2) next to this window, at 110 $\mu g/ft^2$, almost met HUD clearance standards at the time (100 $\mu g/ft^2$). Nevertheless the recommendation by the environmental health personnel was to control both paints and perform thorough cleaning and vacuuming of horizontal surfaces.

Lead Isotopes Results. The lead isotopes results indicate that one of the main suspected sources of exposure (paint #8) was an unlikely contributor to the child's elevated blood lead level because its isotopic composition is distant from the blood isotopic compositions at either time t_0 (PbB = 16 μg/dl), or 3 months later at time t_1 (PbB = 14 μg/dl) (Fig. 3c). Major isotope ratios of paint sample #3 are close to the blood ratio (Fig. 3b); however, when the minor ratios ($^{206}Pb/^{204}Pb$ vs. $^{207}Pb/^{204}Pb$) are considered neither paint sample (#3 or #8) matches the blood ratios (Fig. 3c). Other than direct paint ingestion, the likely pathway of

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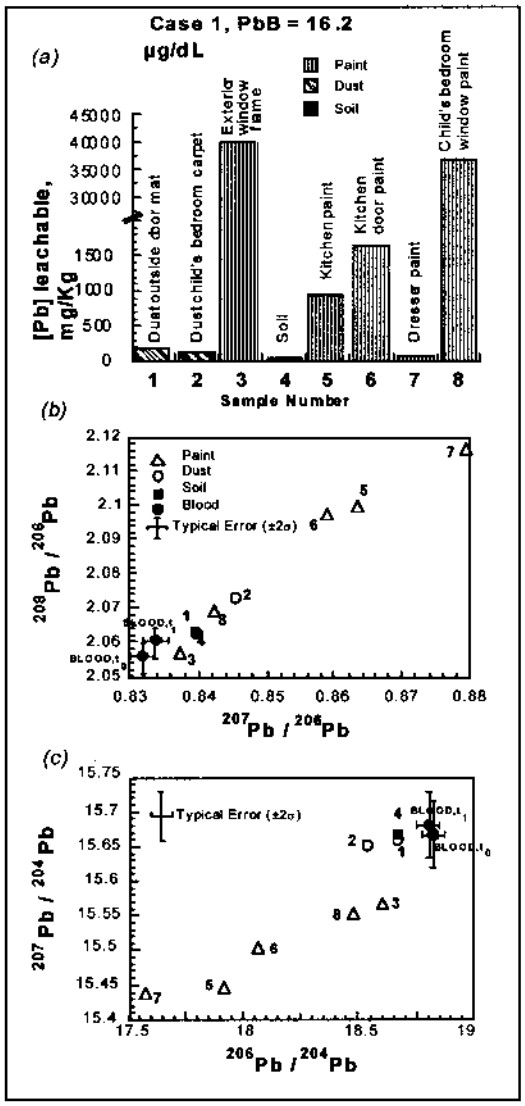

FIGURE 3 Lead concentration (a) and isotopic ratios (b, c) of environmental and blood samples from Case 1, reported by Gwiazda and Smith (Ref. 39). Sample identification numbers listed on X axis of figure (a) are used to identify the samples in (b) and (c). Leachable lead in (a) refers to mg of lead leached per Kg of sample. Blood lead level was 16.2 µg/dl at the time of environmental sampling (t_0) and 14 µg/dl three months later (t_1). Figure reproduced from Gwiazda and Smith (Ref. 39), with permission.

exposure from these sources to the child would be through household dust (#1 and/or #2). However, the isotopic composition of either dust sample does not match the blood isotopic composition in either isotope plot (Fig. 3b,c). Isotopically, the closest samples to blood are soil (#4) and dust from the exterior doormat (#1), both isotopically indistinguishable. Thus, no single sample or combination of samples could account for the extreme isotopic composition of the blood. Alternatively, the blood isotopic value could be a result of lead intake from an even more extreme nonsampled source (i.e., one with lower $^{207}Pb/^{206}Pb$ and higher $^{206}Pb/^{204}Pb$ ratios) combined with any of the samples on the opposite side of the blood (e.g., soil, bedroom dust, with their higher $^{207}Pb/^{206}Pb$ ratios) in Fig. 3 b,c.

Conclusions. The preferred interpretation based on lead isotopes is that a source with a $^{206}Pb/^{204}Pb$ ratio higher than blood either from outside the home or from endogenous (skeletal) origin was supporting the high blood lead level of the child. Additional, although minor, intake from any of the other measured sources in the house is possible and could not be discarded.

The above case study raises an important consideration for the use of stable lead isotopes in lead hazard assessments. The fact that potential lead sources (e.g., paint, soil, dusts) plot in linear arrangements with the biological (e.g., blood) samples does not necessarily allow the distinct identification of the actual lead sources to the child (39). This is because there is no certainty that all actual sources have been sampled, and because there are multiple hypothetical combinations of sources that could yield the isotopic value of the biological sample. Even if the isotopic compositions of a single potential source and a biological sample agree, the isotopic composition of this biological sample could still be explained as the result of mixing of lead from two or more sources along the continuum of the linear arrangement.

There are two main reasons for the covariation of major isotopic ratios of industrially processed lead found in households (52,60): (a) The isotopic compositions of all major ores, the ultimate source from which environmental lead was derived, fall very close to an average line in $^{208}Pb/^{206}Pb$ versus $^{207}Pb/^{206}Pb$ coordinates, and (b) the increased use of recycled lead metal in the manufacturing of paint pigments and other industrial leads throughout the latter 1900s has gradually blurred the isotopic differences of lead across different batches of paint or other materials. Nonetheless, there still are often measurable deviations from colinearity in the lead isotopic distribution of the minor ratios (vs. ^{204}Pb) observed in industrially processed lead found in households. Thus the utilization of *all* lead isotopes in lead hazard assessment, and not just the major ratios, maximizes the information that can be gained with the application of this technique (39).

Even with a full characterization of the relative contribution of all external lead sources to the child, the isotopic composition of the integrated external input and the isotopic composition of lead in blood may not agree owing to the presence

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of endogenous lead sources. In other words, lead in the bloodstream may also be comprised of lead that has been remobilized from endogenous sources such as the skeleton that have accumulated lead over medium to long periods of exposure (e.g., >6 months). The importance of mobilized skeletal lead as a contributor to blood lead levels has been shown in adults (35,36,61), and in children (62). As a result of the mixing of lead from these different exogenous and endogenous sources, there may occur a shift in the blood isotopic ratios away from the isotopic ratios of the main exogenous source(s) of exposure toward the isotopic values of lead released from the endogenous (skeletal) source(s).

2.4 Administered Stable Lead Isotopic Tracers in Metabolic Studies

Stable lead isotope tracer methodologies, in which a tracer enriched in a stable lead isotope (e.g., ^{204}Pb) has been administered to a human or animal model, have provided the most definitive measurements of lead absorption and metabolism in humans and animal models (13,18,19,48,63–65). In fact, a stable lead isotope tracer study performed by Rabinowitz and colleagues in the mid-1970s still represents some of the most accurate and well-cited data on lead metabolism in humans (13,63). Moreover, comparable studies in children have not been conducted, though it is well recognized that lead toxicodynamics in children may differ markedly from that in adults (2).

The stable lead isotope tracer approach for evaluating lead metabolism overcomes many of the analytical (poor precision and sensitivity) and health (exposure to radioactivity) limitations encountered in studies employing simple lead concentration measurements or radiolead isotopes. For example, several studies from our laboratory (DRS) have demonstrated the utility of ultraclean stable lead isotope techniques using ICP-MS and TIMS to investigate lead metabolism and treatments with therapeutic chelating agents in both laboratory animal models and humans (18,19,26,64–66). These studies have provided a means to investigate the metabolism and chelation of lead from chronic versus short-term exposures (18,19,66), as well as to assess the endogenous tissue sources of lead removed with chelation (18,65,66). Other studies using multiple stable lead isotope tracers in nonhuman primates have provided elegant data on the uptake and release of lead from the skeleton (48,67). Collectively, these studies have evidenced the minor error inherent in stable isotope tracer techniques using mass spectrometry relative to other sources of error (e.g., biological variability).

2.5 X-Ray Fluorescence Analysis

X-ray fluorescence (XRF) has received substantial attention as a viable method for noninvasive lead analyses. The clinical use of XRF became feasible in the 1960s with the development of semiconductor technologies (68). However, it

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was not until the mid- to late 1970s and later that it started to receive more widespread use to measure in vivo bone lead levels as a means to develop estimates of long-term lead exposure (61,69–76). X-ray fluorescence has also been used for the in vivo measurement of soft tissue (e.g., kidney) lead levels (77,78), as well as for the assessment of environmental samples (paint, dust) (79–82).

XRF instruments used for in vivo bone lead measurements can be divided into two general classes, those that measure K-shell X-rays and those that measure L-shell X-rays (76). Although some consider the utility and efficacy of both methods to be similar, the K-shell instruments measure lead to a greater depth within the tissue than do the L-shell instruments. This is primarily due to differences in the attenuation of the characteristic X-rays emerging from the tissue; thus L-XRF and K-XRF measurements sample different lead pools within the same measurement region. X-ray fluorescence measurements of bone lead levels have typically been calibrated and validated in past studies using lead-doped phantoms, which are used to derive external standard curves of lead concentration (e.g., μg Pb/g mineral for K-XRF measurements). More recently, Aro et al. (83) performed K-XRF validation using intact cadaver limbs (with skin and soft tissue) that were subsequently analyzed by ICP-MS to determine the actual bone lead levels. They observed close agreement between K-XRF and ICP-MS, with correlation coefficients for tibia and patella lead measured with the two methods greater than 0.9, thereby further validating K-XRF measurements for in vivo bone lead assessments.

Although many past studies with XRF have focused on occupationally exposed adults, several recent studies have used K-XRF to investigate bone lead levels in environmentally exposed populations of young adults and teenagers (84–86). For example, in a study of environmentally exposed young adults (18–21 years, $n = 23$), Hoppin et al. (84) detected a group mean tibia bone lead level of 3 μg/g bone mineral, which they determined was significantly different from zero given their measurement error. They concluded that bone lead levels of 2–3 μg/g bone mineral can be detected in groups of 100 or smaller, although they also point out that because of the large within-person variation in young adults, K-XRF may not yet be a useful tool for individual subjects. In a subsequent study, Hoppin et al. (85) attempted to identify the distribution of bone lead in a teenage population and to explore environmental and demographic factors associated with bone lead concentrations in young nonoccupationally exposed subjects ($n = 168$, age 13.5–19 years). They reported mean tibia bone lead levels of 4.0 μg/g bone mineral (±4.4 SD), and that bone lead levels were not associated with age, sex, race, or current home condition. They concluded that while bone lead levels were measurable in this age group, the common predictors of blood and bone lead levels were not explanatory for bone lead levels.

More recently, McNeill et al. (86) investigated various factors affecting the precision and accuracy of in vivo K-XRF measurements of tibia in a population of 530 young adults with low mean bone lead levels of around 2.5 μg Pb/g bone

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mineral. This study found that measurement uncertainties for young adults were poorer overall than uncertainties for a reference population of occupationally exposed men, which was partly attributed to the presence of obese subjects and women who were included in the group of young adults. In fact, regressions of precision against body mass index (BMI, defined as weight/ht^2) determined that the measurement uncertainties increased with BMI and were poorer for women than men (e.g., measurement uncertainties of 1 sigma were >8 μg Pb/g bone mineral for women with a BMI >0.004 kg/cm^2). They concluded that obese subjects should be excluded from K-XRF studies, since the measurement provides limited information and may be inaccurate. Overall, it remains apparent that while the in vivo measurements of lead in bone will not replace any existing measurement of lead exposure in humans, they have greatly supplemented the existing tests by providing information on lead pools that cannot be otherwise sampled except by biopsy (61,73,76).

These latter studies in young adults substantiate concerns regarding the sensitivity, accuracy, and precision of XRF for assessing bone lead stores in environmentally exposed persons (2,61,75,76,85,86). Concerns surrounding K-XRF measurement sensitivity and precision have been specifically addressed by several studies. Kim et al. (87) reported a methodological approach for more accurately evaluating low-bone-lead data measured by K-XRF, showing that the retention of all point estimates of measured bone lead concentrations provided less bias and greater efficiency in comparing the mean or median levels of bone lead of different populations. This was further suggested by Hu et al. (75), who argued for abandoning use of the minimum detectable limit (MDL) in favor of using all point estimates associated with each bone lead measurement in applications of K-XRF in epidemiological studies, and using the MDL in comparisons of instruments within or between laboratories, as well as in the interpretation of measurements of individual subjects.

2.6 Other Methods

2.6.1 Electrochemical Methods

A number of recent advances have been made in electrochemical methods for the measurement of lead concentrations in biological samples. Electrochemical techniques, which have proven particularly useful for measuring lead concentrations in blood, are based on quantitation of the current produced as lead is reduced or oxidized by varying the potential of a working electrode in an electrochemical cell containing the sample. While several electrochemical methods exist for measuring lead concentrations in biological and environmental matrices (e.g., differential pulse polarography, anodic stripping voltametry), anodic stripping voltametry (ASV) is the preferred method for blood lead analyses owing to its relatively high level of sensitivity (88–91).

The development of disposable carbon microarray electrodes in ASV measurements of low blood lead levels (<10 μg/dl) has proven useful because they require minimal sample pretreatment, and they provide a 10-fold improvement in the signal/noise ratio over single carbon disk electrodes, resulting in detection limits of <5 μg/L (91,92). More recently, similar methods [e.g., LeadCare System (LCS), ESA, Inc.] for the routine measurement of blood lead levels in clinical and field settings have been developed and validated. For example, Counter et al. (93) did a field screening of blood lead levels in a remote Andean village, using the LCS. They also performed intercalibration between the LCS and ICP-MS and AAS for blood lead measurements, reporting generally good correlations between LCS and ICP-MS ($r = 0.913$), and LCS and AAS ($r = 0.829$). While additional studies using the LCS are needed, this initial report substantiates that the portable LCS technique is a viable tool for screening and monitoring of relatively low (>7 μg/dl) and high (>60 μg/dl) blood lead levels in remote locations.

3. CHELATION TREATMENT FOR LEAD POISONING

The need to develop improved intervention strategies, particularly for moderately lead-poisoned children with blood lead levels between 20 and 45 μg/dl, is underscored by the large number of children estimated to be at risk for cognitive impairment (16,94–96). The clinical management of lead poisoning in children may utilize treatment with a number of possible therapeutic chelating agents, such as succimer (meso-2,3-dimercaptosuccinic acid, DMSA, Chemet) or $CaNa_2EDTA$ ($CaNa_2$ethylenediaminetetraacetic acid, Versenate). Succimer is considered to be a particularly safe and effective treatment for lowering blood lead levels, as well as some symptoms of lead intoxication (64,95,97–99). Moreover, it offers some advantages over $CaNa_2EDTA$ chelation, since it is administered orally and it does not appear to cause a significant diuresis of other essential elements.

Numerous clinical and animal model studies have shown that succimer reduces lead levels in blood and many other soft tissues (17,18,99,100), as well as some symptoms of lead intoxication (101). However, it is apparent that succimer treatment may be less effective in removing lead from the brain and skeleton than from blood (17–19,65,100,102). Moreover, few studies have specifically evaluated the efficacy of chelation treatment for reducing lead-induced cognitive deficits, although a recent randomized, placebo-controlled, double-blind clinical trial found that while succimer lowered blood lead levels, it did not improve scores on tests of cognition, behavior, or neuropsychological function in children with blood levels below 45 μg/dl (103,104). Studies of succimer treatment are also addressing this issue in nonhuman primate (105) and rodent (106) models.

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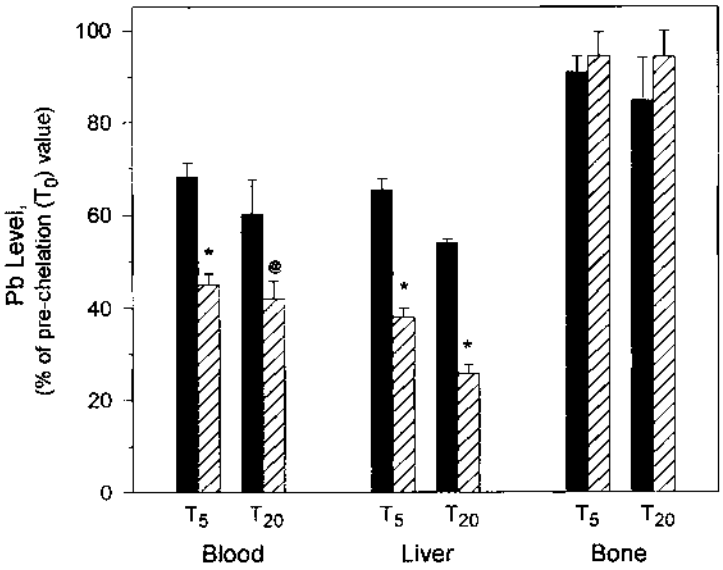

FIGURE 4 Mean (±SE) total lead levels in blood, liver, and bone tissues of the placebo (solid bars) and succimer (hatched bars) treated 1 yr. lead exposed juvenile (age 1 year) monkeys on days 5 and 20 of the first chelation treatment. Levels are expressed as percent (%) of the pre-treatment (day 0) values within each animal. Placebo and succimer 'n' = 22-23/group for day 5, and n = 7–8/group for day 20. A 2 × 2 repeated measures ANOVA indicated that treatment and treatment day were significant ($p < 0.05$) for blood and liver, but not bone lead levels. * = Statistically different ($P < 0.05$) from time-matched placebo group, based on post hoc Tukey's test ('@' = marginally not significant, p = 0.089). For reference, mean (±SE) tissue lead concentrations on day 0 (pre-treatment) were as follows: Blood placebo = 43.0 μg/dl (±2.2), succimer = 50.4 (±3.0); Liver placebo = 16.7 μg/g dw (±1.2), succimer = 18.7 (±1.9); Bone placebo μg/g dw = 57.1 (±5.6), succimer = 62.8 (±8.8). Figure reproduced from Smith et al. (Ref. 18), with permission.

Several recent placebo-matched studies in nonhuman primates have attempted to better clarify the efficacy of succimer chelation for reducing body lead levels, including lead in brain, liver, and bone (18,19). These studies have shown that a treatment regimen comparable to that used clinically significantly increased (>3-fold) the diuresis of lead, and significantly reduced blood and liver lead levels, but not bone lead of juvenile animals, relative to the placebo group (Fig. 4). Furthermore, a comparable study in adult monkeys demonstrated that

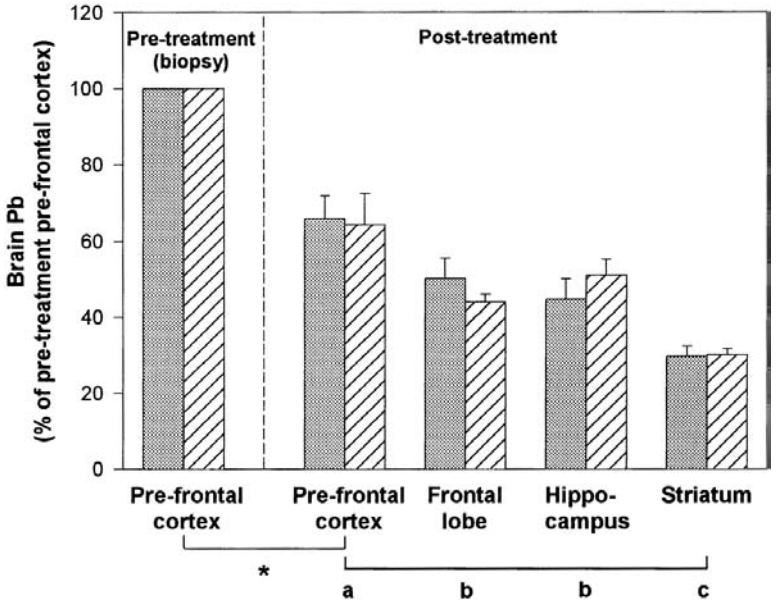

FIGURE 5 Lead levels in brain pre-frontal cortex (PFC, pre- and post-treatment), frontal lobe, hippocampus, and striatum of the vehicle (solid bars, n = 5) and succimer (hatched bars, n = 6) treated adult monkeys (lead levels expressed as a percentage of the pre-treatment PFC level within each animal). Animals were exposed to lead for a duration of five weeks prior to chelation. Values are means (± standard error). Mean (±SE) lead concentrations in the pre-treatment PFC biopsy for the vehicle and succimer treated groups were 1,980 ± 511 and 1,410 ± 207 ng/g dry weight, respectively. Symbols below the x-axis indicate: '*' = Pre- and post-treatment mean values were significantly different; 'a, b, c' = Comparisons of post-treatment brain regions. Means with different letters were significantly different from one another. Figure reproduced from Cremin et al. (Ref. 19), with permission.

succimer did not measurably reduce brain lead levels beyond the reduction achieved with the cessation of lead exposure alone (~34% reduction observed in prefrontal cortex) (19; Fig. 5). Notably, the ~60% reduction (relative to day 0) in blood lead levels of both juvenile and adult monkeys after succimer treatment for 19 days (i.e., succimer + cessation of lead exposure, Figs. 4 and 5) was similar to the 50–78% reductions in blood lead levels observed in clinical studies with children (95,107,108) and adults (108) treated for comparable durations.

This substantiates the effects of succimer on the other tissues not measured in those clinical studies.

However, reductions in blood lead levels reported by Smith et al. (18) are qualified by the rebound in blood lead levels that occurred within a week after completion of treatment (Fig. 6). Because of that rebound, there was no measurable benefit of succimer compared to placebo when assessed using blood lead levels measured on day 24, 30, and 60 (i.e., 5, 11, and 41 days after the completion of treatment on day 19) (Fig. 6). These data are consistent with several recent placebo-controlled clinical trials in children (104,110), both of which observed a significant rebound in blood lead levels over several months following the completion of chelation treatment. Overall, these studies demonstrated the relative benefit of eliminating lead exposures, which serves to underscore the importance of primary prevention of lead exposure. They also suggest that blood lead levels may be a relatively poor surrogate of brain lead, and that the clinical use of blood lead to dictate succimer treatment may not reliably reflect outcomes in the brain.

4. ANIMAL STUDIES

4.1 Rodents

Several high-affinity lead-binding proteins (PbBPs) from the rat kidney and brain have been identified and appear to act as molecular receptors for lead and may mediate its activity within these target tissues. For the rat kidney, a number of studies have shown that lead is bound to the protein α-2 microglobulin, a member of the retinol-binding protein supergene family, and is synthesized in the liver and transported to and taken up by the renal proximal tubule (5). This may explain the molecular mechanisms of how lead would be rapidly transported from the liver to the kidney.

The ability of chronic high-dose lead exposures to induce renal adenocarcinoma in rats and mice has been extensively studied for many years but the underlying molecular mechanisms are not clearly understood. Choie and Richter have shown marked increases in mitosis of renal proximal tubule cells, as well as increases in total DNA, RNA, and protein synthesis, suggesting that lead may influence the nuclear machinery at low dose levels (111,112). Recent studies have indicated that there is a relationship between the formation of intranuclear inclusion bodies associated with chronic lead exposures and the distinct alterations of renal gene expression often observed. Studies in rats have shown that lead-binding proteins may facilitate the intranuclear movement of lead in vitro followed by chromatin binding. This indicates the protein may influence pathognomonic lead intranuclear inclusion bodies in renal gene expression associated

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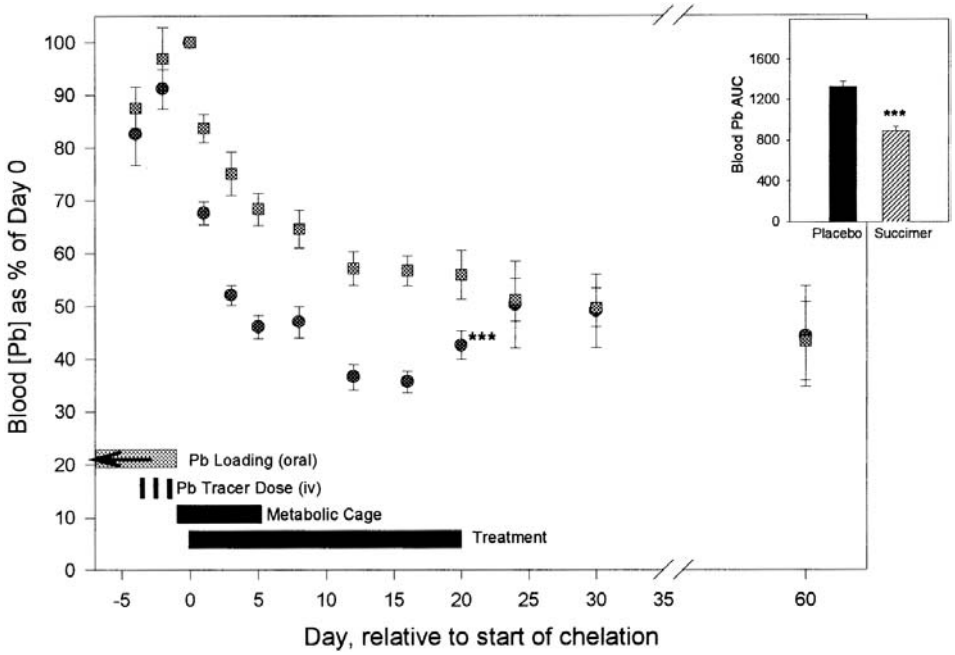

FIGURE 6 Mean (±SE) blood total lead levels (as % of day 0 pre-treatment values) in succimer (n) and placebo (■) treated 1 yr. lead exposed juvenile (age 1 year) monkeys over the course of the first chelation treatment (day 0–day 20) and beyond. Placebo and succimer group n = 21–23/group for days $-4 \rightarrow 20$, and n = 11/group for days $24 \rightarrow 60$. Inset: The mean (±SE) integrated area-under-the-curve (AUC, day 0 to day 20) for the placebo (solid bar) and succimer (hatched bar) groups. *** = Statistically different ($P < 0.001$) from placebo group (comparisons performed only on treatment day 20, and on AUC data). For reference, blood lead levels on day 0 were 43.0 μg/dl (±2.2 SE) for the placebo group and 50.3 μg/dl (±3.0 SE) for the succimer group ($p = 0.08$). Figure reproduced from Smith et al. (Ref. 18), with permission.

with the mitogenic effects observed of lead exposures in the kidney (6). Intravenous injection studies have shown that there is both an up- and down-regulation of a number of renal gene products in the rat kidney, which was found to be temporally linked to the reversible formation of lead-induced intranuclear inclusion bodies (113).

The effects of lead on the heme biosynthetic pathway have been extensively studied in vivo in the rat. Parameters of heme effects that been monitored include

ALAS, ALAD, and ferrochelatase activity, erythocytic Zn-protoporphyrin, and blood and urinary porphyrin levels (114).

4.2 Nonhuman Primates

Lead is probably the most frequently studied of the neurotoxic agents, both in humans and in various animal models. The monkey may be the best model with which to identify the low-level neurotoxic effects of lead since the nervous system of the monkey is very comparable to that of humans and often responds to toxic insults like that of humans. In addition, like humans, monkeys have a long period of gestation, infancy, and sexual immaturity during which the nervous system continues to develop. Therefore, this particular species may be a suitable model for providing a long period of vulnerability to study the sensitive effects of developmental exposure to lead (115,116). Intellectual functions such as learning, memory, adaptability, and distractibility have been investigated by numerous techniques, including discrimination reversal, matching to sample, and delayed alternation, and such various techniques have revealed impairments produced by lead similar to those observed in lead-exposed children (115,116). The behavioral characteristics of the monkey are also more like the human than those of any other laboratory species and research has mainly focused on impairment of learning and other behavioral outcomes that are produced by low-level exposures to lead during development (116,117). Results of primate research have clearly demonstrated learning and memory deficits as a consequence of developmental exposure to lead and implicate the same behavioral problems often observed in children. For example, lead-treated monkeys displayed memory impairment on a series of tasks requiring them to remember a previously observed stimulus, and they have also exhibited a higher rate of response on an intermittent schedule of reinforcement and had difficulty inhibiting responding when required for the task (117).

5. IN VITRO SYSTEMS

In vitro systems have been utilized to isolate, identify, and sequence proteins that have high affinity for lead from various target tissues and to study the biological function and chemistry of PbBPs. For example, PbBPs were observed to undergo aggregation after the addition of Pb^{2+} in vitro suggesting that these proteins may be involved in the formation of inclusion bodies found in the target tissues such as the kidney and the brain (118). In addition, PbBPs have been shown in vitro to facilitate the intranuclear movement of lead and its subsequent binding to chromatin, suggesting that these proteins may be involved in the alterations of renal gene expression and mitogenic effects often observed of Pb^{2+} in the kidney (6).

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PbBPs appear to mediate the bioavailability of lead to sensitive enzymatic processes such as the enzyme ALAD in the heme biosynthetic pathway. In vitro studies have shown that PbBP was able to attenuate lead inhibition of the heme pathway enzyme ALAD both by lead chelation and by zinc donation to this highly Zn^{2+}-sensitive enzyme (119,120). In addition, in vitro metal competition assays demonstrated that cadmium and zinc were the most effective competitors for inhibiting lead binding to PbBP (121).

6. MOLECULAR FACTORS

6.1 Lead-Binding Proteins

Low-molecular-weight proteins with high affinity for lead have been isolated from the specific tissues of rat, monkey, and human. These proteins are not identical across species but they do share common chemical characteristics, including a highly anionic nature and a high content of aspartic and glutamic amino acids. The highest concentrations of these proteins have been found in the two primary target organs for lead, the kidney and the brain. It has been proposed that these molecular factors may play a major role in mediating the intracellular bioavailability of this metal and hence toxicity to several essential cellular processes in these target tissues at low dose levels (122).

Data from numerous studies and epidemiological surveys indicate that nephropathological effects of chronic lead exposures involve tubular toxicity, interstitial disease, and renal adenocarcinoma, and that renal lead-binding proteins may play an important role in these pathological processes (123). The lead-binding proteins (PbBPs), in all species studied to date, are low-molecular-weight proteins rich in aspartic and glutamic dicarboxyl amino acids, have dissociation constants on the order of 10^{-8} M for Pb, and appear to influence the intracellular bioavailability of Pb in the kidney (6,124). Numerous studies have been performed to identify these PbBPs and renal PbBP from rat has been shown to be a cleavage product of α_2-microglobulin, a member of the retinol-binding protein family. Histochemical studies have shown it to be localized within lysosomes of S_2-segment proximal tubule cells (5). It is hypothesized that this protein is synthesized then released from the liver, reabsorbed by proximal tubule cells by normal protein reabsorption mechanisms, and the uptake may be due to specific receptors present on the membranes of S_2 segments of renal tubules (124). Western blot and immunohistochemical studies have shown that a polyclonal antibody to the rat renal PbBP does not recognize the brain PbBP, which appears to be a chemically similar but distinct molecule (5). Recently, two human renal PbBPs have been identified as the 9-kDa acyl-CoA binding protein (ACBP, also known as diazepam binding inhibitor, DBI) and the 5-kDa thymosin beta-4 (8,9). This study was unique in that these human lead-binding proteins were isolated based

on physiological lead that was discovered presently bound in vivo, using the ultraclean laboratory techniques noted above. These proteins were found to exhibit dissociation constants of approximately 10^{-8} M for lead and both ACBP and thymosin beta-4 occur across animal species from invertebrates to mammals in major tissues, indicating possible multiple functions for these proteins (8,9,124).

6.2 Lead Inclusion Bodies

It has been hypothesized that PbBPs may play a role in the formation of pathognomonic nuclear lead inclusion bodies (113,124). Lead inclusion bodies are characterized as lead-protein aggregations within the nucleus of renal proximal tubule cells and the liver parenchyma, and are a morphologically discernible feature of chronic lead exposures (125). When observed by light microscopy, these inclusions are dense, homogeneous eosinophilic bodies, and are acid-fast when stained with carbolfuchsin. Ultrastructurally, the inclusion bodies are distinct from nucleolar structures, having a dense central core and an outer radiating fibrillary region (126). Goyer et al. have partially isolated inclusion bodies and found them to be composed mainly of acidic proteins and to be enriched in lead compared to other nuclear fractions (127). It has been suggested that lead may bind loosely to the carboxyl groups of the acidic aspartic and glutamic acids amino acids (126). PbBP isolated from rat kidneys has been found to aggregate in the presence of lead and zinc in vitro, forming tetramers with two metal ions bound per tetramer (118). In addition, lead has been shown to induce the formation of inclusions in the cytoplasm of cultured rat kidney cells, and these lead inclusion bodies may then migrate into the nuclei of the exposed cells (125). Addition of the protein synthesis inhibitors actinomycin D or cycloheximide prior to lead exposure resulted in a marked decrease of inclusion bodies (where lead uptake by cells was not affected), indicating that active protein synthesis is required for the formation of lead-induced inclusion bodies.

6.3 Sensitive Enzyme Systems

6.3.1 Heme Pathway Enzymes

Lead intoxication has multiple hematological effects, including lead-induced anemia where red blood cells are microcytic and hypochromic as seen in iron deficiency (126). A number of mitochondria and cytosolic enzymes that are part of the heme biosynthetic pathway are known to be highly sensitive to alterations by lead, and the degree of disturbance of heme biosynthesis depends on the duration and the severity of lead exposure. The mitochondrial enzyme δ-aminolevulinic acid synthetase (ALAS), the first and rate-limiting enzyme in heme biosynthesis, is particularly sensitive to direct inhibition by lead. In addition, ALAS

enzyme synthesis is regulated by feedback inhibition such that inhibitory effects of lead on other enzymes downstream in the heme biosynthetic pathway can lead to increased synthesis of ALAS. The association of these two alterations by lead on ALAS has led to different findings concerning the effects of lead on overall ALAS activity, including increases, decreases, and no changes of activity (128). Another mitochondrial enzyme inhibited by lead is ferrochelatase, the terminal enzyme in the heme pathway that catalyzes the incorporation of ferrous ion into the porphyrin ring structure of the heme (126,128). The deleterious effects of lead on mitochondrial inner membrane structure and function and the requirement for transmembrane movement and the concomitant reduction of Fe^{3+} to Fe^{2+} for ferrochelatase activity may possibly explain the observed inhibition of this enzyme by lead (128). Alterations in normal mitochondrial structure/function relationships by lead may be represented as an important aspect of the lead-induced effects on mitochondrial heme enzymes ALAS and ferrochelatase.

The cytosolic enzyme δ-aminolevulinic acid dehydratase (ALAD), which catalyzes the second step in the heme biosynthetic pathway, is highly sensitive to inhibition by lead in most tissues except the brain and kidney. ALAD resistance to lead inhibition in these tissues may be mediated by the presence of endogenous lead-binding proteins. Conversely, the mitochondrial enzymes ALAS and ferrochelatase have been shown to be inhibited in brain and kidney tissues following lead exposure both in vivo and in vitro (123). It is hypothesized that lead-binding proteins may protect ALAD by sequestering lead, donating zinc to ALAD with subsequent enzyme activation, and allowing movement of lead across mitochondrial membranes (119,120,129). The latter effect by PbBPs may explain the inhibition of mitochondrial enzymes ALAS and ferrochelatase that is observed following lead exposure. The molecular mechanisms of this process still remain unclear and the role of lead-binding proteins requires further study.

6.3.2 Alterations in Gene Expression

Studies suggest that PbBPs may play a major role in lead-induced renal cancers, as well as mediating the known alterations in gene expression associated with chronic lead exposures. Lead-induced changes in renal gene expression associated with the formation of intranuclear inclusion bodies have been known for years and these factors may mediate carcinogenesis (7,124). Numerous studies of lead-induced renal gene expression have demonstrated both up- and downregulation of a number of gene products, suggesting that prolonged exposure to lead may alter normal gene expression and this may mediate the carcinogenic process (123). Studies based on rodent models suggest that PbBP facilitates the transport of lead into the nucleus and a study by Mistry and co-workers has shown that cytosolic PbBPs facilitated the cell-free nuclear uptake of lead by a series of saturation and sedimentation analysis techniques (113). It is hypothesized that this lead-protein complex may bind to chromatin and interact with specific 5′

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flanking regions of regulatory/promotion sites of those genes exhibiting altered expression patterns from lead exposure (6,123,124). This mechanism may also activate the expression of oncogenes leading to cellular transformation and carcinogenesis (6). The precise molecular biology of these interactions and whether human PbBPs behave in a similar manner as in the rodent model is unclear. Further studies are needed in this area to determine the mechanism of PbBPs' influence on the development of renal adenocarcinoma that is associated with chronic lead exposures.

6.4 Toxicological End Points

6.4.1 Neurobehavioral

IQ. The most sensitive or critical effects from lead exposure to infants and children involve the nervous system. Lead toxicity to the central nervous system may cause delayed development, diminished intelligence, and altered behavior, and these effects have been demonstrated to occur at blood levels between 10 and 20 μg/dl (130). Studies conducted in the 1970s revealed that children with blood lead levels ranging from 50 to 70 μg/dl may have decrements in cognitive abilities by as much as 5 or more IQ points, but without any overt clinical symptoms (131). Deficits in psychometric intelligence, speech and language processing, attention, and classroom performance were reported in first- and second-grade children with elevated lead dentin levels but showing no overt clinical symptoms (132). Children at the fifth-grade level with higher dentin lead levels had lower IQ scores and required special education services (133). A 10-year follow-up study of these same children found the neurobehavioral deficits to persist well into young adulthood (134). For blood lead levels below 30 μg/dl, the reports of IQ decrements are quite mixed, and some studies showed no changes of IQ when all variables were taken into account (135,136).

Hyperactivity. Neurobehavioral function of rat pups was tested in a study where dam rats were given lead-containing drinking water (0.58, 1.76, and 5.27 mmol/L) 16 days from gestation to the weaning of offspring 21 days postpartum. The pups were then given the same drinking water 30 days postnatally (137). The number of ambulations and rearings was found to be increased in locomotor activity tests, indicating that lead exerts induced hyperactivity in young rat pups.

It has been noted that experimental animals exposed to lead have exhibited behaviors attributed to ''impulsivity'' and ''inability'' to inhibit inappropriate responses, similar effects observed in children diagnosed with attention deficit–hyperactivity disorder (ADHD), leading some to suggest that lead exposure may be associated with attention deficit. One study, based on the hypothesis that attention deficits are related to the ineffectiveness of delayed reinforcement, found that rats chronically exposed from 40 days postweaning to lead acetate in drinking

water (0, 50, or 150 ppm), and trained on a fixed-ratio (FR) wait behavioral baseline, exhibited increased response rates on the FR schedule and a decreased mean longest waiting time, but also a higher number of responses per reinforcer over that of unexposed controls (138). These results suggest that lead-induced differences are consistent with an inability to manage delays of reinforcement. A number of animal models for ADHD syndrome have been developed, but many believe that none of these models are truly representative of naturally occurring developmental ADHD, so care must be taken when determining the characteristics representative of ADHD with certain experimental models, especially those pertaining to both hyperactivity and attentional problems (139,140). For example, Kohlert and Bloch were able to demonstrate that hyposexual rats observed to be hyperactive may serve as a suitable model for the study of ADHD syndrome (139).

The Centers for Disease Control, using data from many studies of neurobehavioral effects of lead in children and animals, has redefined the lead exposure levels for clinical intervention from 25 to 10μg/dl (141). It is evident that screening programs and preventive strategies are necessary, as well as the responsibility of physicians, teachers, and child care personnel for identifying children with behavioral signs of subclinical lead intoxication as early as possible.

Learning Disorders. Chronic lead exposure during brain development is known to affect functions of the central nervous system and one study assessed the most sensitive periods of exposure at different developmental stages of rats exposed chronically to low levels of lead (142). Exposure periods at the prenatal and the early postnatal phase and continued well into adulthood impaired learning as well as hippocampal long-term potentiation, while exposures starting not before 16 days postnatally were found not to affect these end points reflecting the higher vulnerability of the immature hippocampus to lead-induced deficits. Another study of 11-day-old Sprague-Dawley rat pups whose mothers were exposed continually to lead acetate (200 mg/L) in their drinking water from breeding through gestation exhibited differences in a learning paradigm compared to control pups, the data indicating that low-level lead exposures can induce significant behavioral deficits in the young pups (143). Groups of male rats exposed to lead during different periods of development, such as through gestation to lactation (maternal exposure) or continuously through conception to adulthood, exhibited highly significant impairment in water maze performance tests, whereas post-weaning lead exposure periods did not result in impaired performance despite elevated blood and brain lead levels (144). And another study found that the learning ability of rat offspring from dual parental exposure was more impaired than that from single parental exposure, which itself was detrimental compared to unexposed controls (145). These results indicate that a window of susceptibility

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to lead neorotoxicity exists for the development of the brain and lead exposures that may result in long-term cognitive or spatial learning deficits.

6.4.2 Heme Pathway Alterations

Blood ALAD. Lead exposure results in a number of disturbances of heme metabolism. The degree of these disturbances depends on the severity and duration of exposure. As noted earlier, ALAD is very sensitive to inhibition by lead (IC_{50} of 10^{-7} mol/L) in most tissues except the kidney and brain owing to the presence of endogenous cytosolic Pb, Zn-binding proteins that may protect this enzyme from lead inhibition via Pb sequestration, and donation of Zn^{2+} to the ALAD enzyme. However, blood ALAD is highly sensitive to lead inhibition and the measurement of erythrocyte ALAD activity has been used as a monitoring test of lead exposure. One study has found that erythrocyte ALAD activity in a group of lead-exposed male workers was remarkably reduced while erythrocyte protoporphyrin levels were increased (146). Genetic polymorphisms of the heme molecule among the general population may be related to the sensitivity of specific individuals to lead disturbance of heme metabolism (1). There are two common alleles for the ALAD gene, known as $ALAD^1$ and $ALAD^2$, which results in a polymorphic system with three possible isozyme phenotypes: ALAD 1-1, ALAD 1-2, and ALAD 2-2. It has been suggested that individuals with the ALAD 2-2 phenotype may be more sensitive to lead accumulation and its associated biological effects (147), and that the $ALAD^2$ allele may be the cause for the increased sensitivity to lead (148).

Porphyrinuria. Depressed heme synthesis brought about by lead exposures is thought to be the stimulus for the increased rate of activity for the first step in the heme biosynthetic pathway involving the enzyme ALAS. The inhibition of ALAD enzyme activity in the blood by lead and the increased synthesis of ALAS by virtue of negative feedback control due to depressed heme synthesis can lead to an accumulation of large amounts of the heme precursor δ-aminolevulinic acid (ALA) in the blood depending on the severity of lead intoxication. This eventually leads to the increased excretion of ALA in the urine, and this phenomenon is used as an indicator of lead exposure. Changes in ALAD activity in peripheral blood and the excretion of urinary ALA have been found to correlate closely with blood lead levels and can serve as early biochemical indices of lead exposure (1). Increased excretion of coproporphyrin III in the urine may occur as a result of depressed coproporphyrinogen oxidase activity, while porphobilinogen excretion is not usually increased (149).

Zn Protoporphyrin. As noted earlier, the enzyme ferrochelatase is essential for the insertion of ferrous ion into the precursor protoporphyrin IX, and the inhibition of ferrochelatase by lead results in depressed heme formation and an

excess of protoporphyrin in the place of heme in the hemoglobin molecule. As a result, there is an increase in erythrocytes containing protoporphyrin, and with time Zn is chelated into the molecule usually occupied by iron forming zinc protoporphyrin (ZnPP). Red blood cells containing ZnPP are highly fluorescent and this property has been used to diagnose increased lead exposures (126). Some studies have found that protoporphyrin concentrations in the blood are the best risk indicator of lead exposure compared to other diagnostic tests for lead toxicity (150), with the accumulation of ZnPP in erythrocytes being one of the earliest effects of lead-induced disruption of heme biosynthesis. One study utilizing a fluorescent assay to measure erythrocyte protoporphyrin in the blood of occupationally lead-exposed workers found the log of erythrocyte protoporphyrin levels to correlate closely with blood lead levels ($r = 0.72$) (151). Piomelli and coworkers have shown that measurement of erythrocyte protoporphyrin in erythrocytes is useful for lead exposures producing blood lead levels in the range of 25–50 μg/dl (152). However, erythrocyte protoporhyrin measurements may not be sensitive to lower lead exposure levels and such results can be confounded by iron deficiency, which is widespread in the U.S. population, especially among pregnant women (153,154). It has been estimated that as many as 30% of children might be misclassified by erythrocyte protoporphyrin screening owing to iron deficiency as a confounding factor (153). It is clear that a more sensitive and specific biological marker is needed to screen for lower levels of lead exposure producing blood levels of lead of <10 μg/dl.

7. REPRODUCTIVE OUTCOMES

The reproductive effects of lead have long been suspected, but there are limited studies regarding the effects of low-level lead exposure on the male and female reproductive systems (155). Acute lead toxicity has been known to cause sterility, abortion, and neonatal mortality and morbidity, and a number of studies concerning the gametotoxic effects in both male and female animals have been carried out (126). Occupational exposures to lead in a number of industries may pose potential adverse effects on the reproductive capacity of exposed male workers. Clinical and animal studies have indicated that abnormalities of the hypothalamic-pituitary-testicular axis and spermatogenesis can result from chronic lead exposures although the mechanisms of such adverse effects are still unclear and conflicting. One animal study has found that as the levels of lead treatment increased, intratesticular sperm counts significantly decreased, along with suppressed sperm follicle-stimulating hormone (FSH) values and decreased ventral prostate weights accompanied by no changes of serum lutenizing hormone (LH) levels. These results may indicate a possible inhibition of testicular function from lead exposures (156). There is evidence from past evaluations of occupationally exposed workers that moderate exposures to lead may be associated with small

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changes in male endocrine functions that may possibly reflect both primary and secondary effects of lead on the testes and the hypothalamic-pituitary-testicular axis (157). A group of 23 occupationally exposed men who worked in a lead-smelting industry were studied and the results suggest that prolonged exposures may initially produce direct testicular toxicity, followed by disturbances of hypothalamic and pituitary functions with longer periods of lead exposure (158). Another study of 90 occupationally exposed men in the United Kingdom found that lead exposures that were encountered may cause subclinical primary damage to the seminiferous tubules in the testes as evidenced by subclinical increases of FSH that were related to blood lead levels (159). In addition, a significant positive correlation was found between LH levels and the length of occupational lead exposure within the exposed group. Today the toxicological impact of chronic environmental and occupational lead exposures on testicular endocrine function and the male reproductive system is still unclear.

8. RISK ASSESSMENTS FOR LEAD

Lead poisoning is the most prevalent disease of environmental origin in the United States today and continues to be a source of concern for the U.S. Food and Drug Administration and other federal regulatory agencies. Today many uncertainties still remain concerning effects of lead on the nervous system causing minimal brain dysfunction in children. Lead poisoning in children results primarily from exposure by ingestion of lead from environmental media, including paint chips, soil, dust, ceramics, drinking water, and certain medications (130). Past studies in children have failed to define adequately the mechanism of effects, the critical period and duration of lead exposure, and the role of other factors in rendering the development and growth of the child susceptible to lead. Prospective studies are now underway to assist the resolution of these resolutions (160). It is currently believed that blood lead levels as low as 10 μg/dl have been associated with impaired neurobehavioral and cognitive development and electrophysiological deficits in children, and in reduced birth weights and reduced gestational age in infants (161). Lead-induced toxicity to the central nervous system has been found to cause delayed development, diminished intelligence, and altered behavior, and has been demonstrated to occur at blood lead levels between 10 and 20 μg/dl (130). The Centers for Disease Control and Prevention and the National Academy of Sciences have recommended that a blood lead of 10 μg/dl or higher be considered evidence of increased lead absorption. And blood lead levels of 10 μg/dl are also of interest in pregnant women because of exposure to the developing fetus, where ingestion levels of 250 μg lead/day were estimated to result in this blood level (161).

Adult lead poisoning results basically from exposure by inhalation in the workplace, the air of heavy traffic and industrial areas, and ingestion of lead from

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food (162). Blood lead levels of 30 μg/dl have been associated with elevated blood pressure and other adverse health effects in adults (161). Effects on blood pressure from low-level lead exposure, especially in adult males, may be related to the effect of lead on calcium-mediated control of vascular smooth muscle contraction and on the renin-angiotensin system (155). Therefore, the blood levels of 10 and 30 μg Pb/dl may represent the lowest-observed-effects levels for young and adult populations, respectively (161).

Unsettled issues in need of additional study include the frequency of screening young children for lead, the question of whether women should be offered screening for lead before conceiving a pregnancy, the important role of X-ray fluorescence analysis in assessing lead in bone, and the relevant legislative response of the U.S. government to the lead-based-paint abatement.

9. HUMAN EPIDEMIOLOGY STUDIES

9.1 Workers

That occupationally related exposure to lead can result in severe damage to the health of workers has been recognized for centuries (2,163–165). In more recent times a number of protective measures have been instituted, ranging from use of respirators and worker and plant monitoring to exclusion of reproductive-age women and of contaminated workers from certain jobs. Despite these public health concerns and measures, some jobs, such as battery recycling, lead smelting, alkyl lead manufacturing, plumbing, and pipe fitting, can still place workers at higher-than-average risk of excessive exposure to lead. Lead smelting and refining are among the jobs that put workers at the highest risk of occupationally related exposure to lead (166). Additionally, exposure can come from other jobs, such as spray painting, repairing automobile radiators, or welding, that are less directly identified as being at high risk to the workers (1,167).

Studies have shown only a moderate to weak correlation between measurements of lead in the work environment and in the body fluids of workers. Cable-producing workers, for example, were found to have a mean blood level of around 50 μg/ml when the exposure level was near 60 $\mu g/m^3$ (168). Richter et al. reported mean blood leads of 55, 59.5, and 58.4 μg/ml for workers in environments that measured 50, 85, and 190 $\mu g/m^3$, respectively (169). Wu et al. (170) studied over 220 battery-factory workers and reported an average blood lead level of 56.9 μg/dl and an average concentration of ambient lead of 0.190 mg/m^3 in their work environment, but the standard deviations, 25.5 and 0.331, respectively, were quite large for both measurements. Both the weak correlations and the large standard deviations suggest that a major challenge in advancing the understanding of exposure and health consequences is the identification of more specific subgroups of individuals who are the ones who will suffer adverse health at different

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levels of exposure. There remains a lack of agreement among investigators regarding the best methods of obtaining precise measurements of either the individual workers or their work environments. Because of the difficulty of obtaining precise measurements of exposure from the work environment, especially over long periods, and of the sensitivity status of each individual, the results from epidemiological investigations have not always produced consistent results. Traditionally the body burden of lead has been captured by the level of blood lead, but more recently the feasibility of using X-ray fluorescence techniques for measuring lead in the bone has been an improvement in assessing an individual's long-term body burden (171).

Although variation in susceptibility to the effects of lead exposure at an individual level remains poorly understood, the general clinical presentation from lead poisoning has been recognized and well documented from individuals with short-term massive exposure to lead. The major symptoms of lead poisoning include gastrointestinal symptoms of loss of appetite, dyspepsia, constipation, and colic; neurological symptoms of irritability, coma, and convulsions; and muscle and joint symptoms of pain; fatigue, tremor, and wrist or foot drop (172–175).

A shift from a focus on the health consequences of workers experiencing large doses of lead to the health of those experiencing low to moderate levels of exposure has occurred (176). As knowledge has accumulated about adverse health effects at lower and lower levels of lead exposure, workers have been increasingly protected, as a whole, primarily from laws and regulations (167,177) and have been identified at risk and intervention instituted at lower levels. Thus most workers today in developed countries experience relatively low to moderate exposure compared with the previously much higher levels. The study of worker populations has become more complex as investigations have moved to the inclusion of asymptomatic workers and conditions. Furthermore, industry has been generally less cooperative in the pursuit to identify health problems than that to understand or resolve problems that are known to exist (167).

Current studies have benefited from directions provided by the clinical manifestations in workers exposed to very high levels of lead and by the establishment of biomarkers related to such exposure. While a detailed discussion of biomarkers will not be included in this section, it nevertheless should be recognized that there has been an increasingly greater understanding of the changes in specific organ systems, and these changes or biomarkers are precursors of overt clinical symptoms or disease (177). Not only can biomarkers be used in studies in which they are linked to exposure but also they can be linked to clinical health outcomes. Thus the clarification of the causal links between exposure and disease will be increasingly possible as will the use of biomarkers to identify the more sensitive individuals to lead exposure regardless of the level of exposure.

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The current major public health concern is to identify both short-term and long-term risks from low levels of exposure over a long period (176,178,179). The following sections indicate the key health areas that are under investigation to establish or refute an association with low to moderate lead exposures experienced by workers.

9.1.1 Reproductive Functioning

Animal studies have shown that reproduction is affected by lead and early documentation suggested that the number of successful pregnancies decreased among women workers exposed to lead. Until recently, some caution has been exercised in assigning women of childbearing age to high-risk jobs (2,167,176,177,180). Despite these precautions, Savitz et al. (181) reported that women employed, as late as 1980, in the lead industry had an increased odds ratio of 1.6 for having a pregnancy that ended in stillbirth and an odds ratio of 2.3 for a pregnancy that ended in preterm birth. While future studies may turn once again to female workers as they assume the same jobs as men, current studies have addressed the risk of compromised reproductive functioning, such as a decreased number of sperm, less motility, and changes in morphology, resulting from male workers' exposure to lead. A number of studies have reported possible decreases in reproductive functioning of male workers in the lead industry (182–184). Assennato et al. (185), for example, reported that fewer sperm were measured in lead-battery workers with blood lead values greater than 60 μg/dl, and the investigations of Rodamilans et al. (158) and Lerda (186) suggested that reproductive dysfunction is probably associated with the length of time of the occupational exposure. In a recent study of men with moderate exposure, Telisman et al. (187) found corroborating evidence of reduced reproductive capacity and that there were better correlations of reproductive factors with ALAD than with blood lead levels. Their conclusion was that ALAD is an inducator of long-term cumulative lead exposure and that no threshold of exposure was observed in the decrease in semen quality (187). Viskum et al. (188), in their study of men employed at a car battery plant that subsequently closed, demonstrated that lead effect on sperm quality could, at least partly, be reversed. Their findings strengthen the position that lead is the causal agent in the observed adverse reproductive effects.

The issue of whether the results of pregnancy in women with partners working in the lead industry is less favorable has been addressed by examining several types of outcomes. Data to date suggest that paternal exposure at low to moderate levels could be related to an increase in spontaneous abortions. Lindbohm et al. conducted a case/control study of 213 spontaneous abortions and found that there was some evidence of an association between spontaneous abortions and paternal lead exposure, though not a statistically significant one (189). Anttila and Sallmen (190) reviewed articles examining the same relationship and found a total of 340 spontaneous abortions that had been reported. They concluded that the weight

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of evidence suggested an association between paternal exposure to lead and an increased risk of spontaneous abortion. However, they also called for confirmatory studies to be conducted. The authors of the case/control study on spontaneous abortions used a similar study design to investigate paternal lead exposure and congenital malformations. They found an odds ratio of 2.4 for a congenital malformation to be present if there was a father who had been exposed to lead because of his work (191). Although adjustment for potential confounding factors reduced this estimate very little, it did not reach statistical significance. Lin et al. (192) compared birth outcomes of the children of over 4000 lead-exposed male workers to those of the children of over 2000 births to nonexposed and found that workers who had elevated blood lead levels for more than 5 years had a risk ratio of 3.85 and of 2.45 for having a child with low birth weight or prematurity, respectively. Both estimates were statistically significant. In sum, there is some evidence, albeit not very strong, that paternal exposure to lead adversely affects reproductive functioning and viability of the pregnancy. Findings from the human studies, while not convincingly strong by themselves, are supported by animal studies; thus better evidence to substantiate the role of paternal lead exposure and reproduction is needed.

9.1.2 Heme Biosynthesis, Renal and Cardiovascular Effects

Substantial documentation from animal and mechanistic studies (2) have established plausible biological pathways of diminished hemoglobin biosynthesis. The disturbances of the heme pathway by lead ingestion are consistent with observed clinical effects, such as renal dysfunction and anemia (2,176,193–195). The issues of whether damage similar to that manifest in workers exposed to high levels of lead is present at low and moderate levels of exposure and whether subclinical damage is sustained at such levels that then manifests itself in subsequent health problems have continued to be of concern. Often exposed individuals remain asymptomatic, for example, until significant reductions in renal function have occurred. Of particular concern has been the possibility that asymptomatic renal damage or other vascular damage could lead to elevated blood pressure (196), a precursor of serious health problems. Such concerns are well founded in light of the observed cellular and clinical data (128,197). The implication of an association between lead exposure and blood pressure is that excess morbidity and mortality from cardiovascular and cerebrovascular disease would be experienced from occupational exposures to lead (198–200). Because elevated blood pressure is known to increase risk of cardiovascular events, establishing a link with lead exposure would provide an intermediate health outcome of considerable significance not only with respect to the levels at which occupational exposure can be tolerated but also because early treatment of hypertension is highly effective. Moreover, blood pressure measurements are, in principle, objective and feasible data to obtain. Until recently a number of individual small studies (177,199) led

to the conclusion that there was no association between low or moderate lead exposure and elevated blood pressure. A number of problems, such as a lack of a standardized protocol for obtaining the measurements of blood pressure that were analyzed, small sample sizes, and failure to control for important cofactors like age, have added to the uncertainty regarding a lead–blood pressure effect and whether the effect, even if present, is clinically significant (170,201–203). However, when the question of elevated blood pressure was examined in large, carefully conducted studies, the results established a positive association between blood lead levels and blood pressure (2,196,198).

Interestingly, it has been large community studies that have been able to establish the presence of a positive association. In an analysis of data from the second National Health and Nutrition Examination Survey (NHANES), Harlan et al. found a direct relationship between blood lead levels and systolic and diastolic pressures (204). When significant cofactors, such as age, race, and body mass index, were controlled, the relationship remained significant only for males for both systolic and diastolic pressures (205). An alternative explanation that concurrent secular trends both in blood lead levels and in blood pressure, rather than a causal relationship, could account for the observed association does not appear to be plausible (206). Pocock et al., in a survey of over 7000 men, found a positive association between blood lead and both systolic and diastolic blood pressure but concluded that blood lead exposure was unlikely to be a major risk for subsequent cardiovascular events (207). Consistent with the findings from the U.S. and British studies, Neri et al. reported that an analysis of the Canada Health Survey data indicated, overall, a small, positive relationship between blood lead and diastolic blood pressure but that a blood lead level over 10 μg/dl conferred a higher risk of 37% in having a diastolic blood pressure over 90 mmHg, a level considered to be clinically significant and usually that at which treatment intervention is initiated (208). In a recent publication, Korrick et al. reported that an increase in patella bone lead of about 25 μg/g was significantly associated with a twofold risk of hypertension in women; however, no association was found with blood or tibia lead concentrations (209). Adding to the weight of evidence are the findings from a longitudinal study reported by Weiss et al. that showed that an elevated blood lead level, even within the normal range, was a significant predictor of subsequent systolic blood pressure even after adjustment for key cofactors that included baseline systolic blood pressure (210). Investigations have found that blood lead was associated with other significant cardiovascular changes, such as left ventricular hypertrophy, serum cholesterol, and electrocardiographic conduction disturbances (211–213). In sum, while uncertainties remain regarding whether the size of the effect of lead exposure on blood pressure is about the same for diastolic as for systolic or for women as for men, findings from well-conducted studies have established a small positive association. While the effect size is small, from a public health perspective, it is important. The findings indi-

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cate that a reduction to half of the blood lead level would overall reduce the incidence of cardiovascular disease by over 100,000 events per year (211).

9.1.3 Cancer

Animal studies have shown that high levels of exposure to lead can produce renal cancer (214,215). Human studies, however, have been inconsistent in their findings. Elevated risk of renal cancer among occupationally exposed workers was reported by Selevan et al. (216) and by Cantor et al. (217), but no association was reported by Cooper et al. (218). Elevation of other types of cancer as a result of lead exposure from work has not been established. Cocco et al. (219) examined the level of lead exposure in over 25,000 brain cancer deaths and 100,000 deaths from nonmaligant diseases. They concluded that brain cancer risk was increased with exposure to lead. The measurement of exposure was based on occupation and industry codes from the death certificates and could have resulted in some misclassifications of exposure. Fu and Boffetta (220) in a review article concluded that the evidence combined from different studies in a meta-analysis showed a significant increase from lead exposure in stomach, lung, and bladder cancer as well as overall cancer. The numbers for most specific cancers made it impossible to examine them individually. Furthermore, these findings are weakened because few cofactors were available for control in the analysis. Overall, there is some suggestion that an elevation in cancer risk might be associated with occupational exposure to lead, but the accumulated evidence to date is weak (2).

9.1.4 Neurological Functioning

Because of the neurotoxicity of lead at high levels (2,177), evidence relating to possible dysfunction associated with low to moderate levels of exposure is beginning to be accumulated. Kuhn et al. recently reported an elevated risk of Parkinson's disease associated with occupational exposure (221), and a review of the epidemiological studies led Gorell et al. to conclude not only that some risk might be present but also that flawed study methods precluded a clear understanding of the role that exposure might have (222). Conclusions from several reviews of neurobehavioral assessments in workers have indicated a decrease in one or more performance tasks. Based on evidence from 14 studies, Ehle and McKee reported a lower ability to process and integrate information among workers exposed to lead (223). Balbus-Kornfield et al., based on findings from 21 studies, concluded that workers exposed to lead had a reduction in both dexterity and other types of psychomotor functions (224). Meyer-Baron and Seeber analyzed the data from 12 studies and concluded that occupational exposure to lead was related to impairments of central informational processing and of manual dexterity (225). Additional studies that assess a dose-response relationship of neurological functioning within the lower range of occupational exposure to lead are needed to clarify the extent and type of impairment.

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9.2 Pregnant Women

A pregnancy in the face of a low to moderate lead burden may put the health of the woman or her fetus at risk. Blood lead levels change during the course of the pregnancy (226). Because of this and because bone lead seems to be mobilized during pregnancy and passes through the placenta, pregnant women have been identified as a group of special concern (2). Most studies have addressed the viability of the pregnancy itself or the health of the newborn with little focus on the subsequent health of the woman. An area that has been studied is pregnancy hypertension. Rabinowitz et al. studied almost 4000 women with an average blood lead of 6.9 ($\pm$3.3 standard deviation) and found that lead levels correlated with several indicators of elevated blood pressure: systolic blood pressure, diastolic blood pressure, and incidence of pregnancy hypertension (227). Rothenberg et al., in a study of women with blood lead levels within those currently considered acceptable, found the relationship between blood lead and blood pressure to be more pronounced among immigrants, possibly because of higher prior exposure (228). Regardless of the cause, hypertension during pregnancy increases the risk of the woman being hypertensive after the pregnancy as well as of the baby having a serious health problem. As noted above for women with occupationally related exposure to lead, a greater risk of pregnancy loss has been reported. In examinations of this relationship in women with low levels of blood lead regardless of the source of lead exposure, several investigations have failed to find a significant association between blood lead and the pregnancy's ending in a live-born infant (229,230). Only a few investigators (231–233) have examined the issue of congenital malformations associated with lead, given the low incidence of specific malformations and the need to have lead measurements on the mothers. The findings have not been consistent and clear enough to establish a difference by lead levels.

Although the health of the mother and of the child are inextricably linked, the next section deals with children since they seem to be the most susceptible of all groups to insults from lead, whether from in utero or postnatal exposure. Nevertheless, it should be noted that more comprehensive studies of the health of women themselves during pregnancy and delivery as well as postnatally have not been conducted. It has not been established, for example, whether pregnancy and delivery complications are related to maternal lead burden or whether postpartum depression is linked to lead burden although plausible rationales could be constructed for such associations.

9.3 Children

9.3.1 Physical Growth

In addressing the health status of the newborn, several studies have assessed important growth parameters with measures of presumed in utero exposure, such

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as prenatal maternal blood lead or cord blood lead at delivery. In utero exposure has been associated with reduced gestational age, reduced birth weight, and reduced head circumference. McMichael et al. reported a risk of almost threefold for preterm delivery for every 10 μg/dl increase in maternal blood lead (232). A decrease in birth weight related to low-level lead exposure during pregnancy has been consistently reported (234,235). A similar finding was reported by Gonzalez-Cossio et al. when maternal bone lead was measured (236). An average reduction in head circumference of 0.3 cm was found by Baghurst et al. (230). The deficits in some of these growth areas seen at the time of birth appear to persist in the young child. In an examination of the third National Health and Nutrition Examination Survey (NHANES) data, blood lead levels were significantly associated with reductions in stature of 1.57 cm and in head circumference of 0.52 cm although no association was found with weight (237). The relationships were still observed when key cofactors were controlled. The data reported by Shukla et al. indicated that the growth deficits observed in young children are probably related to an interaction of in utero exposure and the level of postnatal exposure (238,239).

9.3.2 Cognitive Functioning

There are inherent difficulties in establishing a causal link between lead exposure and a child's cognitive functioning. In most instances, the at-risk child has some exposure prenatally as well postnatally. Whether exposure at each of these times is equally important or they combine to create an even more damaging result is still not clear, but investigators have examined both of these times of exposure. A further difficulty is that the at-risk child is usually at a disadvantage for reasons other than lead in his/her cognitive performance. There have been many attempts to examine the cognitive-lead link and assess the plausibility of its being a causal agent in the face of inconsistency in findings and their interpretation.

The question of the validity of the measurement of cognitive functioning in young children is well founded because of their developmental status and their limited expressions and behaviors in the cognitive domain. Nevertheless, a deficit established during the first year or 2 could reasonably be attributed to prenatal exposure and would be important information. The findings from these investigations of very young children have been inconsistent. Bellinger et al. (240,241) studied middle- and upper-class children. They found deficits at both 6 months and 12 months of age in children with cord blood lead between 10 and 25 μg/dl compared with children with cord blood lead below 3 μg/dl (240,241). Since the decrements were related to cord blood lead and not to postnatal blood lead, the implication was that the prenatal exposure was more important at this age of the child. Needless to say, postnatal exposure in children before they are mobile is generally at a very low level. Furthermore, because of the social class of the children in this study there should be fewer alternative explanations for the deficits than the lead exposure. Similar findings were reported in data relating

to children followed in Ohio (233,242). The opposite results were obtained by investigators of children in Australia (243).

That postnatal blood lead levels are inversely related to cognitive functioning has been more consistently reported with older children. Bellinger et al., for example, found about a six-point decrement in the General Cognitive Index Score of the McCarthy in children near 5 years of age with exposure that ranged from 3 μg/dl to around 20 μg/dl measured at 2 years of age. These deficits are observed in preschool children as well as school-aged children. The most consistent results have been reported from the follow-ups conducted in Boston, Cincinnati, and Port Pirie (244–246). These investigators have controlled statistically for key cofactors related to cognition. Despite the variety of measurements and other features of the studies, there is consistent agreement among investigators who have synthesized all of the available evidence: higher lead exposure, even below 25 μg/dl, is associated with a decrement in cognitive functioning, on average, of around 2.5 points (2,247–250).

Some investigations relate to whether or not attentional deficits and behavioral problems are more frequent or pronounced in children with more exposure to lead. Bellinger et al. (251) reported from a study of 19- and 20-year-olds that their data suggested that the cognitive skills that seemed to be most affected were executive and self-regulation functions. If replicated, this finding would be consistent with a higher rate of behavioral disturbances that others have reported. In a follow-up of the Port Pirie children at ages 11 to 13, for example, it was found that behavioral problems, measured by the Child Behavior Checklist, were more frequent among those with higher lifetime average blood lead concentrations (252). Other investigators have found an inverse association between blood lead and performance on tasks such as those requiring attention, eye-hand coordination, or reproduction (253–255), as well as making progress in school (133,255). A National Academy of Science Committee (2) concluded that ''the limited data available are generally consistent with the hypothesis that children with greater lead burdens not only perform worse on laboratory and psychometric tests of cognitive function, but also are more frequently classified as learning-disabled and make slower progress through the grades'' (p. 63).

In summary, the studies that have examined children exposed to low to moderate levels have not found a threshold below which developmental parameters, whether related to physical stature or cognitive functioning, have been unaffected.

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Mercury

Thomas W. Clarkson
University of Rochester School of Medicine, Rochester, New York

1. INTRODUCTION

Several major reviews were useful in the preparation of this review (1–8).

1.1 The Physical and Chemical Forms of Mercury

The element mercury is aptly named after the messenger of the Roman gods, as it is the most mobile of all the metals. In its ground or zero-oxidation state (Hg^0), mercury is the only metal that is liquid at room temperature. Liquid metallic mercury can form stable amalgams with a number of other metals. An amalgam with silver and copper is the basis of dental amalgam tooth fillings. Both the amalgam and liquid phases allow mercury to vaporize as a monatomic gas (usually referred to as mercury vapor). Mercury has two oxidation states each capable of forming a variety of chemical compounds. In the mercurous state, two atoms of mercury, each having lost one electron, form the mercurous ion ($Hg\text{-}Hg^{++}$). Mercuric mercury (Hg^{++}), where two electrons have been lost from one atom of the metal, forms most of the compounds of mercury.

Mercuric mercury can also form a number of ''organic mercury'' compounds by bonding to a carbon atom, for example, the phenyl ($C_{6\,5}\text{-}Hg^+$) and methyl ($CH_3\text{-}Hg^+$) mercuric cations. Methyl mercury compounds, used exten-

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sively in the past as fungicides, have been responsible for several mass outbreaks of poisoning.

1.2 Sources of Human Exposure

In the past, mercury and its compounds found a wide variety of uses in agriculture, industry and medicine. Studies of mercury levels in peat bog in northwest Spain indicate that substantial anthropogenic deposition took place as early as 2500 years ago. This, the authors (9) stated, coincided with the startup of the Almaden mercury mine in central Spain. The authors also concluded that anthropogenic mercury has dominated the deposition record in Spain since the Islamic period (8th–11th centuries, A.D.). Global emissions have increased in the past 100–150 years.

Today, most human exposure to mercury vapor is in the occupational settings and from dental amalgam, and to methyl mercury in diets containing fish and seafood (5). A small amount of inorganic mercury of unknown origin is also present in the diet of the general population. Owing to the introduction, in recent years, of controls over the uses of mercury, occupational exposures have diminished. They mainly involve industrial plants using liquid mercury as an electrode in the electrolysis of brine to produce chlorine and caustic soda (the chloralkali industry), the manufacture of thermometers and other scientific equipment, the production of fluorescent lights, the use of metallic mercury in the extraction and refining of gold and silver, and the use of amalgam fillings in dentistry. In fact, gold mining has become a major source of human exposure in many developing countries in recent years (10–12).

Dental amalgams are the dominant source of exposure to mercury vapor in the general population. It was estimated that from 3 to 17 μg Hg/day was absorbed from amalgams (Table 1), far exceeding other sources such as the ambient atmosphere. More recent reports are in general agreement with this estimated range. For example, Barregård et al. (13) found mercury levels in the kidney cortex taken from living kidney donors in the general Swedish population to be significantly higher (0.47 μg Hg/g wet wt) in people with amalgams as compared to those without amalgams (0.15 μg Hg/g wet wt). Kingman et al. (14), in a study of a large group ($n = 1127$) of U.S. military personnel, found statistically significant correlations between amalgam exposure and urinary mercury concentrations confirming previous reports. Unusually high intakes have been reported in a few individuals. Barregärd et al. (15) reported on three amalgam-bearing individuals who attained urinary excretion rates of about 50 μg Hg/g creatinine in urine. This urinary excretion rate corresponds to a steady daily intake of over 80 μg Hg, perhaps as high as 160 μg Hg. In the case of these individuals, the long-term use of chewing gum may explain the extreme values as chewing accelerates the release of vapor from amalgams. They estimated that about one in

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TABLE 1 Average Daily Intakes in Adults in the General Population of Mercury and Its Compounds

Source of exposure	Mercury vapor	Inorganic mercury compounds	Methyl mercury compounds
Air	0.03 (0.02)	0.002 (0.0001)	0.008 (0.006)
Drinking water	0	0.05 (0.0025)	0
Food			
Fish	0	0.6 (0.04)	2.4 (2.3)[a]
Nonfish	0	3.6 (0.25)	0
Dental amalgam	3.8–21 (3–17)	0	0

[a] Daily intakes are much higher in populations depending on seafood or freshwater fish as the major protein source.
Source: Adapted from refs. 4.5.

2000–10,000 persons in the general population in Sweden may attain urine levels of 50 μg H/g creatinine. These values are at about the threshold limit for adverse effects due to occupational exposures to mercury vapor. Given the worldwide use of amalgam, such estimates indicate that large numbers of people could have such values.

Environmental exposures are mainly to methyl mercury compounds as a result of the biomethylation of inorganic mercury by microorganisms present in aquatic sediments and the subsequent bioaccumulation of methyl mercury in aquatic food chains. The ability to methylate mercury is found in some of the earliest evolutionary life forms such as the methanogenic bacteria. After release from the methylating microorganisms, methyl mercury ascends the aquatic food chain via zooplankton into fish. The highest concentrations of methyl mercury are found in edible tissues in long-lived carnivorous fish and sea mammals at the top of the food chain.

Inorganic mercury that is the substrate for biomethylation may be naturally present in aquatic sediments or deposited via local pollution or widely distributed to bodies of fresh and ocean water though the global cycle (16). The global cycling of mercury involves natural sources such as the degassing of the earth's crust releasing mercury vapor to the atmosphere. Anthropogenic sources include coal-burning power stations and waste incinerators. Mercury vapor is the principal form of mobile mercury in the atmosphere. With a residence time of 1 year or so, it distributes globally from its source. The discovery of mercury in aerosols 19 km above the earth's surface gives further evidence for the long residence time in the atmosphere (17). It is converted to a water-soluble form by processes that are not yet well understood and returned to the earth's surface in rainwater.

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The global cycling of mercury is believed to be responsible for the transport and deposition of mercury in areas remote from the original source whether natural or anthropogenic.

Mercury in the general atmosphere and in unpolluted drinking water is present in such low concentrations as not to amount to a significant source of human exposure (5).

2. DISPOSITION AND TOXIC ACTIONS

Each of the major forms of mercury is characterized by a unique pattern of disposition and toxicity, so each will be treated separately.

2.1 Liquid Metallic Mercury

The occasional breakage of mercury thermometers in the mouth results in liquid mercury entering the gastrointestinal tract. It passes through virtually unabsorbed and unchanged to be excreted in the feces. No adverse effects of such accidents have been reported. Accidental breakage of Miller-Abbott tubes can release liquid mercury into the lungs where it can reside for many years. It is slowly oxidized to ionic mercury that passes into the bloodstream leading to elevated tissue levels. However, no adverse effects have been noted except for mild kidney damage (18). Indeed, in the early years of the nineteenth century, tablespoon quantities of the liquid metal were administered orally in attempts to relieve constipation.

2.2 Mercurous Mercury

Since human exposure to compounds of mercurous mercury now occurs rarely if at all, we have little information on its disposition in the body. Compounds of mercurous mercury, especially the chloride salts, have a low solubility in water and are poorly absorbed from the gastrointestinal tract. In the presence of protein, the mercurous ion disproportionates to one atom of metallic mercury (Hg^0) and one of mercuric mercury (Hg^{++}). Some of the latter will probably be absorbed into the bloodstream and distributed to tissues as discussed below.

Mercurous chloride (calomel) was widely used medicinally in past centuries up to about the middle of the present century. It has a mild laxative action that probably explains why it was added to teething powers. However its medicinal uses were stopped when Warkany and Hubbard (19) connected the childhood disease of acrodynia to presence of calomel in teething powders. This disease is characterized by the infant having pink cheeks and hands, being photophobic, and experiencing joint pain sufficiently severe to cause the child to cry and complain frequently. In fact, the constant crying by the child eventually led distraught mothers to seek medical attention. An interesting characteristic of the disease was that of about 1000 infants taking mercury-containing teething powder only

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one would develop the full-blown syndrome. The disease is reversible after cessation of exposure and can be successfully treated by a mercury complexing agent such as British antilewisite (BAL). Since the discovery that inorganic mercury was the cause (19) cases are now extremely rare.

The mechanism whereby mercury produces this disease is not known. Acrodynia can also be produced by exposure of children to other forms of mercury such as mercuric salts, phenyl mercury compounds, and inhaled mercury vapor. Since all these species of mercury can release mercuric mercury in the body, it seems likely that this form of mercury is the proximate toxic species. It is of interest that this disease has not been reported in adults or after exposure of children to methyl mercury in the diet or after placement of dental amalgam fillings. Mercuric mercury may also be responsible for the laxative action of mercurous compounds.

2.3 Mercuric Mercury

2.3.1 Disposition

The diet is the main source of exposure of the general population. Experimental studies on human subjects indicate that on the average, 15% of an oral dose of mercuric mercury is absorbed whether given as ionic mercury or attached to protein. However, individuals differ considerably in the amount absorbed, ranging from 8% to 25% of the ingested dose (20). When administered in creams used to whiten the skin, some absorption of mercuric mercury must take place as severe systemic toxicity has occurred. Occupational exposure of the mercuric oxide aerosols can occur in the manufacture of mercury batteries and perhaps to mercuric chloride aerosols in the chloralkali industry. As with any aerosol, the retention and pattern of deposition in and degrees of absorption from the lungs will depend on particle size and solubility. Experiments on dogs inhaling mercuric oxide aerosols indicated substantial retention and subsequent distribution to body tissues (21).

Studies on 10 adult volunteers (22) given a single nontoxic oral dose of mercury, either in the ionic form as mercuric nitrate or protein-bound, yielded important data on absorption, distribution, and excretion in humans. On the average 15% (range 8–25%) of the oral dose was absorbed. The blood compartment contained an average of 0.27% ($<$0.07–0.48%) of the ingested dose 24 h later. Levels in plasma were about two and a half times of those in red blood cells. In six volunteers the biological half-time in plasma was 24 days (range 12–40) and in red cells, 28 days (range 13–42). The whole body half-time was longer, 45 days (range 32–60).

According to animal data (18), about 30% of the body burden of inorganic mercury is found in the kidney with the highest levels in the corticomedullary region. A limited degree of penetration of the blood-brain barrier occurs but to a

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far lesser extent than what is seen for inhaled mercury vapor and methyl mercury compounds. Likewise mercuric mercury dose not cross the placenta to any significant extent but, instead, accumulates in placental tissues.

Elimination from the body occurs predominantly via the urine and feces although some excretion in sweat may occur. Fecal excretion, at least in part, starts with secretion in bile, according to animal experiments (23,24). Mercuric mercury is secreted as a complex with glutathione via the glutathione transporter located in the cannicular membrane of the hepatocyte. This mechanism does not operate in suckling animals but switches on abruptly at the time of weaning.

Glutathione conjugates (and perhaps conjugates of other small-molecular-weight thiols) of mercuric mercury may also be involved in kidney uptake and urinary excretion but a detailed mechanism is not yet available (25). Studies on animals with radioisotopes of mercury reveal that all the mercury in urine derives from mercury in kidney tissues as opposed to the filtration and excretion of mercury from the bloodstream (18).

Information is limited on suitable biological monitoring media for mercuric mercury. Plasma should be a useful medium but would be confounded by simultaneous exposure to mercury vapor. Whole blood or red blood cells are less suitable as dietary exposure to methyl mercury would affect the mercury levels. The rate of urinary excretion should reflect kidney levels. Correction for changes in urinary flow rates may be needed, as discussed below. Abrupt increases in urinary excretion may be expected if the toxic action of mercury causes an increase in exfoliation of renal tubular cells. Fecal excretion should represent both the dietary intake (including losses from dental amalgam) and biliary secretion. Scalp hair has been used to indicate a previous acute exposure to inorganic mercury (26). However, the extent of deposition in hair is far less than that of methyl mercury, which, owing to its presence in the diet, may confound attempts to monitor mercuric mercury. There is a danger of external contamination depending upon the circumstances of exposure and some transfer to hair may occur via secretion of mercuric mercury in sweat.

2.3.2 Toxic Actions

The lethal dose of mercuric chloride in humans is about 1 g. In the past mercuric chloride was available as an antiseptic, which led to its misuse as a suicidal agent. The ensuring acute gastrointestinal damage causes the victim to go into cardiovascular shock leading to renal failure and death. Chronic lower doses of inorganic mercury may cause renal damage by one of two different mechanisms: an indirect mechanism involving the immune system and a direct action on cells lining the kidney tubules.

Mercury acts on the immune system leading to the production of antibodies that collect at and interact with the glomerular membrane of the kidney (for review, see ref. 5). The selective filtering action of the glomerulus is damaged

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allowing the passage of albumin into the glomerular filtrate and ultimately into urine. If the damage to the glomerular is sufficiently great, the protein loss results in the development of the full nephrotic syndrome with widespread edema, which can be life threatening.

The nephrotic syndrome has been reported in people using skin whitening creams containing mercuric chloride as the active ingredient (27). Autopsy examination has revealed the presence of antibodies laid down in glomerular tissue. Occupational exposure to high levels of aerosols of inorganic mercury has also produced the nephrotic syndrome (28). This immune-mediated mechanism of kidney damage has been reproduced in experiments, mainly using rats. The phenomenon is highly dependent on the strain of rat, the Brown Norway strain being the most susceptible (29).

For information on exposure to lower levels over long periods, one has to turn to reports on occupational exposure to mercury vapor. At these lower levels, an increase in the urinary excretion of albumin may be detected. The amounts of albumin excreted are far less than those associated in the full-blown nephrotic syndrome. It is assumed that this is produced by the same immune mechanisms as that responsible for the nephrotic syndrome. However, recent experimental studies indicate that mercuric mercury can have direct effects on glomerular cells (30).

Direct action on renal cells from acute doses of mercuric chloride given to rats can cause the loss of cells from the renal tubule especially in the more distal region of the proximal tubule. The original columnar-shaped cells are replaced by cuboid-shaped epithelial cells that resist the action of inorganic mercury. The animal becomes ''tolerant'' to subsequent doses of inorganic mercury. The major site of damage is the pars recta section of the proximal tubule (for review, see ref. 31).

Intracellular thiols including glutathione and metallothionein are probably important defenses against the cytotoxic effects of inorganic mercury. Woods and Ellis (32) have suggested that resistance to the renotoxic effects of inorganic mercury (Hg^{++}) is more closely related to capacity for upregulation of GSH synthesis than are elevated GSH levels per se. Piotrowski and Szumanska (33) demonstrate that inorganic mercury can induce metallothionen in kidney tissue.

Chronic exposure to mercuric chloride given in the drinking water can also lead to kidney damage in rats such as loss in kidney weight. In the chronic toxicity test, no detailed examination of kidney function was undertaken (34). Evidence for direct effects on kidney cells at low chronic exposures comes from studies of occupational exposure to mercury vapor (see below).

Two nonrenal effects of inorganic mercury have been reported. One report of occupational exposure to mercuric oxide aerosols claimed to find an association with effects on the peripheral nervous system with signs and symptoms similar to those of amyotrophic lateral sclerosis (35). It is the only report of its kind.

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A second and much-better-documented effect is acrodynia or pink disease as discussed above. Prenatal damage has not been reported probably because this form of mercury does not cross the placenta.

2.4 Mercury Vapor

2.4.1 Disposition

Mechanisms. Mercury vapor is a monatomic, electrically neutral gas possessing high lipid solubility. Its oil-to-water partition is about 80 to 1 (18). It therefore passes readily across cell membranes and other diffusion barriers in the body in a fashion similar to other lipid-soluble gases such as the anesthetics. However, once inside the cells it is subject to oxidation to mercuric mercury. This oxidation step appears to be accomplished solely by the catalase-hydrogen peroxide reaction as follow:

$$\text{Cat-OH} + \text{H}_2\text{O}_2 = \text{Cat-OOH} + \text{H}_2\text{O} \quad (1)$$

$$\text{Cat-OOH} + \text{Hg}^0 = \text{Cat-OH} + \text{Hg}^{++} + 0^{=} \quad (2)$$

Step (1) is the usual first step in the reaction of catalase (Cat-OH) with hydrogen peroxide to form the oxidant species catalase compound one (Cat-OOH). In step (2), catalase compound one removes two electrons from an atom of dissolved mercury vapor in a single transfer step (for details see ref. 36).

This oxidation of mercury vapor to mercuric has been observed in red cells, liver, and brain homogenates. The availability of hydrogen peroxide is rate determining in red cells. Eventually all the vapor will be converted to mercuric mercury by this process. However, Magos (for a recent summary, see ref. 8) in a series of elegant animal experiments has demonstrated that vapor will persist in the bloodstream for a sufficient period to allow diffusion into all organs and tissues of the body. Observations on human subjects are consistent with this conclusion (37). The persistence of vapor in the bloodstream undoubtedly accounts for marked difference in early tissue distribution as between inhaled vapor and ingested mercuric mercury.

Toxicokinetics. Most of the quantitative data on the disposition of inhaled vapor comes from two studies on volunteers exposed for about 15–20 minutes to radio-labeled (37,38) and to nonlabeled mercury vapor (39).

The retention of inhaled mercury vapor is about 80% of the amount inhaled. This is consistent with observations of occupationally exposed workers (40). According to calculations by Magos (8), most of the retained vapor diffuses immediately into the bloodstream. Approximately 8% of the retained dose is found in the blood compartment 24 h after exposure. Unlike exposure to mercuric mercury, more mercury is found in red cells rather than in plasma after vapor exposure. The red blood cell level is approximately twice the plasma level in early

days following a single exposure. However, as mercury vapor is converted to mercuric mercury, the proportion found in red cells will diminish.

About 7.1% of the inhaled dose is found in the head region according to external radioactive counting. Deposition in the kidneys is about 30% 3 days after, according to animal data exposure (18). As vapor is transformed to mercuric mercury, the proportion of the body burden found in kidneys increases.

Elimination from the body occurs by exhalation of the vapor and via excretion of mercuric mercury in urine, faces, and sweat. Exhalation can account for as much as 7–14% of the inhaled dose. Urinary excretion is relatively low soon after exposure but rises as the amount of mercury in the kidneys increases. Thus urinary excretion is as low as 0.25% of the inhaled dose in the week following exposure as compared to 2% in the feces. In contrast, after long-term occupational exposure, urinary and fecal excretion rates are approximately the same.

The half-times of elimination vary between tissues. Lung tissue has the fastest half-time of 1.7 days. This short half-time presumably involves a substantial proportion lost by exhalation. The blood compartment has two half-times, 2–4 days accounting for 90% and 15–30 days accounting for most of the remainder. The kidney has the longest half-time of about 76 days.

The half-time in the head regions is surprisingly short, of the order of about 19 days. Vapor after crossing the blood-brain barrier is presumable oxidized to mercuric mercury that should be effectively trapped as it passes across the blood-brain barrier much more slowly than does the vapor. Since this half-time was determined by radioactive counting of the head region, radioactivity in cerebral blood vessels may have contributed to this apparently rapid elimination. There is evidence from autopsy data for a much longer half-time in brain tissues, perhaps measured in years. Miners who had been retired for many years still had greatly elevated mercury levels at the time of their death (41,42). In the Kosta et al. study (42), the mercury levels in brain and other tissues from these miners were closely related to selenium levels. A WHO Expert Group (5) has suggested that mercuric mercury, after long-term residence in the body tissues, exists as an inert insoluble complex with selenium.

Whole blood, plasma, and urine have been the most common media used for purposes of biological monitoring. As noted above, the red-cell-to-plasma ratio varies depending on the time after exposure. Having at least two elimination half-times complicates the blood compartment. Several elimination half-times have been reported in urine. As noted for mercuric mercury, urinary mercury derives directly and predominantly from the mercury in kidney tissues. If renal damage occurs leading to exfoliation of mercury-laden cells, urinary excretion might increase abruptly.

For these reasons there is no ideal biological monitoring medium to indicate the body burden of levels of mercury in the target tissues, namely the brain and kidneys (see below). The problem is especially difficult in attempts to recapitulate

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episodic exposures. Hair has been used for mercuric and methyl mercury but, with exposure to vapor, external contamination will always be a problem.

The picture is somewhat brighter for long-term exposures where the individual has achieved steady state. The elimination half-times quoted above would suggest that they should occur after about 1 year's exposure except for the extremely long half-time. However, the latter may reflect a nontoxic form of mercury and this may not be important for biological monitoring to assess risks of toxicity. Thus it has been possible to demonstrate linear quantitative relationship between air levels determined by personal monitors and the corresponding blood levels and urinary excretion rates in chronically exposed workers (for details see ref. 8). These relationships are as follows:

$$\text{B-Hg} = 6.4 + 0.48 \times \text{A-Hg}$$

where B-Hg is the mercury concentration in blood expressed as micrograms per liter and A-Hg is the air concentration determined by personal samplers.

$$\text{U-Hg} = 10.2 + 1.01 \times |\text{A-Hg}$$

where U-Hg is the urinary excretion rate expressed as micrograms of mercury per gram creatinine in urine.

The creatinine correction is frequently applied to measurements of urinary mercury to correct for variations in urinary flow rates. Creatinine is produced at an approximately steady state in muscle tissues, filtered via the glomerulus, and excreted unchanged in urine. Approximated 1.6 g of creatinine are excreted in 24 h in the average adult. Thus the amount of mercury in urine associated with 1 g of creatinine corresponds to the amount of mercury excreted in $24/1.6 = 15$ h. Compared to using the observed concentration of mercury in urine, which may vary according to urinary flow rates, the creatinine correction provides more reliable, less variable data (43). Actually the rate of creatinine excretion is closely proportional to the lean body mass (44). Today, weighing scales are available that will directly determine lean body mass thus opening up the possibility that the true creatinine excretion rate can be determined on an individual basis rather that assuming a constant number for everyone.

One report on workers occupationally exposed to mercury vapor noted that concentrations in saliva parallel blood levels (45). If confirmed, these findings indicate a useful future role for saliva as a biological monitoring medium

2.4.2 Toxic Actions

Mercury vapor can cause acute damage to the lungs and death from pneumonia when inhaled at extremely high concentrations. Goldwater (46) has reviewed numerous case reports of mercury-induced pneumonitis but where accurate data on air levels are lacking. Mercury vapor was inhaled as a treatment for syphilis up to the early years of the twentieth century. Careful measurements by Engelbreth

[reviewed by Goldwater (46)] indicated that air concentrations above 1 mgHg/m^3 are needed. Thus the probable range of concentrations is somewhere in the range of 1–17 mgHg/m^3, the higher value being the concentration in air saturated with mercury vapor at room temperature (Fig. 1). Apparently metallothionein protects against pulmonary toxicity from acute exposure to mercury vapor (47).

At lower air levels, the adverse effects of inhaled vapor derive mainly from the action of inorganic mercury on the nervous system and the kidneys

Effects on the Nervous System. Given the long history of occupational exposure to mercury vapor, ancient medical texts described workers exhibiting signs of mercurialism. The signs and symptoms of this toxic syndrome consist basically of a triad of adverse effects, namely gingivitis, tremor, and erethism. Gingivitis loosens and causes loss of teeth and gives rise to a fetid breath. Tremors of the extremities can be so severe as to be incapacitating. Erethism is a collection of mental disturbances ranging from irritability, excessive shyness, depression, and memory loss to a condition similar to senile dementia [Thompson, quoted by Goldwater (46)].

Mercurialism is now rare owing to much greater control over mercury vapor levels in the working environment. It is difficult to estimate what these early levels were, probably in the range of 1 mgHg/m^3 (Fig. 1).

Of much greater relevance to current exposures are the lower ranges in Figure 1. Ratcliffe and Swanson (6), following a detailed review of the quality of reports on mercury effects on the nervous system, concluded that papers published over 50 years ago (48) to quite recent times, e.g., Echeverria et al. (49), provide ample evidence of such effects usually in the context of occupational exposures. Such effects include intention tremor, sometimes so fine that it had to be detected instrumentally, and various behavioral and psychological changes.

Changes on mood scores, poor mental concentration, emotional lability, and somatosensory irritation correlated with urinary excretion of mercury in a cohort ($n = 19$) of mercury-exposed dentists. The average excretion in the exposed group was 36 μg Hg/g creatinine (49). A more recent study by the same group (50) with a much larger cohort ($n = 230$) found that the scores for an intentional hand steadiness test correlated with urinary mercury levels. The authors made special note that hand unsteadiness is of special concern to this profession ''engaged in the exquisitely challenging manual aspects of restorative dentistry.'' Perhaps so, but one must wonder whether the mercury causes the symptoms or vice versa. Perhaps a clumsy dentist with poor mental concentration might spill more mercury. According to the 1995 report, there were many mercury spills in the exposed group, as one might expect with either explanation. Thus we are left with the chicken-and-egg conundrum: which came first?

The urine and air levels at which such effects were found are summarized in Figure 1. It is difficult to arrive at firm conclusions because, as emphasized

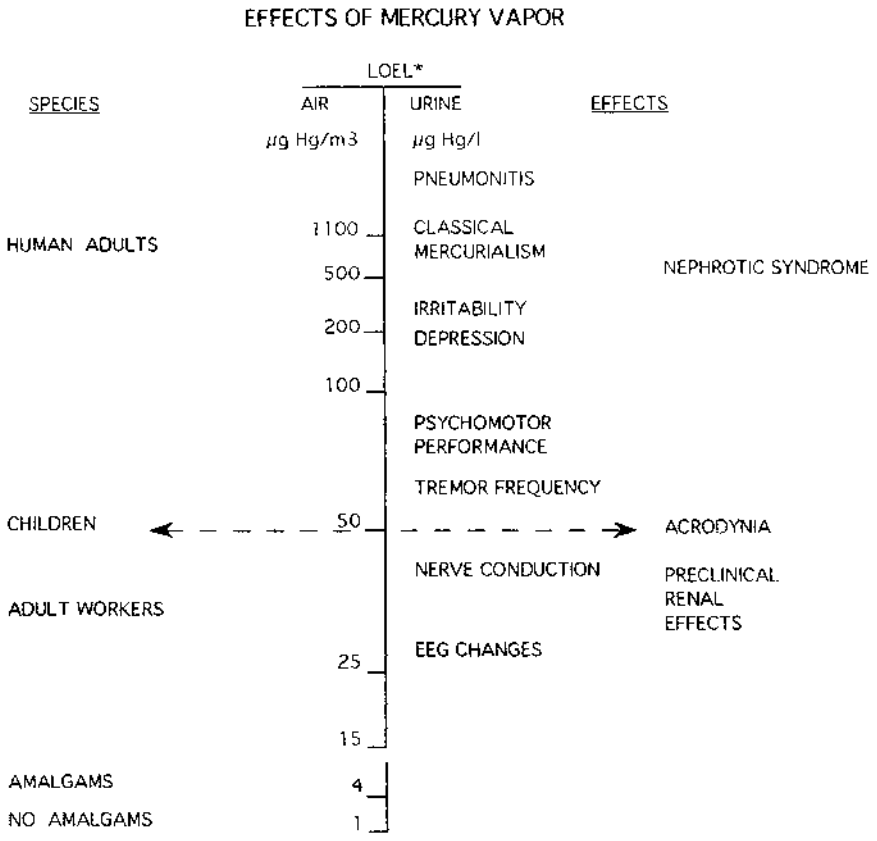

Figure 1 The toxic effects of inhaled mercury vapor are listed according to the urine or air levels. For long-term exposure (about 1 year or so) the urine levels in units of µg Hg/L are numerically similar to the air levels in µg Hg/m^3. All effects listed in this figure arise from long-term exposure except for pneumonitis, which is usually produced by acute exposure to extremely high air levels of the vapor. For details, see text. (Data are from ref. 5.)

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by Magos (8), effects of the central nervous system are persistent. Thus current air or urine levels may not be responsible for the observed effects. In one carefully conducted study, peak urine levels existing in the year prior to the study best correlated with tremor. Several studies of workers occupationally exposed to mercury vapor have indicated that the mildest effects on the central nervous system correlated better with integrated exposures as opposed to current mercury levels (51–53). Therefore, making allowance for the importance of past exposures, it seems unlikely that adverse effects on the nervous system have been observed at urinary excretion rates below 50 μg Hg/g creatinine, equivalent to air levels in the workplace of about 0.05 mg Hg/m^3 (Fig. 1).

Mechanisms: The biochemical and physiological mechanisms underlying the observed adverse effects of the nervous system are still unknown. Mercury vapor itself is believed to be nontoxic, so its metabolite, divalent inorganic mercury, is assumed to be the proximate toxic agent. Recent studies in animals have indicted that exposure to mercury vapor can induce metallothioneins in brain tissue (54). Presumably divalent inorganic mercury is responsible for this effect as it is known to induce metallothioneins in other tissues (55,56). The authors suggest this is a protective mechanism. Studies on metallothionein-null mice also indicate that metallothionein plays a protective role against renotoxicity of inorganic mecury (57).

Renal Effects. The renal effects on humans have been the subject of several recent reviews (5–8). Ratcliffe and Swanson (6) have given a critical evaluation of both quality and relevance to risk assessment. From a total of 91 papers on occupational exposures to inorganic mercury, three papers on Belgian workers (51,59,60) and two papers on Swedish workers (61,62) reporting on renal effects meet the criteria of Ratcliffe and Swanson as suggestive of renal effects associated with exposure to inorganic mercury.

Roels et al. (51) conducted a cross-sectional study of 131 male workers and 54 female workers chronically exposed to mercury vapor in several industries in Belgium using metallic mercury. The observations on these workers were compared to a matched unexposed group of 114 male and 48 female workers, respectively. The mean urine levels in the male and female workers were 52 μg Hg/g creatinine and 37 Hg/g creatinine, respectively. The control groups had mean urine mercury levels of 0.9 μg Hg/g creatinine for men and 1.7 Hg/g creatinine for women. Most measures of renal function did not differ between the exposed and nonexposed groups, e.g., urinary excretion of amino acids, total protein albumin, and β_2-microglobulin, suggesting that both glomerular filtration and resorptive mechanisms were not affected. However, the percentage of abnormal values of β-galactosidase (i.e., those values exceeding the 95th percentile of the control group) were increased at urinary levels above 50 μg Hg/g creatinine for men but not women, whereas abnormal values for retinol-binding protein increased for

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urine mercury levels above 75 μg Hg/g creatinine. Correlation between abnormal values with urinary mercury was most apparent for recent exposures of less than 4 years as opposed to exposure for longer periods.

A study by Barregård et al. (61) on Swedish chloralkali workers indicated an increased excretion of the enzyme *N*-acetyl-glucoseamidase (NAG) down to an estimated threshold urine mercury of 35 μg Hg/g creatinine. No evidence of albuminuria was found

The findings of Langworth et al. (62) on a group of chloralkali workers in Sweden gave support to the findings of Roels et al. (51). Langworth et al. compared findings on 89 chloralkali workers with an unexposed matched control group of 75 people. The mean urinary excretion rate in the exposed group was 25 μg Hg/g creatinine as compared to 1–9 μg Hg/g creatinine in the control group. No differences were found in most of the parameters of renal function including urinary excretion of albumin, orosommucoid, β_2-microglobulin, NAG, and copper. Also no differences were found in serum creatinine clearance and relative clearance of β_2-microglobulin.

A tendency was seen toward increased excretion of NAG in the exposed versus the control group but without statistical significance. However, urinary NAG and mercury excretion correlated significantly ($p < 0.001$). Parameters of immune-mediated effects were normal and not different between the two groups. These included serum globulin concentration and serum titers of autoantibodies.

Taken together the findings of Langworth et al. (62) indicated no inhibition of glomerular filtration or of reabsorption processes in the renal tubules. They do indicate a slight dose-related tubular cell damage, as was the case in the Roels et al. (51) study.

A study on Belgian chloralkali workers (60) compared 44 exposed workers with 49 matched controls. The mean urinary excretion rates in the exposed and controls were 22 and 1.6 μg Hg/g creatinine, respectively. As in the studies discussed above, renal function was unaffected but there was evidence of slight damage to renal tubular cells as urinary excretion of tubular antigens and enzymes was increased in the exposed group. The renal effects were mainly found in workers excreting more than 50 μg Hg/g creatinine.

Mechanisms: These early biochemical markers of kidney function reflect the sub- or preclinical effects of inorganic mercury. They probably result from the action of inorganic mercury on the brush border membranes of the tubular cells leading to loss of membrane-bound enzymes and the leakage of intracellular constituents. They may reflect desquammation of tubular cells. In any event, such effects do not compromise the normal function of the kidney and are almost certainly reversible as damaged tubular cells are easily and readily replaced.

In summary, the evidence from human exposures indicates that the kidney is an important target organ and the one most sensitive to the toxic action of

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inorganic mercury. The effects noted at the lowest exposure levels indicated damage or irritation to the tubular cells of the kidney producing an increase in enzymes, cellular antigens, and biochemical constituents of the cells. Such effects occur before any diminution in kidney function and are reversible. Urinary excretion rates associated with the onset of such effects are usually in excess of 50 μg Hg/g creatinine. Unlike effects on the central nervous system, the renal effects relate to current rather than past exposure levels.

2.5 Methyl Mercury

2.5.1 Disposition

Mechanisms. The methyl mercury cation, like other ions of mercury, reacts rapidly and reversibly with thiol groups ($R\text{-}S^-$). The equilibrium affinity constants are so high that it is unlikely that methyl mercury will bind to other ligands with the possible exception of the selenide form of selenium ($R\text{-}Se^-$) (63). Thus methyl mercury is found in tissues and biological fluids bound to protein- and thiol-containing amino acids and peptides such as cysteine and reduced glutathione (reviewed in ref. 4). Methyl mercury attached to L-cysteine is transported into the endothelial cells of the blood capillaries on the neutral amino acid carrier. The transport mechanism is so specific that the D optical isomer is not transported (64). Methyl mercury is secreted from liver cell into bile as a complex with reduced glutathione on a carrier-mediated mechanism (65). Mounting evidence suggests that methyl mercury enters cells as the cysteine complex, switches to reduced glutathione present at high levels in the cytosol, and is transported out of the cell on glutathione carriers.

The mechanism of elimination of methyl mercury from the body has been the subject of intensive research. After secretion into the bile, the glutathione complex is partially hydrolyzed to its constituent amino acids and peptides. Some reabsorption of methyl mercury may take place in the gallbladder (66). Most of the remainder is reabsorbed in the intestinal tract thus giving rise to an enterohepatic cycle of secretion and reabsorption (67). However, a fraction of the methyl mercury in the intestinal tract is converted to inorganic mercury by the microflora (68). Inorganic mercury is poorly absorbed and therefore carried into the feces. Most mercury in feces following exposure to methyl mercury is in the inorganic form. This complex mechanism is responsible for most of the elimination of methyl mercury from the body as other routes such as urinary excretion account for relatively small amounts.

This fecal mechanism of elimination may not be active in suckling infants. Animal experiments indicated that methyl mercury and reduced glutathione are not secreted in bile during the suckling period (69). The demethylation mechanism of the gut flora is also inoperative during this same developmental period (68). Thus on at least two counts its seems unlikely that mercury will be excreted.

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Methyl mercury is also converted to inorganic mercury by phagocytic cells in the body (70). Thus inorganic mercury is found in autopsy tissues (71,72) and in animal tissues (73) after exposure to methyl mercury.

Toxicokinetics. The main aspects of the disposition of methyl mercury in the body have not changed since previous reviews (4,74). Inhalation of methyl mercury compounds, although never subjected to careful measurement, must be high as cases of poisoning have resulted from this route of entry for both monomethyl (75) and dimethyl forms (76). When volunteer subjects ingested measured amounts of a methyl mercury compound, about 90% was absorbed into the bloodstream whether presented as a simple salt or attached to dietary protein (20).

Methyl mercury is distributed to all parts of the body, the distribution process being completed in about 4 days after a single oral dose (77). About 5% of the absorbed dose goes to the blood compartment. The concentration in red cells is about 20 times greater than in plasma levels in humans. Brain levels are on the average 5 times higher than levels in whole blood. Levels in most tissues are relatively uniform.

Methyl mercury readily crosses the placenta to distribute to fecal tissues. Cord blood levels tend to be higher than maternal blood probably reflecting difference in the degree of binding to hemoglobin. The levels in the fetal brain are similar to those in the mother as determined from animal data.

Methyl mercury is avidly accumulated in human scalp hair during the process of formation of the hair in the follicular cells. Concentrations of methyl mercury in newly formed hair parallel those in blood. Methyl mercury concentrations in the hair strand above the scalp are stable at least for periods of many years (26). Thus the longitudinal concentration profile along the length of the hair strand serves to recapitulate past blood levels. A recent study has shown that levels of mercury in maternal hair predict levels in the brains of infants who died at or within a few days of birth in a population exposed to methyl mercury in their diet (78).

As discussed above, elimination is mainly via the fecal routes. However, methyl mercury is secreted in human milk, a mechanism that can result in substantial exposure to the suckling infant if maternal levels are high. In general, the elimination from the body can be approximately described by a single half-time. Berglund and Berlin (79) interpreted that as indicative that the body was a well-mixed compartment and that excretion from the body is the rate-determining step. This conclusion is consistent from what we know of the high mobility of methyl mercury between tissues and body fluids.

The half-time in the whole body in adult subjects has been determined by radioactive tracer studies to be in the range of 70–80 days (for review see ref. 74). Blood half-times have been determined both by direct measurements on blood and by longitudinal hair analyses. The estimates based on hair analysis

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may be less reliable as a term, for the growth rate of hair must be included. If many strands of hair are included in the longitudinal analysis, artifacts may be introduced (80). Half-times directly determined in blood have averages from each study ranging from 50 to 53 days (74).

All of these observations were based on measurements of total mercury. In the most recent report, in which seven healthy adult male volunteers were given a single dose of radiolabeled methyl mercury, both inorganic and methyl mercury were selectively determined (81,82). The kinetics of methyl mercury in blood and whole body could be described by a single compartment. The half-time of methyl mercury in blood ranged from 32 to 60 days with an average of 44 days. It was the predominant mercury species in the blood compartment. Inorganic mercury gradually became an increasing fraction of total mercury in the body. It was the predominant form in urine and feces.

The pharmacokinetic data on the disposition of methyl mercury in adult humans allows the derivation of a quantitative relationship between the daily ingested dose of methyl mercury and the corresponding hair and blood levels (4). The weight of evidence presented above indicates that the uptake, distribution, and excretion of methyl mercury may be described by a single-compartment model. An individual having a steady daily exposure should attain a steady state where tissue levels have attained maximum levels after exposure for a period equivalent to five half-times. Using the figure of 44 days from the study by Smith et al. (81), a steady state will be attained (i.e., tissue levels will be almost 99% of their final levels) in $44 \times 5 = 220$ days. Under these circumstances, the daily dietary intake d (μg Hg) is related to the concentration in blood C (μg Hg/L) by the expression

$$C = f.d/b.V = \mathrm{A_D} \cdot A_\mathrm{B} \cdot d/b.V$$

where f is fraction of the daily intake deposited in the blood compartment, b (days^{-1}) the elimination constant, A_D the percent of the daily intake that is absorbed, A_B the percent of the absorbed dose deposited in the blood compartment, and V (liters) the volume of the blood compartment (7).

The elimination constant, b, is related to the half-time, $t_{1/2}$ (days), by the equation

$$b = \ln 2/t_{1/2} \text{ (thus } b = 0.016 \text{ for a 44-day half-time).}$$

Published values (4) for the parameters listed in equation 1 indicate a steady-state relationship between daily intake, d, and maximum blood level, C, as

$$C = 0.6.d$$

Up to the publication by Smith et al. (81), a larger value was used for the half-time (69 days) (4), resulting in the relationship $C = 0.95.d$.

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The corresponding hair levels may be calculated from the blood levels using published hair-to-blood ratios. If the hair level is expressed as μg/g and blood concentration as μg/L, the published ratios usually fall in the range of 250–300 to 1 (4).

Likewise the maximum body burden, B μg Hg, may be calculated from the blood level as the blood compartment is assumed to contain 5% of the methyl mercury in the body. Thus the amount in the blood compartment is $C.V$, so that $C.V = 0.05\ B$.

Such relationships are useful in interconverting blood and hair concentrations and body burden (see later).

2.5.2 Toxic Actions

Methyl mercury has the remarkable property of inflicting damage almost exclusively on the nervous system, mainly the central nervous system. Only with doses causing severe toxicity are effects seen in other tissues and some of these may be secondary to damage to the nervous system. The nature of the damage and its severity depend not only on the absorbed dose of methyl mercury but also on the stage of development of the brain. Specifically, prenatal exposure produces a pattern of brain damage different from what is seen after adult exposure. Thus prenatal and adult exposure will be treated separately.

Adult Exposure. Signs and Symptoms of Poisoning: Methyl mercury is a neurological poison affecting mainly the central nervous system. The first symptom is paresthesia, a numbness or tingling sensation in the hands and feet, and circumorally. As the syndrome progresses, the victim exhibits signs of incoordination of movement and speech, constriction of the visual fields, and loss of hearing. Death is often due to a secondary sequala such as pneumonia as the patient becomes completely incapacitated and enters a coma (83).

The signs and symptoms of poisoning result from areas of focal damage in the brain. For example, constriction of the visual fields is a result of destruction of neurons in the calcarine fissures of the visual cortex. The focal nature of the pattern of damage is most dramatically illustrated in the cerebellum where the small granule cells are destroyed whereas the neighboring large Purkinje cells may be hardly affected (75).

The effects of methyl mercury are mainly irreversible as they arise from loss of brain neurons, which cannot be regenerated in the mature brain. Typically the first signs and symptoms may appear after a latent period of weeks or months as illustrated in the Iraq outbreak where no adverse effects were experienced during the period of ingestion of the contaminated bread (83). The longest reported latent period was about 5 months in a victim who had received a brief exposure to dimethyl mercury (76).

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Mechanisms: Methyl mercury is metabolized in the brain to inorganic mercury, This reaction probably takes place in nonneuronal phagocytic cells (70,84). Thus it has been shown (85) that, immediately after a single dose of methyl mercury, inorganic mercury is first seen in the glial cells before moving into the neurons.

The toxicological role of inorganic mercury split off from methyl is not understood. Certainly inorganic mercury persists in the brain of humans (72) and nonhuman primates (86) months to years after methyl mercury has gone. It may have been responsible for an increase in the number of reactive glial cells over an 18-month period in *Macaca* primates subjected to long-term exposure to methyl mercury (87). Astrocytosis in the brain of prenatally poisoned infants in the Iraq outbreak might have been caused by the inorganic metabolite of methyl mercury. The areas of astrocytosis coincided with mercury deposits using a stain that detects only inorganic mercury (88). However, astrocytosis may be secondary to effects in other cells (89). Otherwise inorganic mercury deposits in the brain appear to be inert, probably present as an insoluble complex with selenium (4).

Magos et al. (90), in studies of the comparative toxicity and metabolism of methyl and ethyl mercurials in the rat, indicated that it was the intact organomercurial that was responsible for brain damage and not the inorganic mercury produced as a metabolite. How the intact organomercurial damages brain cells and why damage is localized to certain areas of the brain is still not clear after several decades of research. We still do not know why the brain is the target tissue nor can we explain latent periods of up to several months between cessation of exposure and the onset of signs and symptoms of poisoning.

The selective damage to the brain and to focal areas within the brain cannot be explained by selective deposition of methyl mercury. In general, the brain levels are, if anything, somewhat below the average for other tissues (91). Likewise the same authors reported that levels of methyl mercury within the brain showed no correlation with areas of damage.

The first biochemical evidence of brain damage came from early studies in Japan in the 1960s. Yoshino et al. (92) demonstrated that protein synthesis was depressed in rat brains before neurological signs appeared and when oxygen consumption, aerobic and anaerobic glycolysis, and thiol enzyme activities were unchanged. They concluded, ''The selective inhibition of protein synthesis may have a direct bearing on poisoning by alkyl mercury compounds.'' Their finding was soon confirmed by others (for review see ref. 93).

Syversen (94) found that protein synthesis was depressed in cerebral and cerebellar neurons some of which were targets for methyl mercury whereas others were not. What distinguished target from nontarget cells was the recovery phase. In the nontarget Purkinje cells, protein synthesis recovered and even rose above pretreatment levels. In contrast the target granule cell of the cerebellum did not

exhibit a recovery. According to the author, protein synthesis may be regarded as reflecting a repair process. The target cells have a low repair capacity. This theory, that repair capability determines which cell succumbs to methyl mercury, remains viable to this day (95).

A considerable amount of research has been published since then involving a variety of preparations of brain tissues. Two major classes of effects have been found: (1) that methyl mercury disrupts calcium homeostasis in neurons (96–98) and (2) that methyl mercury leads to a cascade of reactive oxygen species (99–102). These effects were produced with concentrations of methyl mercury (added to the medium) in the micromolar range. One laboratory, however, found oxidative damage at media concentrations of methyl mercury in the submicromolar range (100). They achieved this result by adding copper ions and ascorbate to enhance the production of oxygen radicals. Methyl mercury alone or copper ascorbate alone produced no effects, only the combination. The authors conclude that methyl mercury may be more toxic to cells under proxidant conditions.

It is not known what the relative roles of calcium homeostasis or oxidant injury play in the overall sequence of events in cellular damage developing from methyl mercury. It is an open question whether they are independent mechanisms or lie in a sequence of biochemical and physiological chain of events, or how they relate to inhibition of protein synthesis discussed above. Sarafian et al. (95) further examined the idea that selective toxicity might arise from selective resistance. They noted that many common cellular defense mechanisms against oxidative injury, for example, modulation of cellular levels of glutathione, metallothionein, hemeoxygenase, and other stress proteins, appear to be markedly deficient in neurons suggesting one explanation for the vulnerability of the nervous system to methyl mercury.

A convincing explanation for the long latent period remains elusive. Perhaps damage to DNA (103) or other cellar targets slowly accumulates until the cell can no longer survive. Ganther (104) suggested another possibility. The mercury-carbon bond undergoes homolytic cleavage to release methyl free radicals. These in turn will initiate a chain of events involving peroxidation of the lipid constituents of the cell. The latent period would be the time during which the cell defended itself from peroxidation until finally all defenses were exhausted and a rapid degeneration took place. The problem with his theory is that breakdown of mercury probably does not take place in neuronal cells.

Thus theories of selective and prolonged resistance remain speculative to this day. They do, however, suggest that fruitful research should come from further elucidation of mechanisms of cellular resistance to methyl mercury. However, a few words of caution are in order. In vitro studies where methyl mercury compounds, usually the chloride salt, are added to the incubation media are difficult to extrapolate to in vivo brain levels of mercury. Methyl mercury avidly binds to protein thiol ligands in cells and cellular material. Thus, in any given

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suspension, most of the mercury will move from the medium to bind to the cellular material. The concentration added to the medium may therefore differ greatly from the concentration in the cells or cellular material. The more dilute the suspension, the larger will be the proportion of methyl mercury that is taken up. Unfortunately all the in vitro studies published to date have not measured the mercury attached to the suspended cellular material.

Another difficulty in extrapolation is that the methyl mercury compounds in vitro may not be the same as the in vivo compounds and may differ both in ability to penetrate cells and in toxicity. For example, one in vitro study concluded that methyl mercury was present in the media as methyl mercury chloride (98). This lipid-soluble compound can easily diffuse to all areas of the cell. In vivo methyl mercury is present as water-soluble compounds of protein or amino acids and is carried into and removed from the cell by highly selective transport processes as discussed by Clarkson (105).

Health risks: All studies reported to date are either on populations where overt cases of poisoning have occurred (e.g., Japan and Iraq) or on populations having elevated methyl mercury levels from consumption of fish or sea mammals. Some have been retrospective (e.g., Japan and Iraq); others have been cross-sectional or prospective epidemiological investigations (e.g., fish-eating populations). In most cases, methyl mercury levels in blood and scalp hair have been used as a measure of the dose (e.g., as surrogates for levels in the target organ, the brain). Total mercury is usually measured instead of methyl mercury as the latter, in methyl mercury exposures, accounts for most of the mercury (95% in blood samples and over 80% in hair samples) in the indicator media. The end points used as an indicator of methyl mercury toxicity have ranged from overt clinical signs and symptoms to statistical differences in scores of neuropsychological tests.

The first comprehensive estimate of human health risks was in the late 1960s (1). The Expert Group relied on the two outbreaks in Japan (specially the outbreak in Niigata in 1965 where blood and hair levels of mercury were measured) to establish the lowest blood and hair levels associated with lowest observed adverse effects levels (LOAELs) in adults.

The Niigata outbreak was due to the consumption of contaminated fish by villagers fishing the Agano river in the Niigata prefecture. Contamination of the fish was due to the release of mercury compounds from a factory using mercuric chloride as a catalyst in the manufacture of vinyl chloride and acetaldehyde. Both methyl and inorganic compounds of mercury were discharged to the river. By 1970, 47 cases of poisoning and six deaths were observed (106).

A total of 17 cases were reported that had blood levels measured after the onset of symptoms (1). In a few cases several blood levels were measured over consecutive periods so that back-extrapolation to the time of onset of symptoms

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was possible. A general picture was obtained suggesting that the lowest blood level at the time of onset of symptoms was approximately 200 μg Hg/L. The same approach was taken in interpreting data on hair levels. Here some 35 subjects had hair levels measured after onset of symptoms. They concluded that the lowest hair level at the time of onset of symptoms was 50 μg Hg/g hair.

These LOAELs, the first to be derived for humans, must be regarded at best as approximate. Few subjects were involved. An assumption was made that ingestion of the contaminated fish ceased at the time of onset of symptoms. Also the actual levels of mercury that caused the symptoms may not have been the levels at onset of symptoms if blood or hair levels were still rising or falling. Methyl mercury has a latent period of 1–2 months before onset of symptoms so that an earlier level may have been responsible for the observed effects. A critique by Marsh et al. (107) argued that the LOAELs were underestimated. For example, only one subject with a single hair measurement had a level close to 50 μg Hg/g. All the other subjects had hair levels that were above 100 μg Hg/g.

A subsequent review by the World Health Organization Expert Group (2) agreed with the Swedish conclusions that the LOAELs in blood were 200 μg Hg/L and 50 μg/g in hair. No statistical evaluation of actual risks was possible, but they assumed a 5% risk at the LOAEL as this is the practical limit of detection in such studies. They also applied a ''safety factor'' of 10 to derive maximum tolerable levels for the general population, namely, 20 μg Hg/L in blood and 5 μg/g in hair. The safety factor was applied to allow for a range of susceptibility in the general population including the presumed greater sensitivity of the developing brain. After the passage of almost 30 years and the publication of new epidemiological studies (see below), these conclusions have withstood the test of time.

The outbreak in Iraq gave remarkable confirmation of the Swedish-derived human LOAELs. Large numbers of farmers and their families were exposed to a methyl mercury fungicide that had been used to treat seed wheat. Some of this treated wheat was used directly to make homemade bread. Countless thousands of people were poisoned. Hospital cases recorded throughout the country amounted to about 6000 with 500 deaths in hospital (83). Many more persons were estimated to have been affected but not admitted to hospital (108). The period over which the contaminated bread was consumed was estimated to be about 1–2 months.

Several methods were used to estimate the ingested dose and peak hair and blood levels at the cessation of exposure (83). In some cases hair samples were of sufficient length to recapitulate levels at the end of exposures. For others, successive collection of blood samples allowed back-extrapolation but this required recall by the patient as to the date when consumption of contaminated bread stopped. Information was also obtained on the number of loaves consumed

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and on the methyl mercury content of the loaves. The estimated total ingested dose was then converted to a body burden assuming 95% absorption and a whole-body half-time of 70 days. Figures for body burden, blood, and hair levels were interconverted using average parameters of disposition discussed in the previous section.

It was possible to plot dose-response relationships for a number of signs and symptoms of poisoning (Fig. 2). The shape of the plots for each sign or symptom took the form of a hockey stick. There is a horizontal section indicating a background frequency unrelated to mercury levels, followed by an inclined line indicating the frequency of the sign or symptom increased with increasing mercury levels. The intersection of the two lines was taken as a threshold mercury levels where adverse effects became detectable, i.e., an estimate of a LOAEL for each sign or symptom (83).

It may be seen that the threshold body burden is higher as the sign or symptom increases in severity. The least severe is the onset of paresthesia as compared to a fatal outcome at the highest threshold. The body burden associated with the onset of the first symptom of poisoning in adults (paresthesia) was estimated in the range of 100 μg Hg/g hair as methyl mercury. This figure converts to blood levels of 400 μg/L. Given the uncertainties in numbers derived from

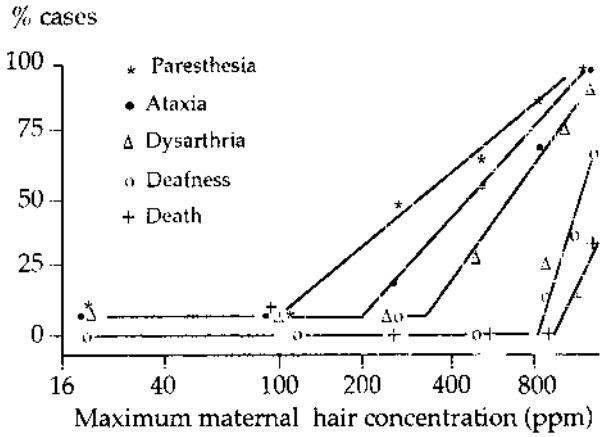

FIGURE 2 The relationship between the frequency of signs and symptoms of methyl mercury poisoning in adults and the corresponding hair concentration. The latter is the estimated maximum hair concentration attained during a brief (few months) exposure to methyl mercury from consuming contaminated homemade bread (for details see ref. 83).

epidemiological investigation, the Iraq numbers are in excellent agreement with the LOAELs derived by the Swedish Expert Group from observations of the Japanese outbreaks.

All subsequent studies have been on fish-consuming populations where dietary intake of methyl mercury presumably was lifelong. This presents a special problem as the peak level of methyl mercury may have been experienced before the studies took place. Nevertheless it is worth examining some typical studies to see what methods were used, the results obtained, and the problems in interpretation.

Adult studies on fish-eating populations have failed to find effects due to mercury at blood or hair levels below the NOAEL derived by the Swedish and WHO Expert groups. A study was conducted by researchers from the University of McGill in the 1970s of adult Cree with elevated blood and hair levels due to consumption of methyl mercury in fish (109). Hair levels in such populations exhibited marked seasonal changes attaining highest levels at the end of the fishing season in late summer (110). Some fish were specially contaminated owing to local pollution by mercury released from a paper pulp plant. The relationship between methyl mercury exposure (hair levels) and neurological abnormalities was tested in two Cree communities by comparing the mercury levels in 41 ''cases'' with selected neurological abnormalities with levels in 179 normal controls. A subject was considered to be a case if he or she exhibited: (1) bilateral symmetrical reduction of visual fields without explanation after ophthalmological examination and/or (2) neurological disease considered by the neurologist to be present in an overall summary of his findings. The hair mercury index of exposure to methyl mercury was the mean concentration of mercury in scalp end hair segments 4 cm in length obtained from each subject during the field study. The average hair levels in the ''cases'' were in the range of 10–16 ppm μg/g. The levels in controls were from 4 to 7 μg/g.

In one community, a significant positive association was found between neurological abnormalities and methyl mercury levels. The main contributing factor was the age of the subjects. Several other factors in this population could also contribute to the observed signs and symptoms. The authors concluded, ''Although allowance was made for confounding variables, it remains possible that the effects are not entirely attributable to methyl mercury. Further, the data do not permit estimation of a threshold level of exposure above which excess neurologic abnormalities might occur because present levels of exposure do not reflect those in the past.''

Spitzer et al. (111) have reported an epidemiological investigation of 321 adult Crees and Algonquins resident in Northwest Quebec to ascertain the health impact from a local industrial source of mercury pollution. Mercury contaminated a freshwater system resulting in elevated levels of methyl mercury in fish and consequent human exposure.

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Several approaches were applied to assess mercury exposure in the study population: (1) the concentration of total mercury in hair (the average for the entire length of the hair sample), the first 9 cm from the scalp and segments greater than 20 cm from the scalp; (2) levels in whole blood; and (3) fish consumption. Only two hair samples were reported to have mercury levels in excess of 40 ppm, four samples were greater than 20 ppm average for the whole length, five for the 9-cm segments, and 28 for segments greater than 20 cm. The correlation coefficient between hair and blood was 0.81 but the hair segment used in this correlation was not stated.

Eleven health outcomes were measured as indicators of neurological damage and general health status of the subjects. Multiple linear regression analysis was used to assess the relationship between health outcomes and hair levels of methyl mercury. The analysis took into account a number of potential confounders including age, sex, diabetes, and alcoholism.

Statistically significant correlations ($p < 0.05$) with one or other or all of the three measures of hair mercury were found for four health outcomes: (1) any neurological or ophthalmological abnormality, (2) coexistent upper- and lower-extremity tremor, (3) abnormalities recorded by the data-gathering technician, and (4) abnormal sensory nerve conduction velocities. The brief description of these health end points does not seem to exclude the possibility of overlap; that is to say that a specific abnormal measure in one category is also responsible for an abnormal score in another category.

This population, as in other freshwater fish consuming First Nations people living in Northern Canada, experienced marked seasonal changes in hair levels. The highest level is achieved in late summer and early fall when weather conditions prevent further fishing. The lowest levels occur just before next year's fishing season (110). Seasonal changes are indicated in this population by differences in average mercury levels in the 9-cm versus the greater-than-20-cm hair segments. Longitudinal analysis was not carried out, so there is no information on peak concentrations in the various hair segments used in this study. The average levels reported in this paper, especially for long hair segments, should be appreciably lower than the monthly peak. Furthermore, there is no information on the magnitude of earlier seasonal peak levels as even the longest hair samples rarely allow recapitulation of exposure for more than 2 years. The lack of information on lifetime exposure and on peak levels precludes the use of this study for risk assessment.

Lebel et al. (112) reported the results of a study on 29 young adults exposed to methyl mercury in freshwater fish caught in the Amazon basin. The hair levels of total mercury were in the range of 5.6–38 μg Hg/g. Results of visual testing showed that color discrimination capacity decreased with increasing mercury in hair, and peripheral field profiles were reduced for those with the highest mercury levels. For women, manual dexterity decreased with increasing mercury in hair.

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Lebel et al. (113) have reported on a larger group ($n = 91$) from the same area, in one of the two villages involved in the first report. Only adults were examined. Hair was used as the biological monitor. Longitudinal analyses were performed on consecutive 1-cm segments. In the case of some female participants, the hair segments' total length extended for 24 cm. Four measures of mercury exposure were used: average total mercury, the highest 1-cm segment (peak value), total mercury, and methyl mercury in the 1-cm segment next to the scalp.

Nine ''neurofunctional'' end points were used including manual dexterity and visual contrast sensitivity at different frequencies outdoors in shaded areas to control the luminance. Multiple-regression analysis was used to correlate these end points with the four measures of exposure giving a minimum of 36 correlations. Contrast sensitivity at the higher frequencies of 6,12, and 18 cps gave statistically significant correlations with peak hair levels and with some of the other measures of dose.

The authors state that age adjustments were made but no details were given. The age range was broad, 15–81 years. Stepwise regression analysis was used to assess the influence of confounders on the neurofunctional outcomes but no explicit list of confounders was given. Sociodemographic data are given that indicate 17% had worked in mercury mining (presumably exposed to mercury vapor), 34% had a history of malaria, and 17% a history of parasitosis. The overlap between these groups was not given.

Also an extensive number of ''clinical'' end points were measured on a randomly chosen subset of 59. Most clinical parameters were normal. However, a high prevalence of disorganized movement, restricted visual fields, and hyperflexia were noted. Mercury (mainly peak levels) related effects were seen for both movement and visual fields but not for reflexes.

All members of the study group had mercury levels that never exceeded 50 ppm, the hair level that is currently regarded as the lowest toxic level in adults. The authors point to the ''particular physiological importance'' of peak levels of mercury. This makes sense as methyl mercury, at least in terms of its overt clinical effects, is believed to be an irreversible poison because it destroys neuronal cells (75). Thus the history of exposure may be key to determining the outcome. When did the peak levels occur in this adult population? The longest hair sample could recapitulate exposure over no more than 2 years. The authors themselves suggest that the adverse effects found in the younger group, aged 15–20 years, may be due to prenatal exposure as mercury releases to the environment due to gold mining started about 20 years ago.

Several cross-sectional studies have also been conducted on populations consuming ocean fish in Peru, American Samoa, and the Mediterranean (for review see refs. 3,4). Regular consumers of ocean fish do not exhibit the high degree of seasonal variation as seen in consumers of freshwater fish. None of these studies was able to detect adverse effects of methyl mercury at hair levels

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ranging up to 50 g Hg/g wet weight or blood levels up to 200 μg Hg/L. Thus they add some confirmation to the original LOAEL estimated by the Swedish Expert Group.

A single cross-sectional evaluation on any adult or even prenatally exposed populations leaves key toxicological questions unanswered, makes risk assessment difficult, and makes the actual lowest-effect levels a matter of conjecture. Unfortunately all studies on chronically exposed adult populations have been cross-sectional. Effects such as paresthesia have been found, for example, in Peru, but they did not correlate with current mercury levels (114). Without longitudinal studies we cannot exclude the possibility that earlier exposure to mercury was responsible.

In conclusion, we must still rely on the two Japanese outbreaks and Iraq because the limited time of exposure allowed estimates of the true maximum blood and hair levels. The conclusion of the Swedish Expert Group and WHO remains our best evaluation of adult risks, namely, that the LOAEL from adult exposure correspond to hair and blood levels of 50 ppm and 200 ppb, respectively.

Prenatal Exposure. Signs and symptoms of poisoning: As demonstrated in the outbreaks in Minamata and Iraq, high prenatal exposure results in signs of poisoning similar to cerebral palsy. The development of the central nervous system is disorganized and inhibited. Studies on both experimental animals and autopsy brain tissue indicated that methyl mercury inhibits neuronal cell division and the migration of neuronal cells in the developing brain. Thus brain size may be abnormally small and microscopic examination of autopsy tissue reveals disorganized development of the cortex and the presence of atopic cells that did not reach their final anatomical destination (115).

Milder cases of prenatal poisoning have been reported in the Iraq outbreak characterized by delayed achievement of developmental milestones and by abnormal reflexes (116). The brain pathology associated with these milder effects is unknown. As discussed in a later section, several epidemiological studies have been undertaken to detect subtle neurobehavioral deficits due to prenatal exposure to methyl mercury. No characteristic signature of methyl mercury as yet emerged from these studies to distinguish the neurobehavioral effects of methyl mercury from those of other developmental neurotic agents such as PCBs and lead (117).

Mechanisms: Studies on the effects of methyl mercury on the cytoskeleton have provided a plausible explanation for the developmental neuropathology (for a review, see refs. 118,119). Methyl mercury causes the depolymerization of microtubules in vitro. Apparently it binds to the thiol ligands of the tubulin monomers and prevents their assembly at the growing end of the microtubule. Microtubules are continuously assembled at one end and depolymerized at the other in a treadmilling process. Blocking only the assembly process allows the

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depolymeriztion process to continue so that the microtubule eventually disappears. The depolymerization of microtubules in turn may lead to the inhibition of synthesis of the tubular monomers (100). The loss of microtubules appears to be the first effect at the lowest concentrations of methyl mercury in tissue culture experiments. The microtubules play a key role in both cell division and neuronal migration. Thus the assumption that the primary action of methyl mercury in vivo is to destroy microtubules would explain the neuropathology and indicate why the action on the developing brain is fundamentally different than in the mature brain where cell division and migration no longer occur.

Health risks: Epidemiological studies of prenatal exposures have an advantage over adult exposures in that the dose can be measured over the period of interest—the 9-month prenatal period as opposed to the lifetime exposure for adults. Nevertheless even prenatal exposures suffer from many complications that make it difficult to establish methyl mercury as the cause of any observed adverse effects. The primary argument for an effect of methyl mercury is a statistical correlation between methyl mercury levels and the prevalence of a given adverse effect. This argument is always open to a scientific challenge, as a correlation, in itself, does not establish causality. This is specially true in the studies to be reviewed where the end points (e.g., delayed development) could have numerous causes and where the effects of methyl mercury, if any, will be subtle and difficult to distinguish from the background noise.

A suspicion that the prenatal period may be the most sensitive stage of the human life cycle to methyl mercury came from clinical observations on a few infant-mother pairs in the *Minamata outbreak in Japan* where the prenatally exposed infants appeared to be more severely affected than their mothers (106). Subsequent animal experiments tended to support these observations. However, such comparisons are of dubious scientific merit as the effects on the mothers are qualitatively different involving different mechanisms than those seen in her offspring.

A study of 83 infant-mother pairs in the *Iraq outbreak* provided the first quantitative dose-response relationship between the peak levels of methyl mercury hair samples from the mother during pregnancy and the risk of adverse effects in her offspring (121). Peak hair levels of mercury were determined by analysis of single strands by X-ray fluorescent spectrometry, which measures the mercury concentration in consecutive 2-mm segments. Since human scalp hair grows at a rate of approximately 1.1 cm per month, each 2-mm segment represents about 5.5 days of exposure. By this means it was possible to make a detailed recapitulation of mercury levels during pregnancy. The individual data points were fitted to a pharmacokinetic model of methyl mercury disposition in the body to allow calculation of the actual peak concentration in hair during the period of pregnancy.

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The reported effects were delayed achievement of developmental milestones (first walking and talking) and the presence of abnormal tendon reflexes. The frequency of offspring having delayed development on abnormal reflexes was plotted against peak hair levels during pregnancy to yield dose-response relationship (Fig. 3). Statistical estimates, based on a ''hockey stick analysis'' indicated a threshold for adverse effects in the range of 10–20 μg/g peak maternal hair in pregnancy. The uncertainties in these estimates were wide, ranging from as low as 7 μg/g to as high as 180 μg/g depending on the assumptions underlying the models used for the statistical estimates.

A recent reanalysis of the same data confirmed the wide range of uncertainty in the estimated threshold (122). These authors also subjected the Iraqi data to benchmark analysis. The benchmark analysis calculates a benchmark dose (BMD) associated with a specified increase in frequency of an abnormal finding. In this analysis, hair levels were used as the surrogate of dose and a 10% increase in response rate was used as the criterion for the lowest detectable change in outcome measure.

The choice of this increase over background frequency is somewhat arbitrary. In general, practical limitations in epidemiological studies make it difficult to detect an increase in abnormal response of 5–10% over the background prevalence of the abnormality. Thus the benchmark may be taken as a measure of the minimal detectable level. A lower limit to the benchmark dose (BMDL), usually calculated as two standard deviations from the BMD, is taken as a measure of the no observed adverse effect level (NOAEL). Crump et al. (122) assumed a

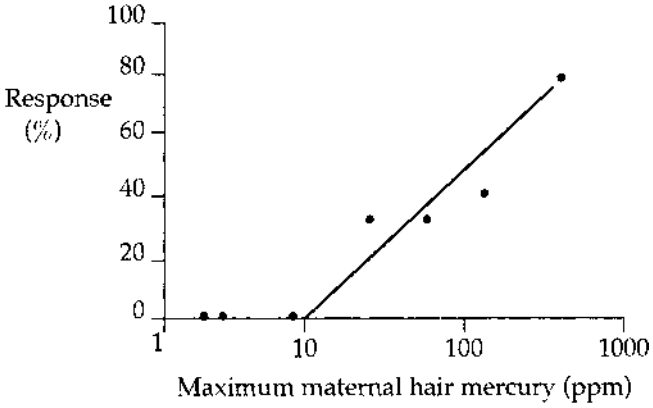

FIGURE 3 The frequency of delayed walking (% response) in prenatal exposed infants to the maximum maternal hair levels during pregnancy. Data are from the Iraq outbreak (for details see ref. 121).

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10% increase in abnormal response and used hair mercury levels as a continuous variable for each of the three end points (delayed walking, delayed talking, and abnormal reflexes). They obtained BMDLs in the range 26–274 ppm.

The U.S. EPA (123) applied a different approach to benchmark analysis of these same data. They grouped the data into three dose categories and defined a dichotomous measure of health outcome as a child being abnormal if an abnormal response appeared in any one of three outcome measures (delayed walking, delayed talking, and neurological abnormalities). This approach resulted in a BMDL of 11 ppm.

Also in the 1970s, an epidemiological study was conducted by the McGill group on prenatal exposures in the same *Cree population* where adult exposures were studied (124). Neurological and developmental abnormalities were examined in 234 Cree children aged 12–30 months from four communities in northern Quebec.

> Four pediatric neurologists documented each child's height, weight and head circumference, assessed dysmorphic and congenital features and reported the presence or absence of acquired disease. A neurological examination was also conducted and included an assessment of special senses, cranial nerves, sensory function, muscle tone, stretch reflexes, coordination, and persistence of the Babinski response, which was judged to be abnormal for the child's age as well as a summary of the presence or absence of neurological abnormality. Finally the neurologist assessed the child's development by use of the Denver Developmental Scale.

Covariates suspected of affecting the results were also measured such as alcohol intake, the use of tobacco, child's age, duration of breast feeding, and mother's age. Data were also obtained on pregnancy, labor, and delivery.

Levels of total mercury were measured in alternate 1-cm segments beginning with the scalp end segment in samples of maternal hair covering the period of 1 month before conception to 1 month after delivery. The index of prenatal exposure was the maximum concentration recorded in these segments. Regression analyses were conducted on both neurological outcomes and the results of the Denver Developmental tests. The outcome measures most closely associated with hair mercury were then subject to a discriminant analysis in which children were classified as cases or controls depending on the presence or absence of abnormality of the relevant outcome measure.

Only one end point—abnormal reflexes—was more prevalent in the highest mercury group. The end point combined both depressed and enhanced reflexes into a single abnormal category. This is not the usual practice in neurology. As the authors themselves point out, the findings are of doubtful clinical significance.

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The authors also note that the statistical method could have led to an association by chance. No dose-response relationship could be found over the entire range of mercury values. No other end point exhibited any association with maternal mercury. The mean maximum mercury levels in maternal hair was 6 μg/g. The highest mercury group had maximum levels in the range of 13–24 μg/g.

In the late 1970s, a study was conducted in *New Zealand* of prenatally exposed infants whose mothers consumed ocean fish. Hair samples and the results of a diet survey were obtained from 10,970 mothers at delivery during 1977–78 in the north island of New Zealand. A total of 73 mothers with average hair levels during pregnancy of above 6 ppm were identified out of over 1000 who consumed ocean fish more than three times a week.

In stage 1 of the study (125) 31 infant-mother pairs in the high-exposure group (mean maternal hair methyl mercury 8.8 μg/g) were compared with a low-exposure group (mean maternal methyl mercury 1.9 μg/g). Statistically significant differences were seen in the Denver Developmental Screening Test (DDST) indicating developmental delays in the high-exposure group. Thus 50% of the high-mercury group had questionable or abnormal DDST results as compared to only 17% in the reference group, a large and statistically significant difference. However, Marsh (115) has argued that age difference between the groups may account for the results. The DDST is not sufficiently discriminating to account for age differences of a few months. The results of these tests were highly dependent on the age of the child.

In 1985 stage 2 (126), 61 children of the original 73 infant-mother pairs were identified. Three control children taken from the original 10,970 were matched against each of the high-mercury children. Matching was on the basis of ethnicity (European, Maori, or Pacific Islander), smoking, place of residence, and the time the mother lived in New Zealand prior to the child's birth. One group of control children had average maternal hair levels in the range of 3–6 ppm and two other control groups, differing in maternal fish consumption, had hair levels in the range of 0–3 ppm.

The children at ages 6–7 years were subjected to a wide range of psychological and developmental tests. Statistical analyses of the results of five test scores indicated that high prenatal exposure (average mercury levels during pregnancy 13–15 μg Hg/g) was associated with decreased performance. The contribution of methyl mercury to the decrease in test performance was much less than that of other factors such as ethnic differences. Even when all known or suspected confounding variables were taken into account, only a small fraction of the decrease in performance could be accounted for.

As Marsh (115) noted in his critique, this was a difficult study to completely control as the study population came from three distinct ethnic groups with social, linguistic, and scholastic differences. Adjustments were made for several covariables but two covariables known to affect neuropsychological tests, namely ma-

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ternal education and intelligence, were omitted. This was the first study of prenatal exposure where psychological and educational tests were applied. If methyl mercury did indeed have an adverse effect in this population, the LOAEL of 13–15 μg Hg/g is consistent with conclusions from Iraq and Canada.

A reanalysis of these data has recently been published (127). In addition to the five tests analyzed by Kjellström et al. (126), Crump et al. (127) included multiple-regression analysis of all 26 scholastic and psychological tests. No association was found between maternal mercury hair levels and decreased test performance when all the subjects were included. However, the results of these regression analyses were greatly influenced by the result from one infant-mother pair where the maternal hair level of 86 ppm was four times higher than any other hair level in the whole study group. When this infant-mother pair was removed from the analysis, statistically significant correlations were seen between mercury and decreased performance in six outcome measures.

Crump et al. (127) conducted a benchmark analysis on five of these tests based on average hair levels in pregnancy. The BMDs estimated from five tests ranged from 32 to 73 ppm with corresponding BMDLs from 17 to 24 ppm When the results from the child with the highest mercury level were omitted, the range in BMDs was 13–21 ppm and for the BMDLs was 7.4–10 ppm.

The justification for omitting the infant-mother pair with the highest mercury levels is not clear. The authors point out that ''none of the test scores on this child were identified as outliers, and we have no information that would indicate that his tests results or his mother's hair mercury level were flawed.'' As pointed out recently by a group reviewing health risks from methyl mercury (117), data should never be omitted on the basis that correlation with mercury levels might thereby be improved. Indeed, on a toxicological basis, the highest mercury point should be the one most likely to reveal a mercury effect.

The maternal mercury levels used by Crump et al. (127) as well as in the original Kjellström papers were average levels during pregnancy. However, as reported by Kjellström et al. (126) based on 1-cm longitudinal analyses of maternal hair samples, the maximum level in pregnancy is about 50% higher than the average values. Thus even if one allowed the omission of the high infant-mother pair as legitimate scientifically, the lowest BMDL would still be in excess of 10 ppm.

Another study on an ocean-fish-eating population was also conducted in the 1980s in *coastal villages in Peru* (128). The villages were isolated by a coastal desert from large towns and industries, so local pollution was minimal. Ocean fish, consumed regularly, was the main source of protein. This study involved 131 infant-mother pairs having mean methyl mercury levels in pregnancy of 8.3 μg/g with a range of 1.2–30 μg/g. These elevated mercury levels were due to a high rate of consumption of fish having normal levels of mercury.

The frequency of neurodevelopmental abnormalities did not correlate with

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maternal exposure to methyl mercury. The authors stated that few confounding factors existed in this population as the mothers did not smoke and consumed little or no alcohol. There was no evidence of malnutrition and socioeconomic differences were small.

Two major studies are now in progress, one in the *Faroe Islands*, where methyl mercury exposure comes primarily from the consumption of pilot whale, and the other in the *Seychelles Islands*, where ocean fish is the source. A study in the Faroe Islands on pre- and postnatal exposure to methyl mercury established a cohort of 1023 infant-mother pairs between March 1986 and the end of 1987 (129). Umbilical cord blood samples had a median level of total mercury of 24.2 μg Hg/L with 25% in excess of 40 μg Hg/L. Maternal hair concentration was measured as the average level in segments of hair that grew during the period of pregnancy. The length of the segments varied but all were longer than 5 cm. Correlation of these average hair levels with cord blood levels was statistically significant but some scatter was apparent. The average hair-to-blood ratio was 191, somewhat less than most published levels (4).

Pilot whale meat was the major dietary source of methyl mercury. The average concentration of mercury was quoted as 3.3 μg Hg/g with about 50% present as methyl mercury. In contrast, codfish, the most popular dietary item of fish consumed, has an average methyl mercury level of only 0.07 ppm.

A total of 523 infants selected as consecutive births from this cohort were followed for three developmental milestones during the first 12 months (130). The development milestones usually attained between the ages of 5 and 12 months were sitting, creeping, and standing. A surprising finding was that early achievement of these milestones was associated with higher mercury levels in the infants' hair. However, early milestone development was also associated with the length of breast feeding, which in turn was associated with infant hair levels. No other potential confounders could be found. It was concluded, therefore, that the correlation of development with infant hair mercury was spurious and represented a beneficial effect of breast feeding. The authors noted that this beneficial effect took place despite high levels of PCBs in maternal milk.

Dahl et al. (131) reported on computer-assisted neurobehavioral tests on 917 children at 7 years of age. Three tests from the Neurobehavioral Examination System were administered. Slight but statistically significant decrements were seen with increased levels of prenatal exposure to neurotoxicants as evidenced by the mercury concentration in cord blood. However, the authors noted that whale blubber contains other neurotoxicants including PCBs. The latter could not be excluded as the underlying cause of the observed correlation between mercury exposure and neurobehavioral dysfunction.

The more extensive report on the Faroes study (132) summarizes the results of all the tests on the 7-year-old cohort of 917. Clinical examination and neurophysiological tests did not reveal any clear-cut abnormalities related to mercury.

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However, several neuropsychological tests showed slight but statistically significant negative associations with cord blood mercury levels. Linear regression analysis revealed a statistically significant ($p = 0.05$ or less) negative linkage with mercury in eight out of 20 such tests, This proportion was increased to 10 out of 20 when other statistical calculations were applied. Cord blood mercury was used as the independent variable.

Linear regression analyses were also conducted after deletion of those children, approximately 15% of the cohort, with maternal hair levels of 10 ppm or higher. Again cord blood mercury was used as the independent variable. With this restriction, significant *p*-values were seen in seven of the 20 tests. In one test a *p*-value attained significance ($p < 0.02$) whereas with the full group it was nonsignificant ($p < 0.1$). In two other tests the *p*-value attained greater significance than in the full cohort.

Cord tissue concentrations for certain PCB congeners were available for 435 of the children in the cohort (the median and range of the cord blood mercury in this group are not given). Four of the psychological tests showed significant negative correlation with the logarithmic transformation of the PCB wet weight concentrations. Nine tests showed significant negative correlations with cord mercury levels (it is not stated whether these are the same tests as in the complete cohort). In the four tests that showed correlations with both mercury and PCBs, the significance of the mercury correlation disappeared in three of the four tests when adjustments for PCBs were made. As the authors point out, PCBs may be an important factor in these tests.

This paper cannot be used to estimate a no-effect level. Only summary statistical data are given such as regression coefficients and *p*-values. The actual data, for example, in the form of scatter plots, are not given.

Further analysis of the data from the 7-year examination has been reported (133). In this analysis, a case-control comparison was made. A case group of 120 children, with maternal hair in pregnancy between 10 and 20 μg Hg/g, was matched with a control group of 312 children, with maternal hair less than 3 μg Hg/g, using age, sex, time of examination, and the mother's score on the Raven's Progressive matrices as matching criteria. The authors stated that the two groups were almost identical with regard to other factors that might affect neurobehavioral performance. Mild decrements were found in the case versus the control group in motor function, language, and memory.

Despite the fact that this appears to be a well-conducted study on a large group of children with extensive neuropsychological testing, the interpretation of these findings is not straightforward with regard to effects of methyl mercury. This population consumes whale blubber along with the meat. Pilot whale blubber has high concentrations of PCBs and other organochlorine contaminants (134,135). A statistical analysis including PCB levels measured in cord tissue revealed that PCBs may have influenced some of the tests but the mercury effect

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remained after correction for PCBs. However, it still remains an open question as to what are the relative contributions of methyl mercury, PCBs, and other contaminants to the mild changes seen in the 7-year-old tests.

Although limited evaluations were made on these children at an earlier age, e.g., milestones of development, the only substantial evaluation took place at the 7-year examination. Without repeated longitudinal evaluation, we have no information on the permanence of the mild effects that were claimed to be associated with mercury. Indeed, the milestone study found beneficial effects correlated with mercury levels as discussed above. Moreover, such mild effects are specially susceptible to confounder influence as the magnitude of the observed changes is close to background noise. A second confirmatory evaluation of this cohort is needed.

The *Seychelles Child Development Study* is a prospective investigation based in an Island population having high daily consumption of ocean fish. The site has several attractive features for a study assessing human health risk from methyl mercury in fish. The Seychellois Islanders regularly consume high quantities of ocean fish, an average of 12 meals per week. The levels recorded in 350 samples of 20 species of fish consumed in the Island range from 0.004 to 0.75 ppm, overlapping most of the concentrations in both freshwater and ocean fish consumed in Europe and North America. The Seychelles is largely a pristine environment with little industrialization. Such neurotoxicants as lead and PCBs were found to be low or undetectable in blood samples collected from the Islanders. The population is literate, cooperative, with minimal emigration or immigration. The socialized welfare system minimizes the impact of economic status on health care. The population is healthy, with low maternal alcohol consumption and tobacco use ($<2\%$). The maternal hair concentrations have a mean value of about 7 ppm with a range of 0.5–27 ppm, thus covering the same range of hair levels as previous studies on fish-consuming populations.

A ''pilot'' cohort of over 700 infant-mother pairs was recruited in 1987–88. The recruitment of the main study cohort is of similar size as the pilot group was started and completed in 1989. The experience with the pilot group was used to guide the experimental design of the main cohort. The offspring of the mothers in each cohort have been subjected to a range of developmental tests appropriate to the age of the child. In the main study the children were examined at the same age (within a few weeks) at 6, 19, 30, and 66 months. The study on the children, now 10 years of age, is in progress. The results of the tests on the pilot group and on the main cohort up to 66 months of age have been published (136–138).

The first results from the pilot study indicated that developmental delays, as determined by the Denver Developmental Screening Test (DDST), were associated with mean maternal hair levels during pregnancy (139). The DDST has three outcomes—normal, questionable, and abnormal. In both the New Zealand and Seychelles pilot studies, a correlation with maternal methyl mercury was

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seen only when the ''questionable'' outcomes were included in the ''abnormal'' category. Combining these categories is not done in the normal clinical application of the test. When similar tests were repeated on the main cohort with careful control of age at testing and using additional covariates (mother's intelligence, maternal and paternal educational levels, history of breast feeding, language spoken at home, and family income), no correlation was found with maternal mercury levels (140).

Neurobehavioral tests were also carried out on a subset of the pilot cohort at 66 months of age. A correlation was seen in some of these tests between decreased performance and prenatal maternal hair levels. However, the results of the linear regression analyses were highly dependent on a few influential points, some of which were outliers. The statistical significance of the correlations disappeared when these points were not included in the regression analyses. A repeat of these same neurobevioral tests on the main cohort at 66 months found no correlations with prenatal mercury levels (see below).

The results of performance tests on the main cohort have been published for ages 6, 19, 29, and 66 months. Myers et al. (137) described the results of tests designed as a direct comparison of the Iraqi prenatal study. The same developmental milestones were measured by the same examiner in children at the same age. No adverse effects attributable to prenatal exposure to methyl mercury could be detected in the Seychelles. As discussed, the original statistical analyses of the Iraqi study (116,121) raised the possibility of adverse developmental effects down to maternal hair levels as low as 7 ppm, with a wide margin of uncertainty as confirmed by subsequent analyses (122,144). The median hair level in the Seychelles was close to 7 ppm with the highest level at 26 ppm. Thus, the lower extreme projected from Iraq is not confirmed.

The primary analyses of tests at 19 and 29 months revealed no connection with mercury levels except that the child's activity score, based on a subjective rating by the examiner during the Bayley tests (Bayley Scales of Infant Development, BSID), declined with increasing prenatal mercury levels. The significance of this finding is not clear as hyperactivity is usually associated with effects of neurotoxicants on children, for example, with lead exposure (142).

A secondary statistical evaluation of these same data revealed that improved scores significantly correlated with prenatal mercury levels when children were grouped by sex either according the maternal intelligence (maternal Raven scores) or according to the household income (143). This was the first indication of an apparent beneficial effect associated with increasing mercury levels. As discussed below, this is believed to be due to a confounding influence of beneficial nutrients in fish.

Davidson et al. (138) have reported on the results of testing on the main cohort at 66 months of age. Six age-appropriate neurodevelopmental tests were administered: the McCarthy Scales of Children's Abilities, the Preschool Lan-

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guage Scale, the Johnson Woodcock Applied Problems and Letter and Word Recognition Achievement Tests, the Bender Gestalt, and the Child Behavior Checklist. The outcomes of these tests were compared by multilinear regression analyses to both prenatal (maternal hair mercury) and postnatal (child's hair mercury) measures of exposure to methyl mercury.

Scores on the Preschool Language Scale increased with higher levels of pre- and postnatal mercury exposures. The Johnson Woodcock test also showed enhanced performance with respect to postnatal exposure levels indicating a 10% improvement over the full range of exposures. The Bender Gestalt test indicated a substantial reduction in error scores for increasing postnatal exposure of males. The overall reduction in errors was 40%.

Thus the results of the 66-month evaluation indicated the children were developing well. Evidence was seen of a beneficial factor that covaries with the mercury levels. The authors suggested that this may be due to intake of beneficial constituents of fish such as the n-3 fatty acids that are known to enhance brain development. Since methyl mercury levels in hair also correlate with fish consumption, a confounding effect is possible from nutritional benefits of eating fish.

The fact that the two most comprehensive and sophisticated investigations on the effects of methyl mercury in seafood on child development come to opposite conclusions is not yet explained. This apparent discrepancy was the subject of a special workshop sponsored by U.S. federal agencies having responsibilities for controlling human exposure to methyl mercury and/or funding research in the area (117). The group suggested several reasons for the divergent outcomes of the two studies:

(1) The concomitant exposure to PCBs in whale blubber may have confounded the correlations reported between decreased performance on neurobehavioral tests and cord blood levels on mercury. PCBs were measured in cord tissue, a medium not yet validated as a biological marker for the body burden. Furthermore, the PCB concentrations in cord tissue were expressed on a wet weight basis whereas the usual procedure is to use fat as the medium of comparison. Unfortunately, cord tissue does not contain sufficient quantities of fat to allow accurate measurement. Only a few congeners out of over 200 congeners suspected to be present in whale blubber were measured. Samples were available for about half the study population.

(2) Other fat-soluble environmental pollutants are known to be present in whale blubber. It is not clear that PCB levels will act as a surrogate for all of them. Thus the confounding influence of organochlorine contaminants remains unresolved.

(3) Other potential confounders may not have been fully addressed in the Faroes such as cigarette smoking, extensive travel to the test site on the day of testing, and town versus rural residence. Such confounders if not adequately addressed might lead to a false-positive outcome. On the other hand, some covari-

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ables were used in the Seychelles study that may overlap and lead to a false-negative result.

(4) The two studies used different approaches to biological monitoring of methyl mercury. The Seychelles used primarily average hair levels during pregnancy as the index of methyl mercury levels in the target tissues. In fact, autopsy data were reported indicating close correlation between maternal hair and brain levels in neonates during within a week or so after birth (78). The Seychelles also used hair levels in the infants as a measure of postnatal brain levels. On the other hand, the Faroes used both maternal hair and cord blood. The most robust correlations with decreased performance scores were found with cord blood mercury levels, but hair was a better predictor of tests on motor performance. It is a moot question as to whether or not the biological monitoring medium is chosen on the basis of correlation with outcome measures unless one knew a priori that mercury was producing performance deficits in the Faroese population.

Neither the Faroes nor the Seychelles studies reported maximum mercury levels during pregnancy. Levels in each trimester were highly correlated in the Seychelles consistent with a population close to steady-state levels. Nevertheless, the average of the pregnancy period must be less than the maximum level. In an ocean-fish-consuming population in New Zealand, the maximum levels in pregnancy were 1.5 times the average levels. In the Faroes the peak levels might be expected to be higher as the level of methyl mercury in whale meat is about 10 times higher than average levels in ocean fish. Thus a few meals of whale meat should have a much more pronounced effect on mercury levels than a similar number of meals of ocean fish. A few longitudinal hair analyses have been reported (129) but not sufficient to draw conclusions about the influence of peak levels during pregnancy.

(5) Whale meat contains substantial amounts of inorganic mercury accounting for an average of 50% of total mercury whereas the muscle of ocean fish contains mercury virtually all in the form of methyl mercury. Inorganic mercury is not absorbed as well as methyl mercury. Studies on volunteers taking radioactively labeled inorganic mercury compounds indicated an average absorption of only 15% but with a considerable range. Some subjects absorbed more than 20%. It is not known what this additional body burden in the mother during pregnancy might have on the fetus. Inorganic mercury affects mainly kidney function. Although transported to a limited extent across the placenta, inorganic mercury is secreted in breast milk. Studies in animals indicted that inorganic mercury is well absorbed in the immature gut. Thus the potential exists for postnatal effects but no measures have yet been reported on inorganic mercury levels in suckling infants whose mothers continue to consume whale meat.

(6) Both Faroes and the Seychelles studies differ in age of assessment and in the outcome measures. However, the expert panel (117) concluded that these differences should not account for the divergent results of the two studies. Thus

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its seems most likely that the underlying cause of the different results are the two different sources of mercury, whale meat versus ocean fish. Indeed it may be argued that the two studies are complementary rather than contradictory, one showing the combined effect of methyl and inorganic mercury and the fat-soluble pollutants, and the other reflecting methyl mercury alone where the beneficial effects of ocean fish consumption appear to be predominant.

3. CONCLUSIONS

The overall results of all epidemiological studies of human prenatal exposure to methyl mercury in fish confirm the original evaluation of the Swedish and WHO expert groups that the adverse effects should not be expected to occur from either adult or prenatal exposure at blood levels below 20 μg/L or hair levels below 5 μg/g. No overt cases of methyl mercury poisoning have ever been reported from consumption of freshwater or ocean fish with the exception of the extreme cases of pollution in Japan.

The natural processes of biomethylation and bioaccumulation of environmental mercury, dating back to the earliest evolutionary stages of life on this planet, produce levels in fish substantially lower than maximum levels reported in the Japanese outbreaks where methyl mercury compounds were manufactured and released into the water system. The epidemiological investigations discussed above looking for subtle changes in the performance of the nervous system in either adult or prenatal-exposed infants so far have given equivocal results. For example, the Spitzer et al. (111) and Lebel et al. (112) studies suggest that neuro-

TABLE 2 Lowest Effect Levels from Prenatal Exposure to Methyl Mercury

Population	Number	LOEL[a]	Media	Ref.
Iraq	83	7–180 (P)	Bread	121
Cree	247	13–24 (A)	Freshwater fish	124
New Zealand	248	25 (P)	Ocean fish	126
Peru	131	>8.3 (P)	Ocean fish	128
Faroes	1023	<10 (A)	Pilot whales	132,133
Seychelles	750	13–15 (A)	Ocean fish	138

A, the average hair level in pregnancy; P, the peak level during pregnancy. [a] The lowest observed effect level is taken as the lowest observed concentration in hair in pregnancy associated with an adverse development effect or, in the case of the Iraq study, the estimated threshold hair level. These numbers are taken directly from the original publications listed below. As discussed in the text, there are doubts that any adverse effect due to methyl mercury was actually detected in these populations with the exception of the high-exposure population in Iraq.

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logical or neurobehavioral adverse effects may be found in populations eating contaminated freshwater fish. Studies on adults consuming ocean fish have been negative. Studies of prenatal exposure to methyl mercury in populations having high daily intakes of either fresh or ocean water fish raise the possibility of subtle developmental adverse effects.

Table 2 summarizes the results of studies on both adult and prenatal effects of methyl mercury. It does not take into account the caveats noted above as to whether there are real adverse effects of methyl mercury. The conclusions of the authors are used without qualification. Even using this uncritical approach it may be seen that none of the studies indicate an effect below 5 ppm in hair.

Some investigators used the highest levels of mercury in pregnancy as the measure of prenatal dose. These peak levels were determined by longitudinal analysis of hair samples. In general, for ocean fish consumers the peak level is about 50% higher than the average during pregnancy. Thus the lowest effect levels for the Seychelles should be increased by 50% to be comparable to the results from Iraq and New Zealand.

An important conclusion to be drawn from the studies reported in Table 2 is that they confirm the original evaluation of the Swedish Expert Group (1) and WHO (2) that adverse effects should not be expected to occur from prenatal exposure to blood levels below 20 μg Hg/L, or hair levels below 5 μg Hg/g.

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Molybdenum

Edward I. Stiefel
Princeton University, Princeton, New Jersey

Henry H. Murray
ExxonMobil Research and Engineering Co., Annandale, New Jersey

1. INTRODUCTION

The chemistry of molybdenum (Mo) is among the most intricate of the transition metals (1,2). The states of oxidation and aggregation available to molybdenum are numerous. Although not high in natural abundance, the element has significant availability and accessibility on earth (3). The great versatility of its chemistry has led to the exploitation of molybdenum by both biological and technological systems (4), making the environmental chemistry of molybdenum complex and multifaceted. Although molybdenum is generally not classified as a toxic heavy metal in the environment, it is heavy, belonging to the second row of the transition period (atomic weight = 95.96). Moreover, molybdenum is ubiquitous in the environment, far more often playing the role of an essential element rather than a toxic one. Nevertheless, as with virtually any chemical entity, there are potentially deleterious aspects of its presence and interactions. In the case of molybdenum, the remarkable copper-molybdenum antagonism shows that both dangerous and beneficial effects can accrue from the interplay of individual components in the biogeosphere.

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2. HISTORY

The word ''molybdenum'' has its origins in the term ''molybdos'' (Greek for lead) or ''molybdaena'' used for soft, lead-like minerals. Indeed, lead, galena (lead sulfide), graphite, and molybdenum disulfide were not clearly distinguished in ancient times and may all have been designated as molybdaena (5). In 1778, the Swedish chemist Karl Wilhelm Scheele (1742–86) showed that the material we now call molybdenite (MoS_2) is a discrete sulfide mineral. In 1782, another Swede, Peter Jacob Hjelm (1746–1813), produced an impure powder of molybdenum metal by reducing the metal oxide and named the metal molybdenum. In 1893, Henri Moissan further purified metallic molybdenum in an electric furnace and began the study of its physical and chemical properties.

3. GEOLOGY AND REFINING

3.1 Geological Sources of Mo

The natural form of molybdenum in the solid earth is MoS_2, properly called molybdenum disulfide or molybdenite (although sometimes referred to as molybdenum sulfide or moly sulfide). Virtually all molybdenum used commercially comes from the mining of molybdenite with major deposits found in Colorado (the Climax and Henderson mines) and in several other locations worldwide (vide infra).

Molybdenite is soft (hardness 1.5–2 on the Mohs scale) and is sometimes confused with graphite. Both molybdenite and graphite have been used in ''lead'' pencils, although only the latter in modern times. In Greek, the word ''molybdos,'' in addition to meaning lead, also designates pencil, reflecting the ability of MoS_2 to be used as a writing implement. The soft molybdenite leaves a slightly bluish-silver streak (on paper or hands) that is barely distinguishable from that of graphite. The difference is sufficiently small that the two streaks must be viewed side by side, wherein the slightly bluish hue of molybdenite allows the visual distinction.

The solid-state structure of molybdenite (6) consists of MoS_2 slabs with close-packed sulfur atoms at the top and the bottom of each slab as shown in Figure 1. The close-packed sulfur layers within a slab are eclipsed with respect to each other and the Mo atoms are sandwiched between layers, occupying alternate trigonal-prismatic interstices. The sulfur atoms are strongly bonded to the molybdenum within the layer, but are only in van der Waals contact with sulfur layers from the next slab. This two-dimensional solid readily cleaves along its basal planes and the individual slabs easily slide over one another. This structure and physical behavior is clearly consistent with the macroscopic flakiness, greasy feel, and lubricity of molybdenite.

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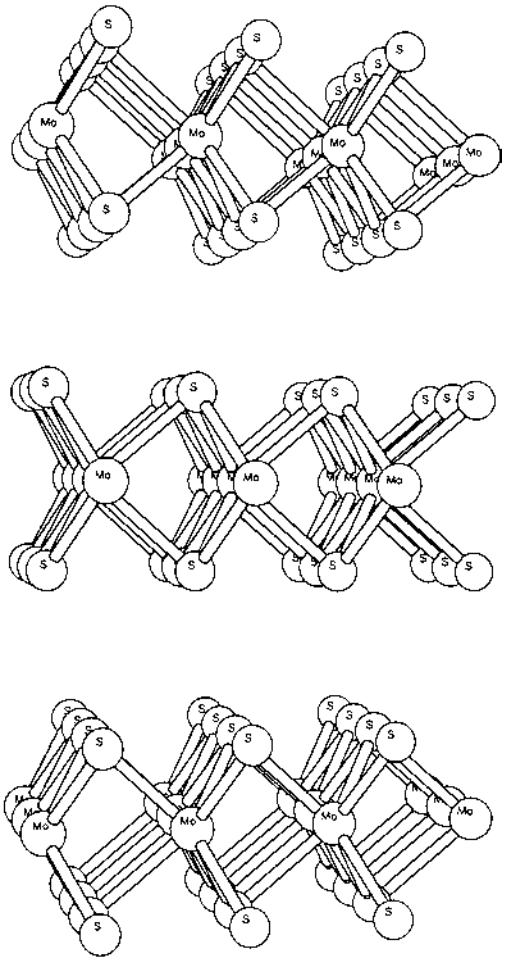

FIGURE 1 The structure of molybdenite, MoS_2, illustrating the layered nature of the crystals. The gap between the sulfur layers of adjacent slabs leads to weak interlayer binding responsible for the lubricity of MoS_2. The edge structures, which are responsible for the catalytic activity, are much more complicated than shown here and not fully defined in most situations (6).

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The molybdenite mineral, with specific gravity of 4.7–4.8, occurs in nature most often in large low-grade porphyry molybdenum deposits, or with copper porphyry deposits. It is also found associated with other minerals including pyrite, chalcopyrite, quartz, fluorite, wolframite, and scheelite.

3.2 Refining of Molybdenum

The first step in the refining of molybdenum consists of crushing and pulverizing the source rock (in which the concentration of molybdenum is only a few percent) to reduce particle size. The molybdenite is separated from the remaining ore through flotation. The flotation agent adheres to the hydrophobic close-packed sulfur basal planes of molybdenite. The resultant complex is highly water repellent and separation is accomplished by aeration in flotation tanks. The molybdenite is attracted to the rising air bubbles and is skimmed off the top of the tank. This process is repeated until molybdenite of 70–90% purity is obtained. The crude MoS_2 is then usually roasted in air to MoO_3, which serves as the raw material for the production of metallic molybdenum (by high-temperature hydrogen reduction) and for the vast majority of molybdenum chemicals.

Details of the formation, nature, and processing of molybdenum deposits are given by Braithwaite (7).

3.3 Reserves and Production

The United States has approximately 50% of the world molybdenum reserve. (i.e., in-place demonstrated resource that could be economically extracted or produced). Other large molybdenum deposits are found in Canada, Chile, China, and in parts of the former Soviet Union. The U.S. government does not maintain a strategic molybdenum reserve. The U.S. Geological Survey (8) reports that in 1999 approximately 44,000 metric tons (based on molybdenum content) of molybdenum worth approximately $250 million (based on average price of oxide) was produced in the United States representing 34% of world production. U.S. production came from eight mines, three primarily producing molybdenum ores (in Colorado, New Mexico, and Idaho), while five recover molybdenum as a by-product of copper mining (in Arizona, Montana, New Mexico, and Utah). Three U.S. plants convert molybdenite concentrate to molybdic oxide from which most molybdenum products are made. The U.S. exports molybdenum ore, concentrates, and specific compounds.

4. MOLYBDENUM MATERIALS AND TECHNOLOGY

4.1 Principal Molybdenum Materials

Commercially, molybdenum-containing materials are classified into four main groups: molybdic oxide (MoO_3); ferromolybdenum (FeMo 60–70% Mo); ammo-

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nium or sodium molybdate [$(NH_4)_2MoO_4$ or Na_2MoO_4]; and ''other'' materials (MoS_2, poly-oxo molybdenum species, and metallic Mo). A brief description of the synthesis of each follows.

4.1.1 Molybdic Oxide

Molybdenite is converted to technical molybdic oxide (''tech-oxide'' MoO_3) through roasting in air at 600–700°C. The principal chemical reactions (7,9) thought to be occurring are:

$$2\ MoS_2 + 7\ O_2 \rightarrow 2\ MoO_3 + 4\ SO_2$$
$$MoS_2 + 6\ MoO_3 \rightarrow 7\ MoO_2 + 2\ SO_2$$
$$2\ MoO_2 + O_2 \rightarrow 2\ MoO_3$$

Very pure molybdic oxide (MoO_3) can be obtained by sublimation.

4.1.2 Ferromolybdenum (10)

Ferromolybdenum (sometimes called FeMo) is an iron-molybdenum alloy (60–70% Mo) used as a molybdenum source in the steel industry (11). It is made by the thermite reduction of a mixture of molybdic oxide and iron oxides with aluminum and silicon acting as the reductants. The reaction is extremely exothermic generating the ferromolybdenum and a silicon-aluminum-oxide (sand) slag. The low carbon levels obtained with the thermite reaction versus a carbon-reduction-based metallothermic process make the thermite reaction practically the only process used commercially.

$$MoO_3 + Fe_2O_3 + Al + Si \rightarrow FeMo + AlO_x/SiO_y + \text{heat}$$

4.1.3 Ammonium and Sodium Molybdate

Ammonium molybdate, $(NH_4)_2MoO_4$, and sodium molybdate, Na_2MoO_4, are obtained by dissolving molybdic oxide, MoO_3, in basic hydroxide solutions of ammonium hydroxide or sodium hydroxide, respectively. The neutral polymeric molybdic oxide is converted into salts of MoO_4^{2-}, with ammonium or sodium serving as the respective counterion (7).

$$MoO_3 + 2\ NH_4OH \rightarrow (NH_4)_2MoO_4 + H_2O$$

$$MoO_3 + 2\ NaOH \rightarrow Na_2MoO_4 + H_2O$$

The molybdate salts are water-soluble and serve as the starting materials for the synthesis of a variety of polyoxo molybdenum compounds (12).

4.1.4 Other Molybdenum Materials (7,13)

The aqueous chemistry of the molybdate anion is sensitive to reaction conditions (primarily pH and temperature) and can be used to generate a variety of polymo-

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lybdate species, specifically heptamolybdate, $Mo_7O_{26}^{6-}$, and octamolybdate, $Mo_8O_{26}^{4-}$. Metallic molybdenum can be obtained from the hydrogen reduction of MoO_3 at 500–1150°C. Although constituting a small portion of the commercial market, owing to its high melting point (2610°C), the base metal is used in filaments, thermovalves, in the glass industry, and for rocket nozzles.

4.2 Molybdenum in Technology (7,13)

By the late 1890s, molybdenum was used in some chemicals and dyes and as a hardener in tool steel. Since the 1930s the application of molybdenum as a preferred or essential alloying element has grown significantly. In addition to its use in metallurgy to increase strength, toughness, and corrosion resistance, molybdenum compounds are used in lubricants, pigments, flame, and smoke retardants (14), catalysts for desulfurization in the petroleum industry, and as an essential element in agriculture.

A survey of the U.S. patent literature, from 1998 through April 2000, indicates that molybdenum is mentioned in about 2% of 345,841 patents (i.e., ~7000 patents) issued since January 1, 1998. The most recent patents relate, in decreasing frequency, to semiconductor or battery devices; organic compounds, catalysts, and colloids; and, to a lesser extent, alloys or metal coatings. It is interesting to note the inverse relationship between the number of patents that discuss molybdenum and the actual commercial usage (percent by weight) of molybdenum. Although approximately 75% of molybdenum is used for steels, alloys, and superalloys, this is not the most frequently cited type of patent. Rather, it is the relatively fledgling molybdenum semiconductor and battery industries that draw the most patent attention. This inverse relationship likely reflects the maturity of the respective technologies (steel as compared to molybdenum semiconductors) rather than the relative amount of molybdenum used.

5. SOURCES OF MOLYBDENUM IN THE ENVIRONMENT

Molybdenum is broadly distributed in the environment owing to its diverse chemistry, its use by a variety of organisms, and its technological applications (15).

5.1 Natural

Molybdenum, as mineral deposits, is most often found in the environment as molybdenite (MoS_2), powellite ($CaMoO_4$), ferrimolybdite ($Fe(MoO_4)_3$), wulfenite ($PbMoO_4 \cdot 8H_2O$), ilsemanite (molybdenum oxysulfate), and jordisite (amorphous MoS_2). Molybdenum is also found naturally (geologically enriched) in hydrocarbon deposits such as asphaltenes, crude oil, and dark shales (16–18). A strong correlation has been observed between molybdenum concentration in samples from rivers in the United States and the hardness of the water (16,17).

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Such a relationship is consistent with weathering of limestone and other calcium- or magnesium-bearing minerals under acid conditions, which exposes trace elements, including molybdenum, to possible leaching (16).

The situation in aerobic and anaerobic environments differs significantly (18,19). In aerobic environments, Mo(VI) is the predominant oxidation state and oxygen coordination of the molybdenum is usual with MoO_4^{2-} often being the single dominant soluble species, for example in seawater (vide infra). In anaerobic environments, the molybdenum can be reduced to Mo(V) or Mo(IV) and sulfur coordination may become critical with binding to organosulfur ligands or MoS_2 formation occurring (18,19).

In certain aerobic environments, leaching of MoS_2 has been reported in which autotrophic thermophilic bacteria oxidize the molybdenum to Mo(VI) (20). The organism tolerates high levels of Mo(VI).

5.2 Soil

The molybdenum content in soils is usually dependent on the content of molybdenum in the parent material (15). Soil concentration varies spatially (laterally) and with depth. Average molybdenum concentration in soil is given in the range of 1–2 mg Mo/kg (i.e., 1–2 ppm) (16,21). However, values from 0.4 to 36 mg/kg have been reported. The form of molybdenum depends on the pH of the soil, the presence of other minerals (Mn, Fe, P, and S), and organic matter. Soil composition also affects molybdenum availability with high phosphate facilitating molybdenum uptake and high sulfur decreasing molybdenum uptake by plants (22,23). Reducing conditions favor the presence of Mo(IV), while oxidizing conditions favor Mo(VI), which generally is the more soluble and available oxidation state.

The speciation of molybdenum(VI) in soil is a complex function of pH and concentration. At the relatively low concentrations typical of most soils the following equations have been used to describe the speciation (24).

$$MoO_4^{2-} + H^+ \rightarrow HMoO_4^- \qquad (\log K = 4.23)$$

$$MoO_4^{2-} + 2H^+ \rightarrow H_2MoO_4 \qquad (\log K = 8.23)$$

$$MoO_4^{2-} + 3H^+ \rightarrow MoO_2(OH)(OH_2)_3^+ \qquad (\log K = 8.17)$$

$$MoO_4^{2-} + 4H^+ \rightarrow MoO_2(OH_2)_4^{2+} \qquad (\log K = 8.64)$$

The products in the first two equations have tetrahedral four-coordinate molybdenum, while the products in the last two equations are octahedral with six-coordinate *cis*-dioxo coordination of the molybdenum. An alternative formulation for ''H_2MoO_4'' involves addition of two water molecules to leave the neutral tris(oxo) tris(aquo) species, $MoO_3(H_2O)_3$ (12). Significantly, molybdate and its first protonation product are anionic while the last two products are cationic. Since the soil is a strong cation-binding medium, molybdate and the forms at

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lower acidity are not strongly absorbed by the soil and are readily available to plants. Indeed, the pH effect is so strong that ''liming'' the soil, that is, addition of CaO, can be undertaken to release molybdate by increasing the pH. The mobility of molybdate is a double-edged sword as its solubility may also allow it to be leached from the soil by excessive drainage.

In addition to the mononuclear Mo(VI) species described above, a variety of di- and polynuclear species can be formed when the molybdenum concentration is sufficiently high (12). Since the concentration of molybdenum in the environment is usually quite low, these polynuclear species are not discussed here.

Evidence has been presented that Mo(VI) can be reduced to Mo(V), detected by EPR spectroscopy, in the presence of humic acid and polysaccharide components of soil or peat (25). Evidence for further reduction to Mo(III) (25) does not appear to be unequivocal.

At the other end of the redox range from Mo(VI) and Mo(V) is Mo(0). While one might not expect Mo(0) to be found in natural systems, there is one report of its occurrence. Remarkably, $Mo(CO)_6$ appears to have been detected in landfills in British Columbia, Canada (26). The samples were taken from anaerobic landfills (atmosphere principally CO_2 and CH_4), some containing 30-year-old waste. Assuming that $Mo(CO)_6$ was generated in the landfill, the source of the molybdenum (steel in pipes, wastes) is not clear, nor is the chemical pathway generating this volatile transition metal species.

5.3 Water

Surface and groundwater have generally been found to contain molybdenum in the 1–2 μg Mo/L range. Exceptions include situations where leaching occurs near anthropogenic sources or molybdenum deposits, such as mines. In such situations, elevated pH levels can increase the concentration of molybdenum in groundwater by as much as 10,000 (2 μg/L to 20 mg/L) owing to the formation of the highly soluble and mobile molybdate ion MoO_4^{2-}.

Molybdenum is the transition metal found in highest abundance in seawater. The average concentration of molybdenum in the oceans is 10 μg/L (3). At the alkaline pH 8.3 of the oceans, the molybdenum in seawater is essentially in the form of molybdate ion. However, molybdenum is also found in colloidal or suspended particles (16).

In anaerobic waters, especially those high in sulfide, the situation can be quite different. First, molybdate is no longer the only form present and may not be present at all depending on the pH and effective sulfide ion concentration. Substitution of oxide by sulfide may occur, which can, in very high sulfide environments, proceed all the way to the formation of tetrathiomolybdate (27,28).

$$MoO_4^{2-} \rightarrow MoO_3S^{2-} \rightarrow MoO_2S_2^{2-} \rightarrow MoOS_3^{2-} \rightarrow MoS_4^{2-}$$

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In addition, once thiomolybdates begin to form, the possibility of internal redox reactions arises (4,29,30). Such reactions can lead to the formation of reduced molybdenum [Mo(V) or Mo(IV)] and/or the precipitation of MoS_2 or MoS_3 (18).

5.4 Air

Urban areas tend to have higher concentrations of molybdenum in the air (0.01–0.03 μg/m^3) compared to rural areas. As is the case for the concentration of molybdenum in soil and water, higher molybdenum concentrations are found in the air near airborne anthropogenic sources. Industrial coal-burning facilities are one such source. The concentration of molybdenum in fly ash has been found to lie between 7 and 160 mg/kg (21).

5.5 Diet

Although we move over ground, breathe air, and drink water that contains molybdenum, the major source of molybdenum for most people is their diet. In the absence of exposure to sources of high molybdenum concentration, such as contaminated water, the average daily intake of molybdenum is 0.1–0.5 mg. Legumes, leafy vegetables, and cauliflower contain relatively high concentrations of molybdenum compared with other foodstuffs. Barceloux (21) reports the recommended daily allowance (RDA) of molybdenum for adults as 0.15–0.6 mg/day. The World Health Organization estimates the minimum daily requirement to be 2 μg of molybdenum per kilogram of body weight.

6. UPTAKE AND TRANSFORMATION BY BIOLOGICAL SYSTEMS

Generally, the form of molybdenum that is taken up by biological systems is molybdate, MoO_4^{2-}, which is a colorless, highly water-soluble dianion in which molybdenum is in its maximum VI oxidation state. The molybdate ion, as shown in Figure 2, strongly resembles other oxy anions, including sulfate, tungstate, and selenate. The need for biological systems to selectively bind to and hence acquire molybdenum requires the ability to discriminate between the naturally occurring oxyanions. The task is difficult for both tungsten and sulfate. For molybdate versus tungstate, the difference in size between the anions is minimal, and hence, separation by selective binding is forbidding. Fortunately, in most cases the concentration of tungstate is quite low and hence this anion does not present a competitive problem. For example, in seawater, the concentration of WO_4^{2-} is two orders of magnitude lower than that of MoO_4^{2-} (3). The differentiation between molybdate and sulfate is troublesome since the concentration of

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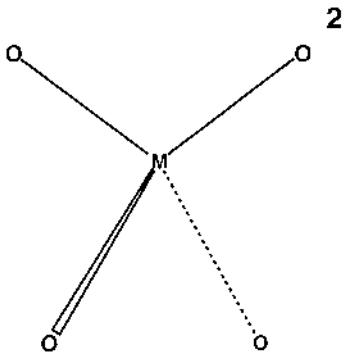

Oxy Anion	M	M-O Distance (Å)
Molybdate	Mo	1.75
Tungstate	W	1.76
Sulfate	S	1.47

FIGURE 2 The tetrahedral structure of MO_4^{2-} ions illustrating the similarities and different bond lengths of the various oxy anions (32,33).

sulfate in seawater, at 885 ppm, is many orders of magnitude higher that that of molybdate (3).

Specific molybdate-binding proteins have been identified by genetic and biochemical approaches (31). Crystallographic analysis indicates that MoO_4^{2-} retains its tetrahedral geometry and binds very specifically in a rigid hydrogen-bonding network of the protein (32,33). The size and anionic nature of the molybdate ion clearly play a key role in the binding of the dianion by the protein. While tungstate, of similar size, binds readily, the far smaller sulfate ion (Fig. 2) does not bind strongly, allowing effective biological discrimination.

The competitive situation is significantly different at the deep-sea hydrothermal vents where molybdenum and tungsten are both nominally available. However, in the vent environment, the high sulfide concentration may precipitate the molybdenum sulfides (MoS_2 and/or MoS_3). Tungsten sulfides do not readily form under these conditions and, in fact, tungsten remains in the vent waters (34). Microorganisms living at the vent sites, in particular hyperthermophilic bacteria and archaea, make use of the available tungsten in enzymes of their central metabolism (35–38). Whether this preference for tungsten over molybdenum is a result of the absence of molybdenum or due to the possibly greater suitability of tungsten enzymes in the higher temperature systems, remains a subject of conjecture (39).

7. ESSENTIALITY, ENZYMES, AND COFACTORS

Molybdenum is essential for most forms of life on earth and, as far as we know, all plants and animals require molybdenum for growth and reproduction. In addi-

tion, most microorganisms that have been studied show a role for molybdenum in their metabolism. The essentiality of molybdenum derives from its presence at the active site of metalloenzymes that execute redox chemistry crucial to the primary metabolism of the cell. The enzymes generally make use of the oxidation-state variability of molybdenum and most work between the Mo(VI) and Mo(IV) states of the element. A list of molybdenum enzymes, their sources, and representative reaction(s) that they catalyze is given in Table 1.

The molybdenum enzymes fall into two distinct categories, which are distinguished by the nature of their respective sites, usually referred to as cofactors. The first category has only one member, nitrogenase, which contains the iron molybdenum cofactor (FeMoco) shown in Figure 3 (40–42). All other enzymes fall into the second category and contain variants of the molybdenum cofactor (Moco) shown in Figure 4 (38,43,44). Many of the molybdenum enzymes have been investigated crystallographically and we now have detailed structural knowledge of a number of the active sites (45–47).

The structure of the nitrogenase iron molybdenum cofactor (Fig. 3), reveals an Fe_7MoS_9 core cluster, which is bound by cysteine and histidine ligands from the protein (at the apical Mo and Fe atoms, respectively) and chelated by the unusual homocitrate ligand at the molybdenum site (48–50). The details of the biosynthesis of the FeMoco have been extensively studied and involve an array of proteins specified by the Nif genes (51–57).

All molybdenum enzymes other than nitrogenase depend on the presence of the molybdenum cofactor, whose family of structures is shown in Figure 4. The molybdenum in this ''cofactor'' is bound by one or two pyranopterin dithiolene ligands as well as by a variety of other groups on the molybdenum center, including oxo, hydroxo, aquo, sulfido, and sulhydryl. In addition, the amino acid side chains of serine, cysteine, or selenocysteine may be present. The particular ligands bound to molybdenum are different for different classes of molybdenum enzymes (38,45). The biosynthesis of the molybdenum cofactor has been intensely studied and involves the expression of genes of the Moa or Cnx gene families (58–60). There is no commonality between the biosynthetic pathways of the iron-molybdenum cofactor and the molybdenum cofactor. This lack of overlap is not surprising in light of their remarkably different chemical structures (Figs. 3 and 4)

We first discuss enzymes active in microorganisms and then proceed to plant and animal molybdenum enzyme systems.

7.1 Microorganisms

Bacteria and archaea play central roles in the biogeochemical cycling of many elements. In several of these elemental cycles, molybdenum plays a key role through its requirement in the metalloenzymes of one or more crucial steps. The

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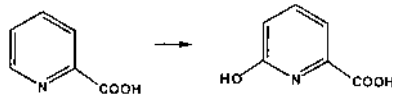
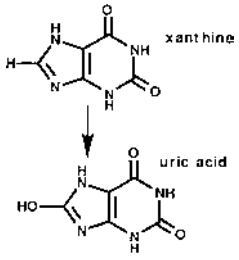
TABLE 1 Molybdenum Enzymes and Representative Reactions Catalyzed (38)

Enzymes	Source	Representative conversions
Nitrogen fixation (39–42)		
Nitrogenase (iron molybdenum and iron proteins)	Bacteria, archaea	$N_2 + 16\ H_2O + 8e^- + 16\ MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16\ Pi$
Oxidations of one-ring *N*-heterocycles		
Isonicotinic acid hydroxylase	Bacteria	
Nicotinic acid hydroxylase	Bacteria	
Nicotine hydroxylase	Bacteria	
Picolinic acid dehydrogenase	Bacteria	
Pyrimidine oxidase	Mammalian liver	
Oxidations of two-ring *N*-heterocycles		
Isoquinoline oxidoreductase	Bacteria	
Quinaldic acid 4-oxidoreductase	Bacteria	
Quinaldine 4-oxidoreductase	Bacteria	
Quinoline oxidoreductase	Bacteria	
Xanthine dehydrogenase	Bacteria, mammalian, fowl	
Xanthine oxidase	Bacteria, mammalian, fowl	

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Oxidations and reductions of oxygen-bearing carbon centers		
Aldehyde dehydrogenase	Mammalian liver	$RCHO \rightarrow RCOOH$
Carbon monoxide oxidoreductase	Bacteria	$CO \rightarrow CO_2$
Formate dehydrogenase	Bacteria	$HCOOH \rightarrow CO_2$
Formylmethanofuran dehydrogenase	Bacteria	
2-Furoyl-coenzyme A dehydrogenase	Bacteria	
Pyridoxal oxidase	Bacteria	
Oxidations and reductions of As, Cl, N, and S centers		
Arsenite oxidase	Bacteria, algae	$AsO_3^{3-} \rightarrow AsO_4^{3-}$
Biotin sulfoxide reductase	Bacteria	
DMSO reductase	Bacteria	$(CH_3)_2SO \rightarrow (CH_3)_2S$
Nitrate reductase (assimilatory)	Plant, fungal, bacteria	
Nitrate reductase (dissimilatory)	Bacteria	$NO_3^- \rightarrow NO_2^-$
Nitrate reductase (periplasmic)	Bacteria	
Nitrite oxidase	Bacteria	$NO_2^- \rightarrow NO_3^-$
Polysulfide reductase	Bacteria	
Sulfite oxidase	Mammalian, liver, bacteria	$SO_3^{2-} \rightarrow SO_4^{2-}$
Selenate reductase	Bacteria	$SeO_3^{2-} \rightarrow SeO_4^{2-}$
Tetrathionite reductase	Bacteria	$S_4O_6^{2-} \rightarrow 2\ S_2O_3^{2-}$
Trimethylamine-*N*-oxide reductase	Bacteria	$(CH_3)_3NO \rightarrow (CH_3)_3N$
Nonredox process		
Pyrogallol transhydroxylase[a]	Bacteria	

[a] Pyrogallol trashydroxylase does catalyze a carbon-based redox process, but is classified as a nonredox enzyme since there is no net electron movement except between substrate molecules.

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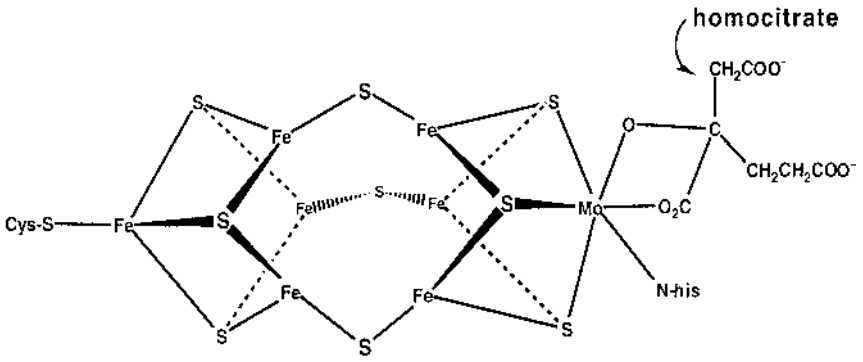

FIGURE 3 The structure of the iron-molybdenum cofactor of nitrogenase, which is the presumed site at which N_2 is bound and reduced in the iron molybdenum protein of nitrogenase (46).

cycles of sulfur and, especially, nitrogen make considerable use of molybdenum enzymes, while the cycles of selenium, arsenic, and anaerobic parts of the carbon cycle also use molybdenum enzymes.

The role of molybdenum in the nitrogen cycle is striking (61). A simplified version of that cycle is shown in Figure 5. The input of inorganic nitrogen occurs at two points. First, nitrogen fixation, an activity associated with a limited number

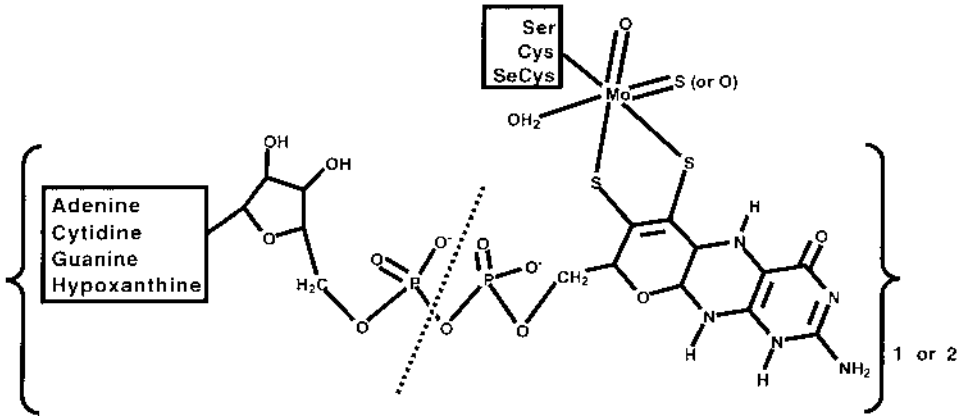

FIGURE 4 The structural family of the molybdenum cofactor, variants of which are present at all molybdenum enzymes except nitrogenase (46,97).

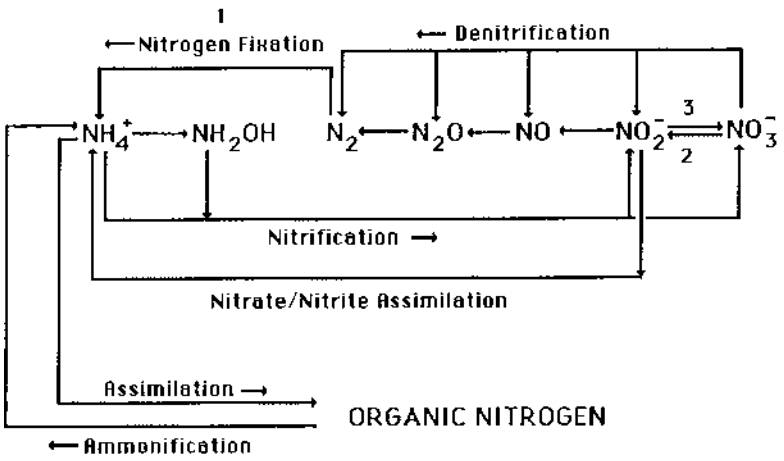

FIGURE 5 The biogeochemical cycle of nitrogen illustrating the importance of molybdenum enzymes in this crucial process on planet earth. The molybdenum enzymes involved are: (1) nitrogenase; (2) nitrate reductase; and (3) nitrite oxidase (61–70).

of bacteria and archaea, converts the abundant N_2 of the atmosphere to the required reduced form of nitrogen, i.e., the ammonia level of reduction (62,63). Once at the ammonia level, the nitrogen is said to be ''fixed.'' The nitrogen is then available to enzymes for biosynthesis of myriad nitrogen-containing molecules in the cell including proteins (amino acids), nucleic acids (purines and pyrimidines), porphyrins, and coenzymes (NADH, flavins, etc.). The second input of nitrogen comes through nitrate reductase, which is found in both microorganisms and plants. Nitrate reductase catalyzes the conversion of nitrate to nitrite, the first step in bringing oxidized nitrogen (whose most abundant aqueous form is nitrate) to a reduced level. Plants and many microorganisms then reduce the nitrite to ammonia in a single step, completing the nitrate assimilation pathway (64,65). Other (micro)organisms are involved in the pathways of denitrification and nitrification, where molybdenum enzymes play key roles in respiratory (dissimilatory) nitrate reduction and nitrite oxidation, respectively (66–70).

7.1.1 Nitrogen Fixation

Nitrogenase is responsible for the reduction of molecular nitrogen to the ammonia level. The enzyme is present only in prokaryotic organisms (bacteria and archaea) but some of these live symbiotically with plants and represent the only input of fixed nitrogen to these systems (63).

The active site of nitrogenase consists of the iron-molybdenum cofactor (FeMoco) and surrounding protein. The site is part of a complex protein system that hydrolyzes ATP (adenosine triphosphate), feeds redox equivalents through an intricate set of redox carriers, and evolves H_2 in addition to reducing N_2. Whether the molybdenum directly or indirectly interacts with the dinitrogen remains a subject of conjecture. The mechanism of the reaction at this active site is not yet understood at the molecular level (40–42).

The need for molybdenum in nitrogen fixation was first established by Bortels (71) and led to fertilization of crops with molybdenum salts in areas where there was evidence of molybdenum insufficiency. Bortels (71) also found that, in some cases, vanadium will stimulate nitrogen fixation. Much subsequent work has shown that there are two alternative nitrogen fixation systems that resemble the molybdenum system chemically, biochemically, and genetically, but which contain vanadium or iron in place of molybdenum (72,73). Under most circumstances, the alternative vanadium and the all-iron nitrogenases are less efficient at nitrogen fixation than the molybdenum enzyme. No known organism possesses only the alternative version. Indeed, organisms do not express (synthesize) the alternate version when there is sufficient molybdenum (i.e., molybdenum represses the synthesis of proteins of the alternative system). Clearly, molybdenum appears to be the preferred element for nitrogen fixation, although the process is sufficiently important that some organisms invest in a backup, albeit less efficient, system. Many nitrogen-fixing organisms do not appear to possess the alternative system.

Some of the nitrogen-fixing bacteria live symbiotically with plants (74,75). These include simple plant systems, such as the anabaena-azolla symbiosis, and higher plants, such as the rhizobium/bradyrhizobium symbioses with legumes and the frankia involvement with alder. The plant provides the reducing equivalents (ultimately from photosynthesis) for nitrogen fixation to the bacteria and the bacteria supply fixed nitrogen to the plant, thereby lessening or eliminating the need for nitrogenous fertilizer in agricultural situations.

7.1.2 Nitrate Reductase/Nitrite Oxidase

A second key role for molybdenum in the nitrogen cycle is in the reduction of nitrate to nitrite catalyzed by the enzyme nitrate reductase (64). The fate of the nitrite product differs in different organisms and, even for the same organism, in different circumstances. For example, in nitrate assimilation, the nitrite produced by nitrate reductase is directly reduced to ammonia using the enzyme nitrite reductase, whereupon it is incorporated into cellular nitrogen molecules. In contrast, in denitrification the nitrite is reduced first to NO, and subsequently to N_2O and N_2 (Fig. 5). Since N_2O is both a greenhouse gas and an ozone-depleting gas, there is considerable concern about the release of this gas by denitrifying bacteria in agricultural situations.

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In nitrification, where ammonia is oxidized to nitrate, the terminal step, the oxidation of nitrite to nitrate, is catalyzed by the molybdenum enzyme nitrite oxidase (76). Indeed when fertilization with reduced nitrogen compounds is used, the nitrification process is essential since most plants cannot directly use reduced nitrogen compounds and require its oxidation to nitrate before they can take it in and use it effectively in their metabolism.

7.1.3 Sulfur Metabolism

As shown in Table 1, the enzymes sulfite oxidase (39,43,60,77,78), tetrathionate reductase (79,80), and polysulfide reductase (81,82) are molybdenum enzymes that participate in the inorganic sulfur cycle. Individual reactions in the cycle are crucial in manipulating the many sulfur species that occur in a variety of aerobic and anaerobic environments (83–86). The only volatile *inorganic* sulfur species is H_2S, which is usually produced by sulfate-reducing bacteria under anaerobic conditions. The presence of H_2S in the atmosphere, while short-lived, is immediately noticeable through its strong odor and extreme toxicity. Interestingly, high concentrations of molybdate inhibit the formation of H_2S by sulfate-reducing bacteria, presumably through inhibition of ATP sulfurylase by molybdate (87).

Dimethyl sulfoxide (DMSO) is found extensively in the marine environment (88). The molybdenum enzyme DMSO reductase (89–93) catalyzes the reduction of dimethylsulfoxide (DMSO) to dimethylsulfide (DMS), thereby participating in a potentially important organic reaction of the sulfur cycle (89). DMS is the major volatile *organic* form of sulfur produced in the environment and is formed by the decomposition of dimethylsulfoniopropionate (DMSP), a component of algae (94). DMS is oxidizable photochemically and biologically to the sulfoxide and many marine bacteria possess DMSO reductase and form (or reform) DMS (94). When DMS enters the atmosphere it is rather rapidly oxidized to methane sulfonic acid salts and the resultant particulate matter can serve as cloud condensation nuclei. Indeed, such particulate matter is required for the formation of clouds, and these sulfur compounds provide one of the few stimuli that lead to cloud formation over open oceans, without the need for land-derived particulates (dust). The resultant cloud cover increases the earth's albedo and may play a role in controlling the temperature of the planet (94).

7.1.4 Other Microbial Enzymes

Microorganisms use a variety of molybdenum enzymes in other reactions tabulated in Table 1. These organisms play critical roles in particular environmental niches where the reaction in question is important to the organism's ability to exploit or survive in that niche. The oxidation of arsenite to arsenate (95), selenate reduction to selenite (96), reduction of trimethylamine *N*-oxide to trimethyl amine (97) (responsible for the smell of rotten fish), and the oxidation of a wide variety

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of *N*-hetercyclic molecules (38,98) are catalyzed by various molybdenum enzymes (Table 1).

7.2 Plants

7.2.1 Enzymes in Plants

The importance of nitrate reductase for plants has already been alluded to in the discussion of bacteria. All plants that do not harbor nitrogen-fixing symbiotic or associated bacteria must obtain their nitrogen from nitrate in the soil or water. The nitrate reductase enzyme that performs the key first step in this reductive transformation (nitrate to nitrite) has therefore been highly studied (99).

Additional critical enzymes found in plants catalyze the critical final steps in the synthesis of plant hormones and growth factors, including abscissic acid (ABA) and indoleacetic acid (IAA). ABA (60,100) is a key plant hormone responsible for the timing of leaf shedding (abscission) among other processes. The enzyme ABA aldehyde oxidase catalyzes the oxidation of the abscissic aldehyde to the carboxylic acid. IAA is an auxin whose synthesis involves an aldehyde oxidase activity, namely, indole-3-acetaldehyde oxidase (60). These molybdenum enzymes, along with xanthine dehydrogenase and other aldehyde oxidases, have now been recognized as key enzymes in plant metabolism (60,100).

7.2.2 Molybdenum in Agriculture and Forests

Long before the recognition (vide supra) of the specific roles of molybdenum in microorganisms, plants, and animals, fertilization with molybdenum was employed in particular environments and on particular crops. The subject has been thoroughly reviewed (23). The micronutrient aspect of the molybdenum requirement means that truly miniscule amounts of molybdenum need be added in many cases. Sometimes it is sufficient to merely coat the seed with a dilute solution of a molybdenum salt. Alternatively, foliar spraying is an effective technique with levels of 100–200 g/hectare being quite effective. Typically application solutions contain $Na_2MoO_4{\cdot}2H_2O$ or $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, while seed coatings may also contain MoO_3 (101,102).

Molybdenum deficiency in legumes was first noted in subterranean clover (*Trifolium subterraneum*) and later in soybeans (*Glycine* max). Deficiencies are usually found in acidic soils where molybdenum is less available (vide supra). While lime alone can produce yield increases, the combination of lime and molybdenum salts can have an even greater effect (23).

The situation in forests is similar to that in agricultural soils (103,104). In particular, liming increases nitrate utilization, which is dependent on the availability and use of molybdenum in the enzyme nitrate reductase.

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7.3 Animals (Including Humans)

7.3.1 Enzymes in Animals

The three main molybdenum enzyme classes known to be involved in animal are xanthine oxidase and dehydrogenase, aldehyde oxidase, and sulfite oxidase. The inability to synthesize the molybdenum cofactor, common to all molybdenum enzymes except nitrogenase, leads to combined molybdenum enzyme deficiency. This condition is often fatal, largely owing to the absence of sulfite oxidase activity (77,105). Such defects have recently been detected prenatally (106,107). We first discuss xanthine oxidase, the most studied of molybdenum enzymes.

As shown in Table 1 (38,43), xanthine oxidase or dehydrogenase catalyzes the conversion of xanthine to uric acid, a key step in purine catabolism, which in primates, including humans, is the final step in this pathway. The disease gout is due to the accumulation of excess uric acid (or sodium urate) that crystallizes in undesirable locations such as the kidneys (stones) or the joint of the big toe (108). Gout is often treated by diet, but intractable cases are treated with drugs such as allopurinol, which bind to xanthine oxidase at its molybdenum active site, inhibiting the binding of the substrate and hence decreasing production of uric acid.

There is a second reason why xanthine oxidase has been extensively studied (109–112). The enzymatic reaction uses O_2 as the oxidant and can produce either superoxide or hydrogen peroxide as the product. If superoxide dismutase and/or catalase cannot disproportionate these entities effectively, then oxidative damage can ensue. The problem is exacerbated during ischemic episodes, such as heart attacks, where flow of blood to the heart is temporarily impeded by a blocked blood vessel. Upon reestablishing the blood flow, called reperfusion, a burst of superoxide (O^-) or hydrogen peroxide (H_2O_2) can be generated by reaction of incoming oxygen with reduced xanthine oxidase (which can store up to 12 redox equivalents per dimeric enzyme unit). The rapid production of O_2^- and H_2O_2 can lead to severe oxidative damage. The conversion of xanthine dehydrogenase to xanthine oxidase involves parts of the enzyme other than the molybdenum site (38,43,109–112). Possible intervention involves administration of inhibitors specific to the molybdenum site of xanthine oxidase/dehydrogenase as early as possible in the ischemic episode.

Aldehyde oxidase resembles xanthine oxidase in many of its properties catalyzing oxidation of aldehydes to carboxylic acids and hydroxylation of a variety of pyrimidines. However, its exact importance in human metabolism remains uncertain.

Perhaps the most critical of all molybdenum enzymes in animals is sulfite oxidase (77,78,105). This enzyme catalyzes the conversion of the toxic sulfite ion to the generally benign sulfate ion. The absence of this enzyme may be due

to mutations in the protein or in the biosynthesis of its molybdenum cofactor. In either case, the results are disastrous. Children born with sulfite oxidase deficiency are severely compromised and generally do not survive. Thus, the essentially of molybdenum for human and animal nutrition is most clearly seen in the sulfite oxidase deficiency of certain animals.

7.3.2 Other Beneficial Aspects

In addition to the low toxicity and essentiality of molybdenum already discussed, additional beneficial aspects are known for various molybdenum compounds. Molybdate must be added during prolonged total parenteral nutrition to avoid amino acid intolerance (113). Studies on rodent teeth suggest beneficial effects of molybdenum on tooth enamel (114,115). Molybdenum-based dental compositions with anticaries properties have been patented (116) and reviewed (114,115). An increased level of molybdenum in the diet of rats appears to be correlated with increase fluoride in bone (117). Optimum levels of molybdenum appear to enhance the immune response of Merino lambs toward nematode parasites (118).

Researchers have recently disclosed (119) the use of tetrathiomolybdate (MoS_4^{2-}) to treat, among other things, aberrant vascularization. Since vascularization is also a prerequisite for metastatic cancer, the use of tetrathiomolybdate (presumably as a copper antagonist, see below) has been proposed as a tumor growth inhibitor (119). Metastable radioisotopes that decay to molybdenum have long been used in nuclear medicine (120,121). The clinical success of such systems is due both to the properties of the parent isotope, ^{99m}Tc, and to the low toxicity of the daughter product, a molybdenum species.

8. TOXICOLOGY

8.1 Toxicity

Generally, molybdenum is considered to be of relatively low toxicity in both plants and animals, including humans. Nevertheless, factors such as age, species, dietary habits, and relative amount of copper, sulfur, and molybdenum in the diet are factors to be considered. One important caveat to low molybdenum toxicity is the copper-molybdenum antagonism (vide infra) experienced most severely by ruminant animals.

Toxicological information (15,21,122), including acute, oral, inhalation, and dermal (LD_{50} and LC_{50}), has been obtained for some of the industrially important compounds, specifically pure MoO_3, technical-grade MoO_3, $(NH_4)_2Mo_2O_7$, $Na_2MoO_4{\cdot}H_2O$, and MoS_2. These molybdenum species are not considered ''toxic'' substances as defined by the Occupational Safety and Health Administration (OSHA) (123). LD_{50} values for all of the compounds are greater than 2000 mg/kg and LC_{50} values are approximately 2 mg/L or higher. At quite high levels

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(millimolar), ammonium salts and MoO_3 can display a modest but statistically significant level of genotoxicity (124).

Quantitative analysis of the toxicological effects of molybdenum on humans is less well established. A tolerable daily intake of 0.009 mg Mo/kg/day for humans was obtained from a risk analysis derived from a survey of the absorption, excretion, uptake, and physiological and toxic effects of molybdenum in humans and animals (122). This tolerable daily intake is greater than the ''adequate'' daily dosage that average adolescents or adults receive in an average diet. In a 1979 study by the EPA (21), no adverse effects were observed for students receiving 8 μg/kg/day. Barceloux (21) gives an account of Russian literature from the early 1960s in which hyperuricemia (gout) and arthralgias (joint inflammation) were observed in Armenians who consumed an estimated 10–15 mg of molybdenum per day. The statistical significance of this study is unclear (21).

8.2 Absorption, Distribution, and Elimination in Animals

Soluble molybdenum complexes are readily absorbed by the gastrointestinal tract with highest levels of molybdenum appearing in the kidney and liver (21). However, net retention of molybdenum in the body appears small as most is excreted through the kidneys. High levels of copper and sulfur in the diet enhance the elimination of molybdenum from the body. This is reported to occur by the blocking a protein carrier and thereby inhibiting reabsorption in the kidneys (21). Insoluble molybdenum compounds such as MoS_2 are generally unavailable to living systems and show limited chemical activity. A similar fate is postulated for the end products associated with the molybdenum-copper antagonism, that is, the generation of insoluble ''$CuMoS_{3 or 4}$'' materials.

8.3 Copper Molybdenum Antagonism

The elements copper and molybdenum have an interesting and long-studied relationship. As mentioned above, copper and molybdenum are often found associated in porphyry mineral deposits. Moreover, chemical studies have revealed an enormous number and variety of copper molybdenum species, especially when sulfide is present as a potential bridging ligand (125,126).

The most spectacular manifestation of Cu-Mo interaction occurs in biological systems. Specifically, it is well established that ruminant animals grazing on soils high in molybdenum, especially those also high in sulfur, become severely ill and often die. The mechanism of this effect is now largely understood, at least at the chemical level (21,126–134). In the anaerobic rumen of the grazing animals, e.g., sheep and cows, sulfate can be reduced to sulfide by sulfate-reducing bacteria. The sulfide reacts with MoO_4^{2-} to produce trithiomolybdate and tetrathiomolybdate, $MoOS_3^{2-}$ and MoS_4^{2-}, respectively, which complex strongly with copper and make it unavailable to the organism. It is thus copper deficiency that

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sickens and kills ruminant organisms when excess molybdenum is available. The equations that describe the chemical conversions are:

$SO_4^{2-} \rightarrow S^{2-}$ (H_2S in various states of ionization depending on the pH)

$S^{2-} + MoO_4^{2-} \rightarrow MoOS_3^{2-}, MoS_4^{2-}$

$MoOS_3^{2-}, MoS_4^{2-} + Cu^{+,2+} \rightarrow$ ''$CuMoS_{3or4}$'' (unavailable Cu/Mo/S compound)

The copper-molybdenum antagonism is dramatically illustrated by contrasting molybdenum tolerance between ruminants (such as goats, sheep, camels, and cows) and other animals studied (such as horses or pigs). Ruminants are adversely affected by feed containing 2–30 ppm of soluble molybdenum, while horses and pigs can tolerate greater than 1000 ppm of molybdenum (131).

The strong affinity of tetrathiomolybdate, MoS_4^{2-}, for copper has also been exploited clinically. Specifially, tetrathiomolybdate has been used in the chelation of copper in the treatment of Wilson's disease (134–137) wherein copper accumulation causes severe damage to the liver and other organs. Moreover, tetrathiomolybdate is also in Phase I clinical trials as an antitumor agent (138). In this latter application, the tetrathiomolybdate apparently ties up the copper to a sufficient extent to inhibit angiogenesis, for which copper is essential.

9. ANALYTICAL CONSIDERATIONS

A number of chemical and instrumental options for the qualitative and quantitative analysis of molybdenum exist due primarily to the large number of accessible oxidation states and the diverse chemical reactivity of molybdenum. The analytical method employed depends upon the available sample size, concentration in the matrix, and nature of the matrix that contains the molybdenum (139–141).

Historically, molybdenum content in ores has been accurately determined gravimetrically as lead molybdate by precipitation and a variety of spectrophotometric methods have been used for molybdenum determination. More recently, X-ray fluorescence spectrometry, inductively coupled argon plasma–atomic emission spectroscopy (ICAP or ICAP-AES), and isotope dilution inductively coupled plasma–mass spectroscopy (ID-FI-ICP-MS) have been employed (142). Mass spectroscopic analysis can make use of the rich isotope distribution that naturally occurs in molybdenum to readily determine the number of molybdenum centers in a complex. GC-MS was used (26) to identify $Mo(CO)_6$ in landfill gases. Extended X-ray absorption fine structure. (EXAFS) has been used to identify the connectivity and coordination environment about molybdenum in biological systems (143). Reasonable structure elucidation is possible when appropriate model compounds are employed for calibration (143,144).

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10. CONCLUSION

Molybdenum compounds are ubiquitous in the environment. Their natural occurrence is augmented by anthropogenic inputs through technology and agriculture. Most often the biological effect of molybdenum is benign or beneficial. Many enzymes that require molybdenum are essential in the physiology and biochemistry of specific organisms. Moreover, the reactions catalyzed are crucial, not only for the metabolism of individual organisms, but also in the global biogeochemical cycling of elements such as nitrogen and sulfur. As with any substance, there are toxicological concerns that for molybdenum come into play only at very high concentrations. However, for certain organisms, under specific conditions, a strong interaction with copper has been established. This interaction can be either antagonistic or beneficial. We hope that this chapter has given a balanced picture of the complex environmental chemistry of molybdenum, a useful and essential element.

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15

Microbial Resistance Mechanisms for Heavy Metals and Metalloids

Mallika Ghosh and Barry P. Rosen
Wayne State University School of Medicine, Detroit, Michigan

1. INTRODUCTION

In this chapter the mechanisms of resistance to ions of the heavy metals zinc, cadmium, lead, copper, arsenic, and antimony in bacteria will be described. In addition, the pathways of arsenical resistance in the prokaryote *Saccharomyces cerevisiae* will be discussed. Although often grouped as *heavy metals*, the ions of these metals are better characterized as *soft metal ions*, which are those with high polarizing power (a large ratio of ionic charge to the radius of the ion), in contrast to the *hard metal ions* of Groups I and II such as Na^+ and Ca^{2+}. This distinction between hard and soft metals is important biologically. When considering how these metals interact with biological molecules such as proteins, hard metals most frequently bind to proteins weakly through ionic interactions with functional groups such as carboxylates of glutamate or aspartate residues. In contrast, soft metals form much stronger, nearly covalent, bonds with functional groups such as the thiolates of cysteine residues and the imidazolium nitrogens of histidine residues. These strong interactions with proteins account for much of the biological toxicity of soft metals.

All of these ions are toxic in excess, and bacterial metal resistances probably arose early in evolution owing to widespread geochemical sources of metals.

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Resistance genes to inorganic salts of soft metals, including arsenic, antimony, lead, cadmium, copper, nickel, zinc, bismuth, and mercury, are found both on extrachromosomal plasmids and in chromosomes of bacteria, archaea, and eukaryotes. For metals such as copper and zinc, which are required in low amounts but toxic in high amounts, the efflux systems are components of the homeostatic mechanisms that maintain intracellular concentrations at optimal levels. Recent reviews have been written on bacterial metal resistances (1–3), which allows this chapter to focus on a few specific mechanisms.

2. P-TYPE ATPases FOR MONOVALENT SOFT METALS: Cu(I) AND Ag(I) PUMPS

P-type ATPases comprise a superfamily of enzymes that transport cations (4). Some pump cations into cells, some into organelles, others pump cations out of cells, and yet others are cation exchangers: it is not possible to predict the directionality of transport from inspection of the sequence. Every member of the superfamily has conserved sequences that include an ATP-binding domain, an aspartate residue that is the site of phosphoenzyme formation, and a phosphatase domain. There are at least five branches to the family (5). One branch includes the hard metal cation-translocating ATPases, and another the soft metal cation-translocating ATPases (Fig. 1). Both branches can be further subdivided into branches that comprise monovalent and divalent pumps (1). The first bacterial P-type ATPase identified was the Kdp K^+-translocating ATPase, a pump for uptake of monovalent ions of the hard metal potassium (6). Other hard metal bacterial P-type ATPases include the MgtA and MgtB pumps for uptake of divalent ions of the soft metal magnesium (7).

The soft metal ATPases were identified more recently. They have common features not present in the hard-metal ATPases, in particular characteristic cysteine- or histidine-rich metal-binding motifs at the N-terminus and a Cys-Pro-Cys (or His) sequence in the sixth membrane-spanning segment (Fig. 2). The presence of the CPC(H) sequence has led to the designation *CPx-type ATPases* (8). However, given that the substrates of this class of enzymes are all soft metal ions, *soft metal P-type ATPases* seems an appropriate designation. The most widely recognized members of the soft metal P-type ATPases are the human copper pumps ATP7A (or MNK) (9) and ATP7B (or WIND) (10). Inheritable mutations in the genes for these pumps produce Menkes or Wilson disease, respectively. Both MNK and WND have six CXXC metal-binding sequences in their N-termini and a CPC sequence in a membrane-spanning segment (11). Peptides corresponding to the N-terminal metal binding domains have been shown to bind a variety of metal ions, including Cu(II), Cu(I), Ag(I), and Zn(II) (12,13). The function of the N-terminal metal binding domains is not known. Possibilities include: (1)

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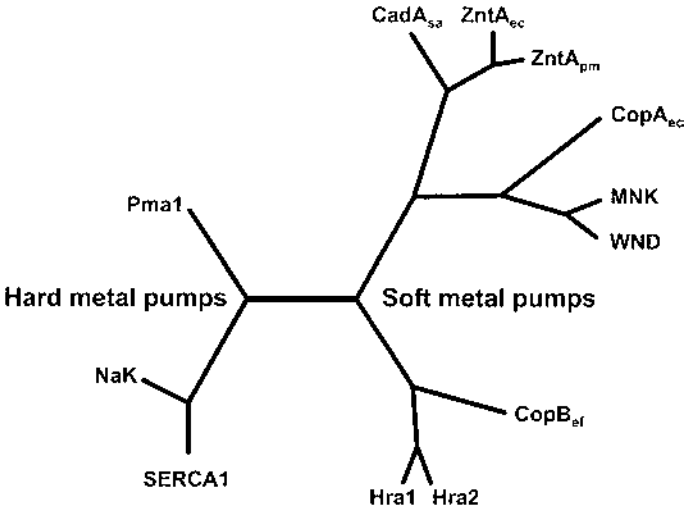

Figure 1 The family of soft metal ion-translocating P-type ATPases. This family is growing rapidly, so only a few representative ones are shown to illustrate the nature of the lineage. The dendograms were made using the CLUSTAL4 algorithm (84) with DNASIS software from Hitachi Software Engineering Co., Ltd. On the left is the hard metal ion ATPase subfamily, represented by Pmal, the fungal H^+-translocation ATPase (85), NaK, the mammalian Na,K- ATPase (86), and SERCA1, the endoplasmic reticulum calcium pump (87). On the right is the soft metal ion ATPase subfamily. Two major branches are shown, those with histidine-rich N-termini such as the Cu(I)/Ag(I)-translocaing CopB ATPase of *E. hirae* (17) and the histine-rich proteins Hra1 and Hra2 of unknown origin (20). The other branch has two groups of proteins, the monovalent copper pumps, including the human enzymes MNK (9) and WND (10) associated with Menkes and Wilson diseases, and the *E. coli* Cu(I)-translocating CopA ATPase (25). The second grouping are the divalent soft metal ion pumps for the metals Zn(II), Pb(II), and Cd(II), including the *S. aureaus* plasmid pI258 CadA ATPase (26), the ZntA pumps of *E. coli* (30,31), and *Proteus mirabilis* (88).

the metal binding domains serve as sensors to activate the pump; (2) they serve as the initial binding site, transferring the metal ion to the translocation domain, which probably includes the CPC motif; (3) in eukaryotes they may be involved in trafficking of the pump to the appropriate membrane. In support of the last possibility, the metal-binding domains of either the Menkes or Wilson proteins do not appear to be essential for copper transport, but their removal appears to

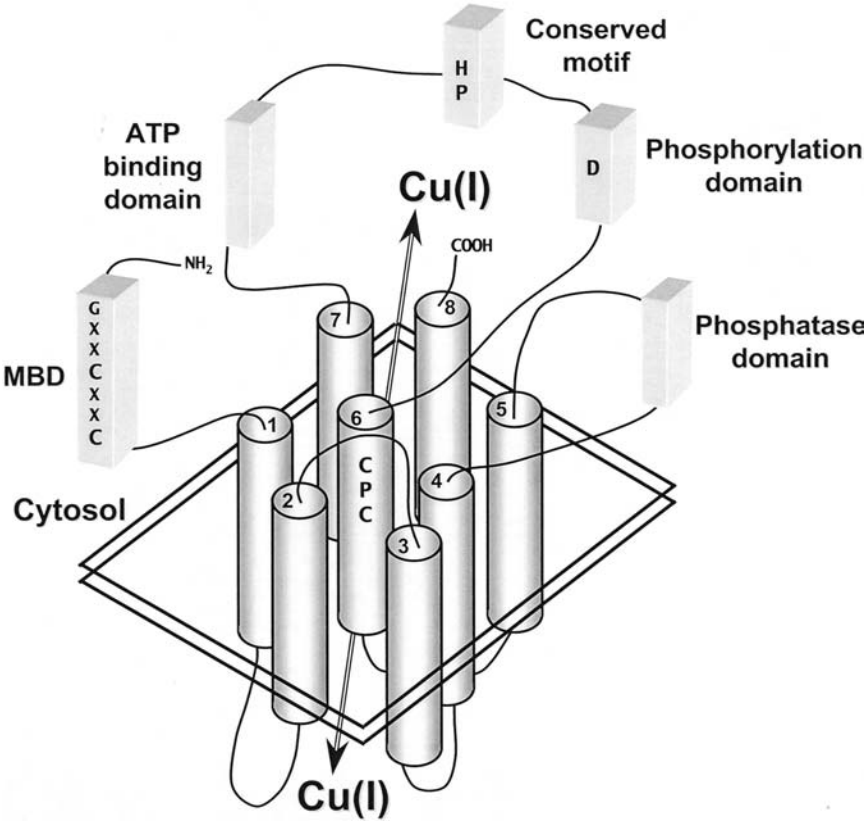

FIGURE 2 Generic model of a soft metal ion-translocating P-type ATPase. This model of a soft metal ion-translocating P-type ATPase illustrates the common features of these pumps (adapted from ref. 11). Most pump either monovalent or divalent soft metals from the cytosol, either into an intracellular compartment or out of the cell. Some, such as *E. hirae* CopA, pump ions into the cell. They have an N-terminal region with one or more cytosolic metal-binding domains, most of which are cysteine-rich motifs but some are histidine-rich sequences. They have eight transmembrane segments (TMS). The cytosolic loop that connects TMS4 and TMS5 is the conserved phosphatase domain. TMS6 has the consensus CPC or CPH sequence, which could be involved in metal ion translocation. In the cytosolic loop that connects TMS6 and TMS7 regions involved in catalysis, the phosphorylation and ATP-binding domains are found. In addition, there is a conserved His-Pro sequence that is found only in soft metal ion-translocating P-type ATPases.

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affect trafficking (14,15). On the other hand, the N-terminal metal-binding domains may have a different function in bacteria, most of which lack intracellular membranes that would require trafficking signals.

The first bacterial proteins identified as members of the monovalent cation-translocating ATPase subfamily were the CopA and CopB copper pumps of *Enterococcus hirae* (16,17). The genes for these two P-type ATPases are in an operon that also contains the gene for a transcriptional regulator, CopY, and a copper ion chaperone, CopZ. While CopA and CopB are cotranscribed, they have considerable differences both structurally and physiologically. A disruption in the *copA* gene renders cells copper-requiring, suggesting that CopA is required for copper import pump. In contrast, a *copB* disruption renders cells copper sensitive, suggesting that the 745-residue CopB protein is responsible for copper efflux from cells. That two such similar pumps transport their substrates in opposite directions illustrates the point that the directionality of transport cannot easily be deduced from inspection of the primary sequence of the proteins. Together the two pumps provide for copper homeostasis.

CopB has a histidine-rich N-terminus. Of the 25 histidine residues in CopB, 16 are located in the first 100 residues, before the first transmembrane segment. It seems reasonable that this region binds copper. In the sixth putative transmembrane region is a CPH sequence that corresponds to the CPC of other soft metal P-type ATPases. CopB has been shown to be an efflux pump involved in resistance to copper in *E. hirae* (18). To demonstrate the direction of transport, Solioz and co-workers prepared everted membrane vesicles and showed that the vesicles accumulated ^{64}Cu(I) and ^{110}Ag(I). The affinity for metal ion was in the micromolar range, and for ATP the K_m was approximately 10 μM. Vanadate, the classical inhibitor of P-type ATPases, inhibited CopB activity. CopB has been purified and reconstituted into proteoliposomes (19). CopB formed an acylphosphate reaction intermediate with the γ-phosphate of ATP, and formation of the phosphorylated intermediate was sensitive to vanadate. The purified protein exhibited a low level of ATPase activity that was also inhibited by vanadate. However, ATP hydrolysis was not stimulated by copper ion, although a P-type ATPase would be expected to require its metal ion substrate for activity.

The sequences of two homologues of CopB, Hral and Hra2, have been reported (20). These were originally described as *Escherichia coli* proteins. However, they are not found in the *E. coli* genome (21), and their derivation is unknown. Another soft metal P-type ATPase with a histidine-rich N-terminus is the Ag(I) resistance pump SilP from *Salmonella typhimurium* (22). Of the 25 histidines in SilP, 15 are located in the N-terminal region, including a highly charged stretch of residues with the sequence EHHHHHDHHE. However, the N-terminus of SilP exhibits little sequence similarity with the N-terminus of other soft metal P-type ATPases, including CopB, and SilP confers resistance to silver but not copper. SilP and CopB are the only two Ag(I) resistance pumps thus far

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identified, and both have histidine-rich (albeit unrelated) N-terminal metal-binding domains. This may indicate that Ag(I) recognition is via histidine residues rather than the CXXC motifs of the majority of Cu(I) pumps.

The 727-residue CopA protein has a single CXXC sequence in the N-terminal region, and a CPC sequence in the putative sixth transmembrane segment (17). Sequences for other bacterial CopA homologs have been identified, and the list is growing daily. However, the physiological function of only a few of these sequences has been described, and even fewer have been investigated biochemically. Two of the best characterized are the CopA pumps of *Helicobacter pylori* (23,24) and *E. coli* (25). As mentioned above, disruption of the *E. hirae copA* results in a copper requirement. In contrast, disruption of either of the *H. pylori* or *E. coli* pumps results in copper sensitivity. This reflects the fact that the *E. hirae* CopA is an uptake system, while the other two homologs are efflux pumps. Again, it is quite remarkable that the direction of ion transport can be inward for one protein and outward for others that are close homologs. The explanation cannot be that the proteins have the opposite orientation in the membrane because each uses ATP, which is found only in the cytosol; the catalytic domains of all three must be exposed in the cytosol. On the other hand, the topological orientation of the protein in the membrane has been determined only for the *H. pylori* enzyme (24). The protein has been shown to have eight transmembrane segments, with cytosolic N- and C-termini. It is reasonable to assume that all soft metal P-type ATPases will have a similar topology.

CopA from *E. coli* is an 834-residue protein with high similarity to copper pumps such as *E. hirae* CopA, the human Menkes and Wilson disease proteins (25). While the CopA homologs from *E. hirae* and *H. pylori* have only a single N-terminal CXXC sequence, *E. coli* CopA has two, G_{11}LSCGHC and G_{107}MSCASC. The presence of multiple metal-binding domains in the *E. coli* protein may make it a better model for the human copper pumps, which have six N-terminal CXXC sequences. CopA can be predicted to have eight transmembrane segments, including C_{479}PC in predicted transmembrane helix 6. In addition, there is a conserved HP motif in the soft metal P-type ATPases that corresponds to H_{562}P in CopA. Regulation and resistance exhibit different metal ion specificities. The *E. coli copA* gene is inducible by addition of either copper or silver salts to the medium. In contrast, disruption of *copA* resulted in sensitivity to copper salts but not Ag(I). Thus there must be an as-yet-unidentified regulatory protein that controls *copA* expression, and that regulator recognizes either Ag(I) or Cu(I), while CopA recognizes only Cu(I). Everted membrane vesicles from cells expressing *copA* accumulated ^{64}Cu. ATP and DTT were both required, and vanadate inhibited transport. Even though ^{64}Cu(II) was added to the uptake assay, it would be reduced to Cu(I) by the strong reductant DTT. The fact that no transport of copper ion was observed without DTT strongly indicates that Cu(I) is a substrate of the pump (25).

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3. P-TYPE ATPases FOR DIVALENT SOFT METALS: PUMPS FOR Zn(II), Pb(II), AND Cd(II)

The second branch of the soft metal P-type ATPases are those for divalent soft metal ions, including Zn(II), Pb(II), and Cd(II). They can be further subgrouped into the CadA ATPases, which are found mainly in gram-positive bacteria, and ZntA ATPases, which are mainly in gram-negative bacteria (1). The first gene for a divalent soft metal P-type ATPase to be identified was *cadA*, a cadmium-resistance determinant on *Staphylococcus aureus* plasmid pI258 (26). The *cadA* gene encodes a 727-residue P-type ATPase that exhibits considerable sequence similarity to the CopA Cu(I)-translocating ATPases. When the *cadA* gene was expressed in *Bacillus subtilis*, the CadA protein was produced as a membrane protein that could be visualized on sodium dodecyl sulfate polyacrylamide gel electrophoresis (27). CadA was shown to form a phosphorylated intermediate during the catalytic cycle (28). The phosphoenzyme intermediate was formed only in the presence of Cd(II) and γ-[^{32}P]ATP and was sensitive to hydroxylamine treatment, which is diagnostic of an acylphosphate bond. Presumably phosphorylation occurs at Asp415, which corresponds to the conserved aspartate residue in all P-type ATPases. Everted membrane vesicles prepared from *B. subtilis* expressing *cadA* accumulate ^{109}Cd(II) in an ATP-dependent manner (27). The topology of a CadA homolog from *H. pylori* has been determined. Like the *H. pylori* CopA, it has eight transmembrane segments (29).

The *zntA* gene was first identified from sequencing of the *E. coli* genome. From its sequence, the *zntA* gene product could not be differentiated from copper P-type ATPases. However, disruption of *zntA* resulted in sensitivity of *E. coli* cells to Zn(II), not Cu(II) (30,31). The fact that *zntA* is a chromosomal gene suggests that it has a function in normal growth of *E. coli*, probably in zinc homeostasis. In everted membrane vesicles of *E. coli*, ZntA catalyzes ATP-coupled accumulation of ^{65}Zn(II) or ^{109}Cd(II) in a vanadate-sensitive reaction (30). Vesicles made from a strain with a *zntA* disruption did not accumulate ^{65}Zn(II). When *zntA* was expressed on a plasmid, vesicles from the disrupted strain accumulated ^{65}Zn(II). Although no isotope of Pb(II) is available, the data suggest that ZntA transports Pb(II). A *zntA*-disrupted strain was nearly three orders of magnitude more sensitive to Pb(II) than the wild-type strain (32). Moreover, ^{65}Zn(II) transport in vesicles was inhibited by either Pb(II) or Cd(II). Similar results were obtained when the *zntA*-disrupted strain expressed the *S. aureus cadA* gene on a plasmid. Neither conferred resistance to Cu(II) nor catalyzed uptake of ^{64}Cu in vesicles. Thus both ZntA and CadA are extrusion pumps for the divalent soft metal ions Zn(II), Pb(II), and Cd(II).

ZntA has been purified and shown to exhibit ATPase activity at rates equivalent to those for the hard metal P-type ATPases (33). ATP hydrolysis required a divalent soft metal ion, the first metal-ion-dependent ATPase activity by a soft

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metal P-type ATPase to be clearly demonstrated. The activity was stimulated by (in order of effectiveness) Pb(II) > Cd(II) ~ Zn(II) ~ Hg(II). Although the free metal ions stimulated ATP hydrolysis, the rates were higher when the soft metal ions were complexed with thiolates of cysteine or glutathione. In fact, free Cd(II) or Hg(II) inhibited activity at neutral and alkaline pHs. In vivo the concentration of glutathione is in the millimolar range (34), and it is likely that soft metal ions do not exist free. These results raise the interesting possibility that the soft metal P-type ATPases recognize metal-glutathione conjugates in vivo.

ZntA and CadA homologs appear to be widespread in nature, although they have not yet been found in animals. It is likely that these evolved for zinc homeostasis: zinc is required for a number of enzymes and transcription factors but is toxic in excess, so cells must have mechanisms to prevent overaccumulation. Although most of the zinc pumps identified to date are prokaryotic, genes for homologs have been identified in other kingdoms, for example in genome of the archeaon *Methanobacterium thermoautotrophicum* and in the genome of the plant *Arabidopsis thaliana*. It would be of interest to know whether humans have a ZntA homolog and, if so, whether there are inheritable diseases related to mutations in zinc pumps. Since these P-type ATPases are also the first Pb(II)-translocating pumps to be identified, it is possible that differential expression in Pb(II)-exposed individuals may produce variations in Pb(II) sensitivity in humans. The answers to questions such as these will become clear as genome projects are completed.

4. THE ArsAB ATPase: AN As(III)/Sb(III) EFFLUX PUMP

In *E. coli* high-level resistance to As(V), As(III), and Sb(III) is conferred by the *arsRDABC* operon of plasmid R773 (35). The *arsC* gene encodes an arsenate reductase that converts As(V) to As(III). As(III) and Sb(III) are the substrates of the ArsAB efflux pump, which is an As(III)/Sb(III)-translocating ATPase. The 429-residue ArsB subunit is an integral membrane protein located in the inner membrane of *E. coli*. The results from construction of a series of gene fusions between *arsB* and *phoA*, *lacZ*, or *blaM* demonstrate that ArsB has 12 membrane-spanning segments, with the N- and C-termini in the cytosol (36). ArsB can function even in the absence of the ArsA subunit to catalyze translocation of the arsenite anion, with energy supplied in the form of a membrane potential (37) (Fig. 3A). The ArsAB complex is able to couple ATP hydrolysis to the transport of arsenite, making it a more efficient efflux system than ArsB alone (Fig. 3B). As a semimetal or metalloid, arsenic can have nonmetallic properties, existing in solution as the oxyanions arsenate and arsenite. Alternatively, As(III) and Sb(III) have properties similar to those of soft metals, interacting with high affinity with thiolates. ArsB has only a single cysteine residue that is not required for

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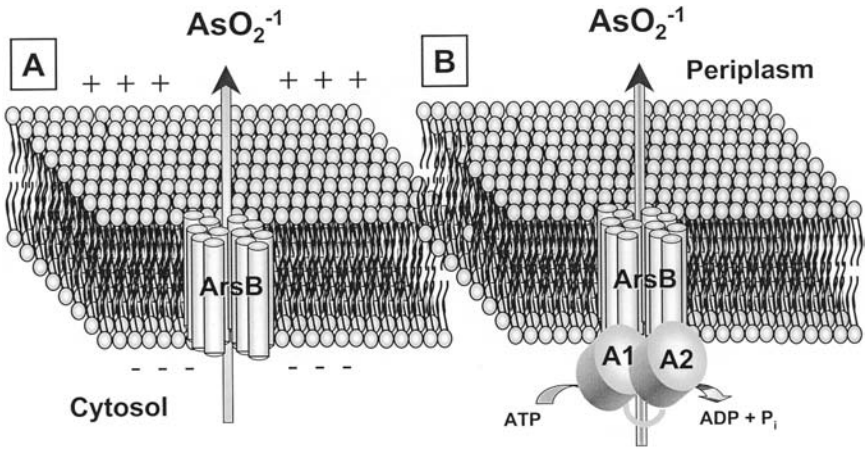

FIGURE 3 The ArsAB pump. The *E. coli* Ars arsenite translocator has two modes of energetics (37). (A) ArsB is an inner-membrane protein that can function as a secondary $\Delta\omega$-coupled uniporter. (B) The ArsAB pump is a complex of the ArsA ATPase and ArsB membrane carrier and functions exclusively as a primary ATP-driven pump. ArsA has two halves, A1 and A2, which are homologous to each other and are connected by a 25-residue linker peptide. Hydrolysis of ATP by ArsA is coupled to arsenite or antimonite translocation by ArsB.

ArsB catalysis; thus ArsB does not interact with As(III) as soft metals but must recognize and transport the nonmetallic, oxyanionic forms of the metalloids (38).

In contrast, the 583 residue ArsA protein, which is the catalytic subunit of the pump, is allosterically activated by the soft metals As(III) or Sb(III) (39). In the absence of its partner, ArsB, ArsA can be expressed and purified as a soluble protein. ArsA has two halves, A1 and A2, connected by a 25-residue linker. A1 and A2 are homologous to each other, clearly the result of an ancient gene duplication and fusion. Both A1 and A2 have a consensus nucleotide-binding domain (NBD), both of which are required for activity (40,41).

As(III) or Sb(III) specifically stimulates ATP hydrolysis (39). It is clear that they do so as soft metals, with the thiolates of Cys113, Cys172, and Cys422 serving as ligands to the metals (42). Those three cysteine residues are located in different regions of the primary sequence, which implies that the protein folds in such a way that the cysteines are brought in proximity to each other, allowing them to coordinate with the metalloid (43). From X-ray diffraction data of crystals of As(III) or Sb(III) complexed to small molecule dithiols, the lengths of an As-

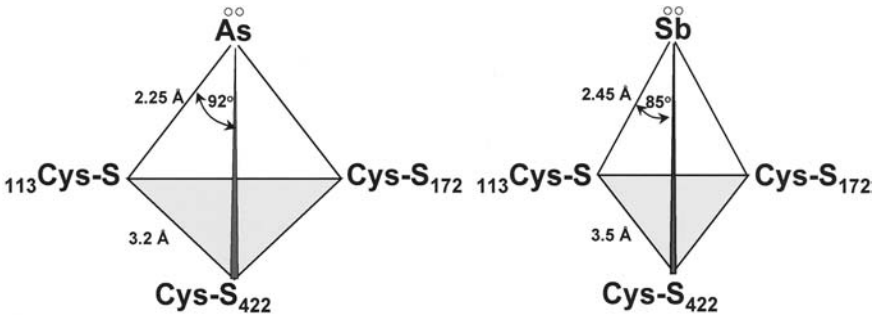

FIGURE 4 Proposed geometry of the ArsA metalloid-binding domain. The metalloid-binding site ArsA is proposed to be a trigonal pyramidal structure containing the three-coordinately liganded sulfur thiolates of Cys113, Cys172, and Cys422, with either As(III) or Sb(III) at the apex. The bond angles and distances are predicted from crystallographic analysis of small arsenic or antimony thiol compounds.

S bond and Sb-S bond have been shown to be 2.23 and 2.45 Å, respectively, with S-As-S and S-Sb-S angles of 92.7° and 84.8°, respectively (44). Extrapolating from the shape of these small molecules, a model of the metal-binding site in ArsA has been proposed (Fig. 4) (43). Filling of the allosteric site with metal results in an increase in the rate of ATP hydrolysis at the NBD. This is associated with conformational changes that occur upon metalloid binding (45). A1 and A2 are tethered by a 25-residue flexible linker peptide (46), but otherwise the NBDs do not interact in the absence of metalloid (47). When As(III) or Sb(III) coordinates with Cys113 and Cys172 in A1 and Cys422 in A2, this draws the two halves of the protein closer, bringing the two nucleotide-binding sites into contact, which increases the rate of ATP hydrolysis (48,49) (Fig. 5).

How activation occurs is an open question, as is whether both NBDs are catalytic. Both sites bind nucleotides, even when the other is inactivated by mutagenesis (50). The two sites do not appear to be equivalent, either in sequence or in binding properties. In the absence of metalloid activator, the A2 site binds the ATP analog 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) but not ATP, leading to the hypothesis that the A1 NBD is a high-affinity binding site but that the A2 site can bind ATP only when the enzyme is activated (47). Although mutations in either NBD eliminate the high, activated rate, ArsAs with inactive A2 sites still hydrolyze ATP at the basal, unactivated rate (48). In contrast, A1 substitutions result in complete inactivation. These results suggest that the A1 site exhibits independent unisite catalysis in the absence of As(III) or Sb(III); participation of the A2 site requires metalloid binding, which produces multisite catalysis (48).

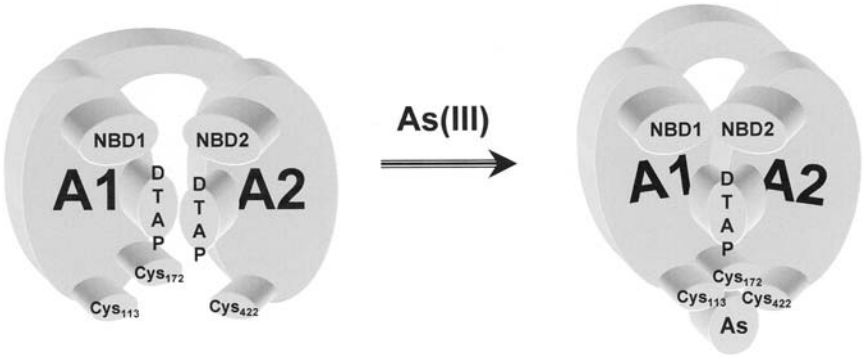

FIGURE 5 Model of ArsA catalysis. In the absence of metalloid activator, the A1 and A2 halves of ArsA are independent of each other, with only the A1 NBD exhibiting a basal rate of ATP hydrolysis (48). Binding of Sb(III) or As(III) to Cys113, Cys172, and Cys422 brings the halves together. This results in interaction of the A1 and A2 NBDs to interact and promotion of catalysis. Conformational changes in the conserved DTAP domains report binding and hydrolysis of nucleotides (51,52).

In both the A1 and A2 halves of the protein there is a 12-residue consensus sequence DTAPTGHTIRLL (the DTAP domain) that undergoes considerable conformational change during catalysis (51). Using intrinsic tryptophan fluorescence from ArsAs in which single typtophan residues had been introduced at specific locations, the movement of domains in ArsA could be monitored during the individual steps of the catalytic cycle. This has allowed modeling of the reaction cycle (52). The results suggest that conformational change is the rate-limiting step in the overall reaction in the unactivated state, and that binding of the allosteric activator in some way overcomes this rate-limiting step. This is reminiscent of the E1-to-E2 transition in P-type ATPases, where the conformational change from one state to the other is a major feature of the mechanism.

5. ARSENICAL RESISTANCE IN EUKARYOTES

Although this chapter has focused on soft metal resistance in bacteria, the prevalence of arsenic in the environment ensures that arsenical resistance mechanisms are universal and ubiquitous (1,35,53). Arsenic is a well-documented human carcinogen, and the adverse effects from exposure to this metalloid are considered among the top priority health hazards in the world. In populations exposed to arsenic, various tumors, including tumors of lung, skin, bladder, and liver, have been shown to be associated with exposure to arsenic (54). In addition to their

natural prevalence, metalloids are also used as chemotherapeutic agents for the treatment of parasitic diseases such as leishmaniasis, and resistance has become a major obstacle in treatment of this tropical disease (55). Recently, arsenite in the form of arsenic trioxide has been shown to be an effective treatment for acute promyelocytic leukemia (56). However, a significant fraction of the patients became unresponsive to this chemotherapeutic agent, suggesting increasing drug resistance.

It is of interest, therefore, to compare and contrast the mechanisms in eukaryotes with those in prokaryotes. Several mechanisms of metalloid resistance in eukaryotes have been proposed. For example, arsenic is methylated in many eukaryotes, and this has been proposed to be a detoxification mechanism (57). More recently transport-related resistances have been described (35). One pathway of metalloid resistance is conferred by members of the MRP group of the ABC superfamily of transport ATPases. In humans, multidrug-resistance-associated protein (MRP1) catalyzes export of GS conjugates such as leukotriene C4 (LTC4) as one of its normal functions (58). The role of homologs such as cMOAT appears to be export of glutathione conjugates of bile salt from liver canaliculi (59,60). The gene for MRP is frequently amplified in small-cell lung carcinomas and has been shown to confer resistance to arsenite and other drugs (61). MRP-catalyzed export of glutathione from cells was increased by arsenite, suggesting that MRP functions as a $As(GS)_3$ carrier (62). More recently, overexpression of MRP1 has been shown to be a pathway for protecting human cells from the cytotoxic effects of heavy metals (63). In this report, Sb(III)-selected lung cancer cell lines, which were also cross-resistant to As(III), showed a correlation between increased efflux of Sb(III) and overexpression of MRP1. A stable and inducible resistance to arsenite and antimonite has also been reported in Chinese hamster cell lines (64). Although the responsible gene(s) and protein(s) have not been identified, resistance is associated with increased efflux of arsenite from the cells (65).

As mentioned above, arsenical and antimonial drugs such as Pentostam, an Sb(V)-containing drug, are still the first line in the treatment of trypanosomiasis and leishmaniasis, and clinical resistance occurs in upward of 25% of all cases. It has been proposed that Sb(V) derivatives are metabolized in vivo into Sb(III), which is the likely active form of the drug (66). Although metalloids are reduced by GSH nonenzymatically, the process is too slow to occur biologically (67). It is likely, then, that enzymatic mechanisms for reduction exist in these organisms. Thus far, no As(V)/Sb(V) reductase has been identified in *Leishmania*, but an arsenate reductase has been identified in the eukaryotic *S. cerevisiae* (68,69).

In vitro strains of *Leishmania* have been selected that exhibit resistance to both the +3 and +5 oxidation states of arsenic and antimony. In these arsenite-resistant strains there is increased expression of *pgpA*, which encodes an MRP

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homolog (70). It has been hypothesized that *pgpA* gene product is a pump that transports As-thiol conjugates into intracellular compartments (71). There are multiple pathways for arsenite resistance in *Leishmania*. While amplification of *pgpA* leads to increased resistance, in cell lines selected for arsenite resistance, disruption of *pgpA* does not eliminate resistance (72). This is consistent with the existence of an independent resistance mechanism, and in the plasma membrane of *Leishmania* there is an arsenite-translocating ATPase (73). Although the gene for this pump has not been identified, it is still present in the *pgpA* disruption and must therefore be a different gene product (74).

Recently, the *S. cerevisiae ACR3* gene was shown to encode a membrane protein that confers arsenite resistance (68,75). Recently Acr3p has been shown to catalyze arsenite extrusion from yeast cells (76). A homolog of Acr3p has been identified in an *ars* operon from *B. subtilis* (77). Interestingly, ArsB, which is present in most bacterial *ars* operons, is not homologous to Acr3p (35). Acr3p and ArsB are both plasma membrane proteins of similar size, both catalyze extrusion of arsenite from cytosol, and both are proposed to be anion uniporters, coupled to the membrane potential. However, they differ in presumed transmembrane topology and in metalloid specificity. ArsB has 12 membrane-spanning segments (36), while Acr3p has been proposed to have only 10 (75). Furthermore, ArsB confers resistance to both arsenite and antimonite (78), while Acr3p is an arsenite-specific resistance protein (76).

When the *S. cerevisiae ACR3* gene was disrupted, the cells were still moderately resistant to arsenite, suggesting that there might be a second pathway for removal of arsenite from the cytosol (76). The yeast cadmium factor protein, Ycflp, a close homolog of human MRP1, had been shown to encode a MgATP-dependent glutathione S-conjugate transporter responsible for vacuolar sequestration of cadmium and other compounds (79,80). A *S. cerevisiae* strain with a disrupted *YCF1* gene could be complemented by expression of either human MRP1 cDNA (81) or the gene for an MRP homolog from *Arabidopsis thaliana* (82). Since MRP1 confers arsenite resistance, it was of interest to examine whether the yeast Ycflp could do so as well. A strain with a *YCF1* disruption was found to be as sensitive to arsenite as a strain with an *ACR3* disruption, and double disruption of both *YCF1* and *ACR3* resulted in hypersensitivity to arsenicals (76). In both cases *ACR2* was required for arsenate resistance, so Acr2p-catalyzed reduction of arsenate to arsenite is a prerequisite for either *ACR3* or *YCF1*. While Acr3p is plasma membrane localized and is responsible for arsenite extrusion from cells, Ycflp is localized in the vacuole and catalyzes ATP-coupled sequestration of $As(GS)_3$, the triglutathione conjugate of As(III). Thus each of the two transporters produces arsenite resistance by independent pathways. While Acr3p appears to be specific for arsenite, Ycflp has broad substrate specificity, providing resistance to As(III), Sb(III), Cd(II), and Hg(II), probably by transport of each of these soft metals as glutathione conjugates. Human MRP and *Leish-*

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mania PgpA, both homologs of Ycflp, probably catalyze the same reaction. No glutathione transferase has been shown to catalyze the formation of soft metal–glutathione conjugates, but such an enzyme can be predicted to exist. While As(III) and GSH react spontaneously to form $As(GS)_{3\,(83)}$, the process is probably too slow to be involved in biological formation of the conjugate.

In conclusion, although the pathways of resistance in prokaryotes and eukaryotes are similar in overall design, most of the prokaryotic proteins are unrelated evolutionarily to their eukaryotic analogs (Fig. 6). In both *E. coli* and yeast, the first step in resistance is detoxification of arsenate. In prokaryotes this reaction is catalyzed by ArsC arsenate reductases, while in eukaryotic yeast Acr2p catalyzes this reaction. Arsenite, the product of the reductase reaction, is then ex-

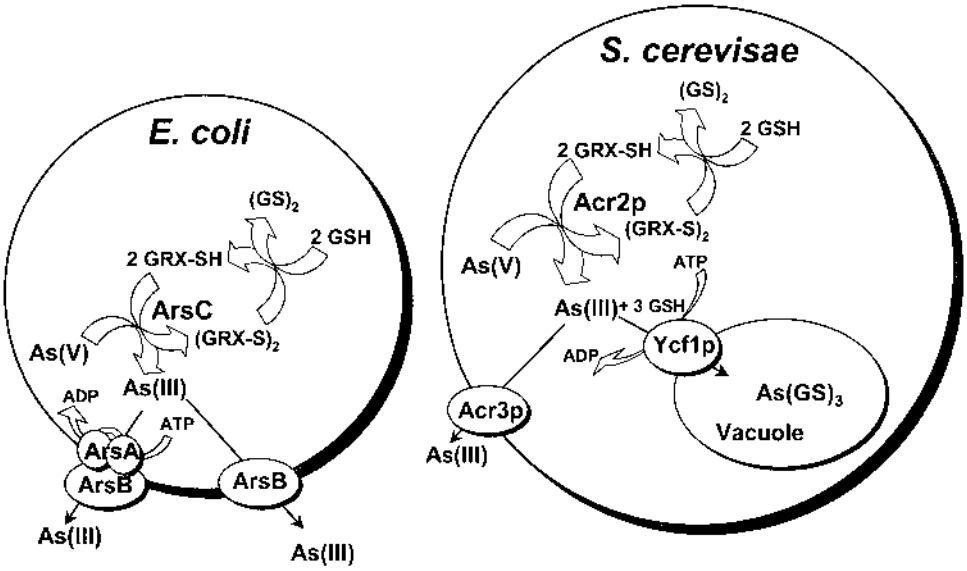

FIGURE 6 Pathways of arsenical detoxification in prokaryotes and eukaryotes. In both *E. coli* and *S. cerevisiae*, the first step in resistance is reduction of arsenate to arsenite by the bacterial ArsC or yeast Acr2p enzymes. In both organisms, glutathione and glutaredoxin serve as the source of reducing potential (89,90). In *E. coli*, the resulting arsenite is extruded from the cells by the Ars efflux system, which, depending on the subunit composition, can be either the ArsB uniporter or the ArsAB ATPase (37). In *S. cerevisiae*, there are two types of arsenite transporters. Acr3p is a plasma membrane arsenite efflux protein that probably functions as a uniporter coupled to the membrane potential. Ycflp, a member of the MRP family of the ABC superfamily of drug resistance pumps, transports $As(GS)_3$ into the vacuole.

truded from cells by a plasma membrane carrier protein. In most bacteria this efflux protein is ArsB, while in yeast it is Acr3p. Both carriers are proposed to be anion uniporters, coupled to the membrane potential. In bacteria ArsB can associate with ArsA to form the ATP-coupled ArsAB pump. In *S. cerevisiae* there is a second, parallel pathway for removal of arsenite from cytosol, the ABC transporter Ycflp. This pump catalyzes ATP-coupled accumulation of $As(GS)_3$ (and the glutathione conjugates of other soft metals) into the vacuole for sequestration, and it is likely that human members of the MRP family, which confer resistance to arsenite, will do so by a similar mechanism. Other components in the pathway for arsenical resistance in humans are not known. Human enzymes that catalyze arsenate reduction and conjugation to GSH have not been identified, nor has an Acr3p homolog been found. It will be of interest to determine whether the pathways of metalloid detoxification in humans are similar to those in yeast.

6. CONCLUSIONS

The intracellular concentrations of heavy metals and metalloids are kept in balance by a variety of transporters. Homeostasis of required metal ions involves pairs of uptake and efflux systems. Resistance to toxic metals requires efflux systems. In bacteria there are families of transporters and pumps that catalyze metal extrusion. One is the superfamily of P-type ATPases, which includes Cu(I) and Ag(I) pumps, and ZntA and CadA for divalent soft metals ions such as Zn(II), Pb(II), and Cd(II). Another family includes the ArsAB ATPase, which catalyzes extrusion of and resistance to metalloid oxyanions. These or analogous pumps are present in most organisms, reflecting the need to prevent the buildup of heavy metal ions.

ACKNOWLEDGMENTS

We thank M. Solioz and P. Kaur for discussions and suggestions. Work from the authors' laboratory was supported by U.S. Public Health Service Grant GM55425.

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Genetic Susceptibility to Heavy Metals in the Environment

Diane W. Cox and Lara M. Cullen
University of Alberta, Edmonton, Alberta, Canada

John R. Forbes
McGill University, Montreal, Quebec, Canada

1. INTRODUCTION

Many metal ions are required for essential functions, yet are toxic in excess. Metalloproteins play important structural and functional roles in cell metabolism. Homeostatic mechanisms maintain a critical balance to avoid toxicity. Such mechanisms must be able to respond to changes in metal concentration in our environment due to diet or environmental pollutants. Maintenance of balance requires regulated absorption, transport, and excretion mechanisms, controlled by a number of genes whose products ensure correct transport of metals to specific sites. Each metal is expected to have its own series of transporters. However, as we will show in the following pages, several metals are highly dependent upon the concentration of other metals. When any gene within the balanced system is nonfunctional, the balance can be upset.

The specific genes involved in human transport are best known for copper and iron. Both metals are associated with diseases involving metal storage, which

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can be affected by excessive metal intake. These two metals have important interactions. Zinc and molybdate, in the form of tetrathiomolybdate, each have an effect on the transport of copper. Knowledge of the copper transport pathway has increased dramatically in less than a decade and is the major focus of this chapter. Copper and iron are chosen for discussion also because they demonstrate genetic disorders and show environmental consequences.

2. OVERVIEW OF COPPER TRANSPORT IN HUMANS

The average daily copper intake is between 1 and 2 mg, and approximately half of this is absorbed (1). This copper plays an essential role in a number of proteins. Cytochrome oxidase is an inner-membrane mitochondrial protein complex, functioning as a key enzyme that catalyzes the reduction of oxygen to water, using the free energy of the reaction to generate a proton gradient required for respiration. Other proteins that require copper for function are superoxide dismutase (SOD1), which converts superoxide anion to hydrogen peroxide and protects against cellular free radical damage; lysyl oxidase, required for collagen and elastase cross-linking, and dopa beta-monooxygenase, which converts dopamine to norepinephrine. In excess, copper generates free radicals and becomes a potent cellular toxin via the Haber-Weiss reaction (2). The consequences of hydroxy radical production in vivo include lipid peroxidation, DNA strand breakage and base oxidation, mitochondrial damage leading to reduced cytochrome oxidase activity, and protein damage.

Copper is widespread in the environment, released particularly through mining operations, incineration, weathering of soil, industrial discharge, and sewage treatment plants. The concentration of copper can be increased during its distribution. Copper, particularly at low pH, can be leeched from copper pipes and plumbing fixtures into the drinking water. When water sits stagnant in pipes, the copper concentration in the water can be markedly increased. Chronic exposure to high doses of copper can cause liver disease in normal individuals, for example, with the use of a high-dose copper supplement 10 or more times the daily requirement (3). The ability to excrete copper through the liver is normally high and copper is unlikely to cause damage except in individuals who have abnormalities of biliary excretion. However, there are genetic situations in which individuals can be susceptible to normal amounts of copper in the diet. Human genetic disorders associated with an imbalance of copper homeostasis include Menkes disease, a disorder resulting in overall copper deficiency, and Wilson disease, a disorder of toxicity due to excess copper storage. Aceruloplasminemia, a complete deficiency of ceruloplasmin, is associated with iron storage. Aceruloplasminemia demonstrates the interplay between iron and copper. Canine copper toxicosis, which occurs particularly in Bedlington terriers, is a different disorder of copper storage.

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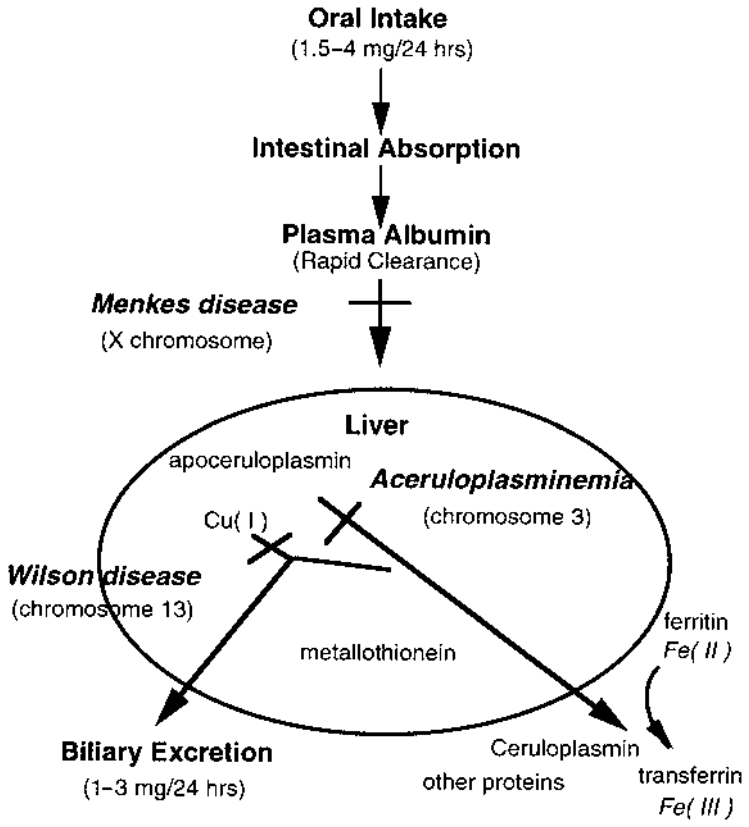

FIGURE 1 Overview of key features of copper transport, showing diseases that can result from defects in three parts of the pathway.

A simplified overview of the mammalian copper pathway is shown in Figure 1. Ingested copper is absorbed via the small intestine (4) and rapidly enters the bloodstream bound to albumin, histidine, and small peptides (1). The absorbed copper is transported to the liver, where it is excreted into the bile. In the hepatocyte, a small amount is incorporated into the plasma protein, ceruloplasmin, a 130-kDa protein containing six atoms of copper. Most of the copper in plasma is bound to ceruloplasmin. Normal concentrations of copper in the serum are 3–10 millimolar in adults. Ceruloplasmin concentration is usually 200–400 mg/L, with higher amounts in young children (5). Ceruloplasmin is an acute-phase reactant and increases in response to inflammation, liver disease, and malignancy. Copper in the plasma is carried to the kidney, where a small amount is resorbed.

If the concentration of copper is very high in the plasma, as indicated by an elevation in ceruloplasmin, adequate resorption may not take place and a slight increase in urinary copper excretion could occur.

Discovery of the genes involved in two copper transport diseases has been important in our understanding both of normal copper transport and of the consequences of abnormal functioning of either of these genes. One of these genes, involved in biliary copper excretion, influences our capacity to rid the body of excess copper from the environment. The second gene, involved in a copper-deficiency state, is highly related.

3. DISORDERS OF COPPER TRANSPORT IN HUMANS

3.1 Menkes Disease

3.1.1 Clinical and Biochemical Features

Menkes disease is an X-linked recessive copper deficiency usually leading to severe disease and death in early childhood (5,6). This disease, while not influenced by environment, is important for the understanding of other aspects of response to copper. The features of the disease are those characteristic of severe copper deficiency. There is a pronounced reduction in all copper-containing enzymes. Resulting features include hypothermia and progressive cerebral degeneration (deficiency of dopamine beta-monooxygenase and cytochrome oxidase), arterial aneurysm, bladder diverticuli, and ligament laxity (deficiency of collagen cross-linking mediated by lysyl-oxidase) and hypopigmentation (deficiency of copper-dependent tyrosinase enzyme activity). Another feature of Menkes disease is distinctive brittle hair with a corkscrew-like appearance. Treatment by copper histidine administration can be effective in part, if started sufficiently early (7,8). The problems with connective tissue begin in utero and are not reversed by this treatment (8). A variant of Menkes disease, occipital horn syndrome, is a mild form of Menkes disease characterized by connective-tissue abnormalities, such as hyperelastic skin, skeletal abnormalities, hernias, bladder diverticuli, and aortic aneurysms, all due to reduced lysyl oxidase activity (9,10). Developmental delay, if present, is less severe than in classical Menkes disease.

Biochemical features of Menkes disease reflect the copper deficiency (11,12). These include reduction of liver copper, and low plasma copper and ceruloplasmin. However, the copper level in the duodenal mucosa is two- to threefold higher than in normal individuals. Copper absorption in the intestine is normal, suggesting that there is defect in copper transport out of the intestinal epithelium and into the circulation. An additional diagnostic feature of Menkes disease is that patient fibroblasts in culture accumulate high levels of copper due to defective copper efflux compared with that of normal controls (13,14).

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The gene for Menkes disease (designated *ATP7A*) was identified in 1993 by positional cloning, in a female patient with a translocation breakpoint at the Menkes locus (15–17). Sequencing of the cDNA revealed a predicted protein (ATP7A) similar to a P-type ATPase previously found to transport copper in bacteria (18). ATP7A is expressed in all tissues including the intestine, but not in the liver. The protein mediates copper efflux from cells in peripheral tissues, particularly intestine, as a mechanism for copper homeostasis. Lack of ATP7A in intestinal cells results in the defect of intestinal copper transport and the resulting widespread copper deficiency seen in Menkes disease. ATP7A may also be involved in copper incorporation into cuproenzymes in peripheral tissues. Numerous *ATP7A* mutations have been identified in Menkes disease patients (19). Deletions, some of many kb in length, have been identified in 15–20% of patients with classic Menkes disease (16,17). Approximately 90% of known mutations are predicted to destroy the protein, causing the severe disease seen in most patients (12,19,20). The few missense mutations or splice site mutations observed are typically found in patients with the milder form, occipital horn syndrome (21,22).

3.1.2 Animal Models of Menkes Disease

The mottled mouse has biochemical and phenotypic signs like those seen in Menkes disease, and the orthologous gene (*Atp7a*) is defective (22–24). More than 20 mutations are identified in *Atp7a* that lead to the mottled (Mo) phenotype. Different alleles of the mottled locus lead to mice with a wide range of disease severity, ranging from prenatal lethality to connective tissue abnormalities as in cutis laxa (23,25,26). For example, the brindled mouse (Mo^{br}) has a 6-bp gene deletion and a phenotype of prenatal lethality, consistent with severe classical Menkes disease (25,27). Although ATP7A protein is expressed in Mo^{br} fibroblasts, the protein is probably inactive, and does not traffic within the cell, as is required for copper export (discussed below) (28). The blotchy mouse (Mo^{bl}) has a splice site mutation that interferes with normal splicing, causing markedly reduced mRNA levels, and features similar to occipital horn syndrome (29,30).

3.2 Wilson Disease

3.2.1 Clinical and Biochemical Features of Wilson Disease

Wilson disease (hepatolenticular degeneration) is an autosomal recessive disorder of hepatic copper transport and storage. The disease locus maps to chromosome 13q14.3. This is a disorder in which excess copper in the environment may play a role. Wilson disease affects approximately 1 in 30,000 individuals in most populations, possibly up to 1 in 10,000 in certain populations such as in China, Japan, and Sardinia (5,31). The characteristic defects observed in Wilson disease are reduced excretion of copper into the bile, resulting in a toxic accumulation of copper in the liver and an increase of copper excretion in the urine. The second

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defect is a reduced incorporation of copper into ceruloplasmin. Although the plasma ceruloplasmin concentration may lie within the normal range, the incorporation of radioactive or stable isotopes of copper always indicates a defect in the incorporation of copper into ceruloplasmin (32,33).

The cloning of the gene in 1993 (34,35) has helped explain the biochemical and clinical changes observed. As a result of mutations in the copper transporter gene, *ATP7B*, copper accumulates in the liver, first inducing the production of metallothionein, which can apparently maintain copper in a relatively harmless state. The accumulation of copper causes damage to mitochondria. Copper is deposited in renal tubules, and kidney damage occurs to varying degrees. Copper also accumulates in the basal ganglia of the brain causing neurological disease. It is not yet clear whether the neurological damage is due to expression of the gene in the basal ganglia, or to an effect of high levels of circulating plasma, or to the combined effects of both.

In Wilson disease, clinical presentation is highly variable (5,31), with age of onset from less than 5 years to greater than 50 years, and clinical manifestations presenting as hepatic or neurological disease. Patients may have chronic or fulminant liver disease, neurological disorder with or without liver involvement, purely psychiatric illness, or isolated acute hemolysis. Renal damage may also occur. Neurological forms manifest as a movement disorder with poor coordination, tremors, and loss of motor control, or with dystonia, with rigidity and gait disturbance (5,31). Patients with neurological Wilson disease have copper accumulation in the liver and reduced plasma ceruloplasmin levels, but may not show clinical evidence of liver damage. Psychiatric disorders can occur in as many as 20% of patients, (5,31). A distinctive feature of patients with Wilson disease is the presence of Kayser-Fleischer rings in the cornea, due to copper deposition in Descemet's membrane at the outer rim of the cornea. This is occasionally easily visible, but usually is observed only by careful slit lamp examination. Kayser-Fleischer rings are frequently absent in patients with hepatic disease (36).

An important biochemical feature of Wilson disease is hepatic copper accumulation due to impaired biliary copper efflux (5). Normal adults typically have 20–50 μg copper/g dry liver, whereas Wilson disease patients have greater that 250 μg/g. Copper also accumulates in the kidney, brain, and cornea. Serum holoceruloplasmin (enzymatically active) levels, and consequently serum copper levels, are usually below normal, although they may be normal in the presence of liver disease (36). Nonceruloplasmin copper (mostly albumin bound) is increased. Urinary copper excretion is greatly elevated. Apoceruloplasmin, encoded by a gene on chromosome 3, has normal biosynthesis in Wilson disease patients, but copper incorporation into the protein during biosynthesis is impaired.

Wilson disease can be effectively treated if diagnosis occurs sufficiently early. Chelating agents are effective for removing excess copper from blood and/or tissues. Penicillamine, the sulfhydryl-containing amino acid cysteine substi-

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tuted with two methyl groups, was introduced by Walshe in 1956, and has rescued many hundreds of patients from this potentially fatal disorder (37). Penicillamine removes copper from plasma, preventing further accumulation by greatly increasing urinary excretion of copper. Penicillamine is not particularly effective at removing liver copper stores. Studies in the LEC rat model indicate that penicillamine inhibits the accumulation of copper in lysosomes, macrophages, and Kupffer cells, and makes copper soluble, for mobilization from these cellular components (38). Hepatic metallothionein is induced and copper is stored in the liver in a relatively nontoxic form. Trientine, or trien, has also been used extensively for chelation (39–41).

The dramatic effect of a high oral ingestion of zinc led to the use of oral zinc sulfate as a treatment for Wilson disease, particularly in Europe, where it has been used since 1979 or earlier (42). The use of zinc, often as zinc acetate, is now becoming more widespread worldwide. Oral zinc has a different mode of action from the chelating agents. The mechanism of action is through induction of metallothionein in enterocytes (43). Metallothionein preferentially binds copper because of its higher affinity. Copper is eliminated through shedding of the enterocytes during normal turnover. This approach offers a cheap alternative treatment. Although the long-term effectiveness and side effects of zinc therapy have not been as well evaluated as for penicillamine, maintenance zinc treatment in follow-up studies of up to 10 years suggest that zinc is effective as maintenance therapy (44). The interaction of copper and zinc is discussed further below.

Ammonium tetrathiomolybdate, one of the newer chelating agents to be used, may be particularly useful for patients with neurological disease, as it does not seem to lead to initial neurological degeneration, as is sometimes observed with penicillamine (45). This reagent is particularly effective at removing copper from the liver, in contrast to trientine or penicillamine. Because of its effectiveness, continuous use could cause copper deficiency. Reversible bone marrow suppression has been noted as an adverse side effect (46). With this agent, as with other chelators, studies must be undertaken to ensure that copper is not mobilized to other tissues. Such studies will be facilitated with the use of proven animal models.

Cloning of the Wilson disease gene (*ATP7B*) was accomplished by conventional linkage analysis (47), by physical mapping of the relevant region of chromosome 13q14, and finally by its high homology with the Menkes disease gene (34,35). *ATP7B* encodes a predicted protein characteristic of copper-transporting P-type ATPases, with 57% identity to *ATP7A*. *ATP7A* and *ATP7B* have different tissue expression profiles, leading to the distinct phenotypes of Menkes and Wilson disease. *ATP7B* is expressed primarily in liver and kidney; and less in the brain and placenta.

Translation of the nucleotide sequence predicted six putative heavy-metal-binding domains, and a mean amino acid identity of 65% between each of the

six domains found at the 5′ end of *ATP7A*. Both *ATP7A* and *ATP7B* also contain highly conserved domains characteristic of the P-type family of cation transporting ATPases. The functionally important regions of the predicted protein, similar in the genes for the two disorders, were predicted to be: 1) a transduction domain, containing a Thr-Gly-Glu (TGE) motif, and 2) cation channel and phosphorylation domains, containing a highly conserved Asp-Lys-Thr-Gly-Thr (DKTGT) motif. The aspartate residue forms a phosphorylated intermediate during the cation transport cycle. An invariant proline residue in a cysteine-proline-cysteine cluster, located 43 residues N-terminal to this aspartate, is within the predicted cation channel. 3) An ATP-binding domain (residues 1240–1291) is a highly conserved region situated at the C-terminal end of a large cytoplasmic ATP-binding domain, including a Gly-Asp-Gly (GDG) motif. Eight hydrophobic regions are predicted to span the cell membrane. In addition to the potential membrane-spanning regions, a small hydrophobic domain is predicted (residues 362–386) including part of copper-binding domain 4. This may affect the tertiary structure of the copper-binding region, forming a pocket.

Over 200 mutations have been found in the *ATP7B* gene of Wilson disease patients (for reference see the Wilson disease database at http://www.medgen.med.ualberta.ca/database.html). The spectrum of known mutations is different from that of *ATP7A* (20,48). The majority of known mutations (51%) in *ATP7B*, as indicated in the database, are single-base-pair missense mutations, which are infrequent (2%) in *ATP7A* (19). The remaining *ATP7B* mutations include nonsense, splice site, and small insertion/deletion mutations, sometimes resulting in frame shifts. No gross gene deletions (common in *ATP7A*) of *ATP7B* have been observed. Differences in the observed mutation spectrum of ATP7A and ATP7B may be biased since Wilson disease mRNA and genomic structure are not routinely analyzed in patients with Wilson disease. Most mutations are very rare in the population; consequently most patients are compound heterozygotes. One mutation, His1069Glu, is found in up to 30% of patients of European origin, up to 65 or 70% in eastern Europe. Homozygotes in several studies have had an onset of about 20 years of age, in the neurological form (49–52). The Arg778Leu mutation is commonly found in Asian populations and is associated with severe early-onset hepatic disease in homozygotes (50,53). The extreme phenotypic variation among Wilson disease patients may be explained in part by allelic heterogeneity of the *ATP7B* gene, but other genetic and environmental factors must also be involved.

3.2.2 Possible Susceptibility of Heterozygotes to Copper in the Environment

There is no evidence reported that heterozygotes, who carry only one mutated gene for Wilson disease, ever develop clinical symptoms. However, they are relatively frequent in the population, on average 1 in 90. Possibly these individu-

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als are more susceptible than normal individuals to an increased concentration of copper, from drinking water or other environmental sources.

3.2.3 Copper and Oxidative Damage

Excess copper in tissues leads to the production of damaging free radicals and to DNA cleavage (54). Copper overload particularly affects mitochondrial respiration and causes a decrease in cytochrome C activity (55). Damage to mitochondria is an early pathological effect in the liver. Damage to the liver has been shown to cause increased lipid peroxidation and abnormal mitochondrial respiration, both in copper overloaded dogs and in patients with Wilson disease (55). The generation of free radicals by the presence of copper could be particularly accelerated in patients with Wilson disease who lack the antioxidant effects of ceruloplasmin. Wilson disease patients have been shown to have low levels, in the liver, of antioxidants, including ascorbate and urate (56) and α-tocopherol (56,57). These observations suggest that antioxidants may be important adjuncts for saving tissue from damage in patients with Wilson disease. Further studies are needed in this area.

3.2.4 Animal Models of Wilson Disease

There are two rodent models of Wilson disease: the LEC rat and the toxic milk (tx) mouse (58,59). Both rodents exhibit hepatic copper accumulation due to reduced biliary copper excretion, and reduced copper incorporation into ceruloplasmin. The LEC rat has a large deletion removing 25% of the *Atp7b* coding region (58). Unlike patients with Wilson disease, the LEC rat develops liver tumors. Copper and iron may both participate in the induction of DNA damage and malignancy in the rat (60). This rat model is being used for many studies of the transport of copper, and for the evaluation of new modes of therapy (61,62). LEC rats offer opportunities for experiments attempted at therapy at the gene level.

The tx autosomal recessive mutation first arose in the inbred DL mouse strain (63), producing homozygous dams unable to secrete copper into milk. Consequently, litters of homozygous tx dams are severely copper deficient and display poor growth, hypopigmentation, hepatic accumulation of copper, and early death (64). In the original tx mouse, the causative mutation is a missense mutation at position 4066 of *Atp7b*, resulting in a methionine-to-valine substitution in the eighth transmembrane region (59). In 1987, a new autosomal recessive mutation (tx^J) arose in the C3H/HeJ strain at the Jackson Laboratory, Bar Harbor, and was shown to be allelic with the original tx mutation (65). The mutation differs from that of the original tx mouse and is found in the second transmembrane domain (V. Coronado, M. Nanji, D. W. Cox, unpublished). The toxic mutations apparently destroy activity of the protein, as their phenotypes are identical to that of a recently described Atp7b knockout mouse (66).

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3.3 Other Copper Diseases

Indian childhood cirrhosis and Tyrolean infantile cirrhosis appear to have a strong environmental component, producing disease when dietary copper is exceptionally high. These diseases may be similar to canine copper toxicosis.

3.3.1 Indian Childhood Cirrhosis

Indian childhood cirrhosis occurs in infants and young children, with a fatal outcome unless copper is removed through chelation therapy. This disease has a strong environmental factor. This disease has been recognized and described in India since the late nineteenth century and much as been written on the subject (see reviews in refs. 67,68). Clinical features are in many respects similar to those seen in Wilson disease, except that the onset is generally earlier. In some of the series of patients reported with Indian childhood cirrhosis, Wilson disease could have been the cause as clinical symptoms can manifest as early as 3 years of age (69). The histological criteria suggested for Indian childhood cirrhosis are necrosis of hepatocytes with ballooning, pericellular collagen disposition, and inflammatory infiltrate (70). These features are not exclusive to Indian childhood cirrhosis. An important difference between Indian childhood cirrhosis and Wilson disease is that copper, demonstrated by automatic absorption spectrometry in Wilson disease, is usually not stainable by copper stains such orcein rhodanine or rubeanic (71). However, orcein staining has been found as a consistent feature of Indian childhood cirrhosis (72). A different distribution of the copper is also observed. In Wilson disease, copper is found in the cytoplasm. In Indian childhood cirrhosis, copper is reported to accumulate in the nuclear fraction of hepatocytes (73). Another difference between Indian childhood cirrhosis and Wilson disease is that the former can be halted by a brief period of penicillamine therapy (74). Once the copper is removed, the children and infants do not have a recurrence of the disease.

Ingested copper appears to play an important role in this disease, although there is probably an underlying genetic predisposition. Large amounts of copper are ingested because of the use of brass pots. The first-born male in the family has been particularly likely to be affected, because of the tendency to favor feeding animal milk, which is boiled in the brass pots (67). The brass pots used for food are generally covered with a thin layer of tin, which is replaced frequently. When this layer wears off, copper becomes accessible from the brass. Experiments have shown that cow's milk, with a copper content of about 19 μg/dl, contained 26 μg/dl after 6 h at room temperature, and about 625 μg/dl after boiling (67). With the alteration of feeding patterns, this disease has almost disappeared in the areas of India in which it was prevalent (68). There are still sporadic cases of Indian childhood cirrhosis, suggesting that the disease is likely heteroge-

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neous. However, in general, it appears that once the high ingestion of copper is stopped, liver disease will not occur.

3.3.2 Tyrolean Infantile Cirrhosis

Another type of liver disease with an environmental component occurred in a high frequency in a rural area of Western Austria between 1900 and 1974. This disorder was clinically and pathologically indistinguishable from Indian childhood cirrhosis, but appeared to be transmitted in an autosomal recessive manner (75). As in India, the tin or brass vessels in which milk came in contact appear to have contributed to the development of excessively high levels of liver copper in these patients. With a change in cooking vessels, the disease has now disappeared. *ATP7B* has been excluded as candidate gene underlying this form of cirrhosis by a study of genetic markers or haplotypes (76). Children with a similar type of disease do occasionally appear in other geographical regions (77). The heterogeneous causes, probably genetic, have yet to be discovered.

Autosomal-recessive non-Indian childhood cirrhosis (NICC) is phenotypically similar to copper toxicosis in Bedlington terriers, but clinically much more severe (78). In both cases, there is no reduced level of ceruloplasmin (>200 mg/L) (79) and neurological damage and Kayser-Fleischer rings have not been reported.

3.3.3 Canine Copper Toxicosis

A disease in dogs is mentioned here, because there is likely to be a similar genetic disease in humans, which we can identify when the causative gene is identified. Copper toxicosis is an autosomal recessive disorder of copper transport, common in Bedlington terriers and seen occasionally in other terrier breeds (80). This copper disorder is phenotypically and biochemically similar to Wilson disease, the LEC rat, and the toxic milk mouse. Liver pathology shows some differences; copper is sequestered in lysosomes in canine copper toxicosis (81,82). In normal Bedlington terriers, the mean level of hepatic copper is 200 μg/g dry weight. In Bedlington terriers affected with copper toxicosis, the mean value of hepatic copper is 6000 μg/g dry weight, and a range of 1900–12000 μg/g dry weight hepatic copper has been observed (81). In the affected dogs, the accumulation of copper in the liver results from the failure of the liver to excrete excess copper into bile (83). This results in chronic hepatitis and eventually liver cirrhosis. Affected dogs can be treated with agents that are effective for the treatment of Wilson disease, such as zinc acetate or penicillamine (84). However, differences at the clinical and biochemical level are also observed. Neurological symptoms and ocular Kayser-Fleischer rings are not observed in affected dogs, but are found in some patients with Wilson disease (82,85). In addition, the affected dogs do not have a low

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plasma concentration of ceruloplasmin (82,85), as observed in most Wilson disease patients.

The copper toxicosis locus in Bedlington terriers has been mapped to chromosome region 10q26 by fluorescence in situ hybridization (FISH) (86) using a BAC clone containing C04107, a microsatellite marker closely linked to canine copper toxicosis (87). In the same study, FISH mapping excluded canine *ATP7B*, *CTR1*, and *CTR2* as candidate genes. Recent mapping studies, and molecular characterization of *ATOX1* in Bedlington terriers, excluded *ATOX1* as a candidate gene (88). The canine copper toxicosis locus lies in a region of conserved synteny with human chromosome 2p21 (88,89). The gene *ATP6H*, encoding vacuolar proton ATPase subunit M9.2 (90), has a human ortholog on chromosome 2p21 and has been suggested as a candidate gene (91). *ATP6H* contains a conserved sequence motif (90), CSVCC, similar to those of metal-binding proteins (MTCXXC). Furthermore, yeast defective in vacuolar ATPase has abnormal copper and iron homeostasis (92). Comparative mapping between human and dog genomes has identified the canine copper toxicosis-candidate homologous region in humans to be between the *SPTBN1* and *SLC1A4* genes, a region of approximately 30 cR (93). In the same study, *ATP6H* was excluded as a candidate gene underlying canine copper toxicosis based on radiation hybrid (RH) mapping. However, the presence of *ATP6H* pseudogene(s) was not considered in the study, and there is no proof that the functional gene was mapped. Pseudogenes have also been identified for *Atox1* (88) and *ATP7B* (86) in the canine genome.

4. INTRACELLULAR COPPER TRANSPORT PATHWAYS

4.1 Yeast Transport Pathways

Copper diseases can be understood only when we understand all of the transport pathways. The copper transport pathway in yeast is described first because it is so similar to that in mammals. Identification of components in this simpler organism has aided in the understanding of transport in mammalian systems, in both normal and disease states. Genetic and biochemical analysis can more easily be carried out in the yeast, *Saccharomyces cerevisiae*, for which many biochemical pathways, and also the complete sequence, are known. Many of the copper transport proteins are functionally conserved between organisms, so yeast can be used as a model for deciphering mammalian copper homeostasis at the cellular level.

The protein primarily responsible for high-affinity copper uptake in yeast is Ctr1p (94). Prior to uptake, copper (and iron) is reduced through the action of reductase proteins in the plasma membrane, Fre1p and Fre2p, which release metal from extracellular ligands and increase bioavailability (95,96). The important role of reduction for copper uptake is demonstrated by the restoration of copper uptake in Fre1p-deficient mutant yeast by the addition of ascorbic acid. Ctr1p, a plasma

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membrane protein, contains 11 repeats of a methionine rich motif (Met-x2-Met) predicted to bind copper (97). Ctr1p appears to be copper specific. A second high-affinity copper transporter, Ctr3p, is postulated to function in copper uptake through an endocytic pathway (98). Although either Ctr1p or Ctr3p will allow sufficient high-affinity copper uptake for growth, both proteins are required for normal cell growth under copper-limited conditions.

In yeast, copper homeostasis is tightly regulated by copper uptake, unlike the situation in mammals in which copper is regulated largely by copper export. Transcription factors coordinately regulate the expression of *CTR1*, *CTR3*, and *FRE1* genes (99,100). In addition to controlling the uptake of copper, yeast cells avoid toxicity by sequestering copper and other heavy metal ions. The proteins that perform this function are the metallothioneins, low-molecular-weight, cysteine-rich proteins that bind copper tightly in the cuprous form, thus protecting cells from metal-induced free radical damage. The genes *CUP1* and *CRS5* encoding the yeast metallothioneins (101,102), are upregulated by a transcription factor in response to copper (103).

Copper is never free within the cell (104). Copper entering the cell is bound by a series of chaperone proteins, each functioning to deliver copper to a distinct copper target. Ccsp, originally called Lys7p (genetic locus *LYS7*), delivers copper to Sod1p (locus *SOD1*) (105); Cox17p (locus *COX17*) delivers copper to mitochondria (106); Atox1p (locus *ATX1*) delivers copper to the membrane-copper-transporting ATPase Ccc2p, and is required for high-affinity uptake of iron (107). Ccc2p is one of the highly conserved transporters that will be discussed in more detail below. Atx1p interacts directly with Ccc2p to transport copper. Ccc2p plays a critical role in iron transport, transporting iron into Fre1p. Deletion of the *ATX1* gene resulted in a mutant yeast defective in iron uptake, and lacking incorporation of copper into Fet3p. The mutant cells cannot grow on iron-limited medium unless supplemented with copper or iron (107). The phenotype is the same when either *ATX1* or *CCC2* is deleted. In addition to targeting of copper to specific proteins, chaperones ensure that copper is delivered to needed sites, and is not randomly sequestered into metallothioneins.

4.2 Copper Homeostasis and Transport in Mammals

Most of the proteins of the transport system in yeast cells are also found in mammals. In mammals, the liver, and to some extent the intestine, are the main control points for the regulation of copper levels.

Based on the role in yeast, the product of the *CTR1* gene is believed to be responsible for the import of copper into cells (108). There are also other proteins, including albumin, histidine, ceruloplasmin, and transcuprein, that may donate copper to cells (1). Details of the entry of copper into hepatocytes are not entirely known; however, in polarized hepatocytes in vivo, copper uptake occurs at the

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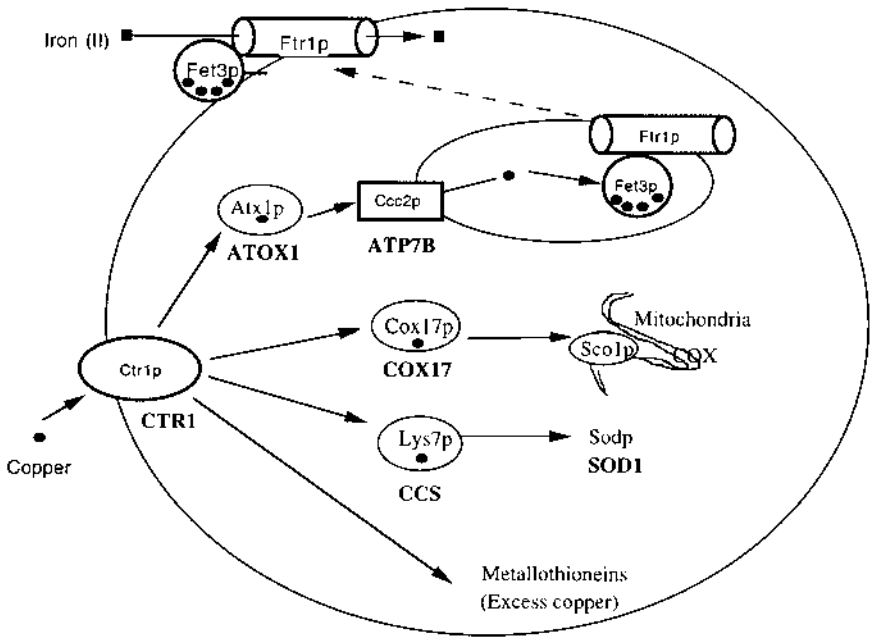

FIGURE 2 Proteins involved in cellular copper transport. Yeast proteins are in lower case, human proteins are shown in bold below the yeast orthologs. (Modified from Ph.D. thesis, J. R. Forbes, 2000.)

basolateral plasma membrane in contact with the sinusoids (109). Transport of copper within the cells is very similar to that described for the yeast cell. The mammalian orthologs are able to functionally replace their yeast counterparts (Fig. 2). The human copper chaperone genes produce proteins that transfer copper to specific targets: *ATOX1* for transport into the membrane copper transporters, *CCS* into superoxide dismutase (*SOD1*), and *COX17* into mitochondria via *SCO1* for incorporation into cytochrome oxidase (COX) (110). In the human genome, there are at least 16 metallothionein genes in a cluster on chromosome 16 (111). Mammalian metallothioneins, unlike the preferential binding in yeast, can bind a variety of metals. The primary role of metallothionein in mammals appears to be detoxification. Mammalian cells expressing excess metallothionein are cadmium resistant (111,112). The metallothioneins are probably required for copper detoxification only when the copper efflux of the cell is impaired. Metallothioneins are thought to be involved in protection against damage from free radicals: mouse cells overproducing metallothioneins resist chemically induced oxidative damage, while mouse cells lacking metallothioneins are more sensitive to oxidative

stress (111). However, mice lacking metallothioneins have no defects in copper transport (111). Glutathione, a cysteine-containing tripeptide, avidly binds copper and is found in many tissues (including liver, brain, kidney, and other tissues). Glutathione has been suggested to be involved in the transfer of copper stored in the protein-bound form, particularly that in metallothioneins, and when excess copper is present (109). As in yeast, metallothioneins bind copper when present in excess, are highly inducible, and are responsive to transcription factors.

Two P-type ATPases are critical for the elimination or transport of copper from cells and are therefore important in handling copper in the environment. The human copper-transporting P-type ATPases (*ATP7A* and *ATP7B*) were discovered because of their involvement in Menkes disease and Wilson disease, respectively. The gene for Menkes disease, cloned first, was identified as encoding a copper transporter because of its similarity to bacterial ATPases.

4.3 Metal-Transporting P-Type ATPases

4.3.1 Features of the P-Type ATPases

Membrane metal transporters play a key role in metal resistance and sensitivity and in metal transport diseases. Cloning and sequencing of the genes encoding the proteins defective for Menkes disease and Wilson disease revealed predicted structures similar in nature to a class of integral membrane proteins known as P-type ATPases (15–17,34,35). P-type ATPases (E1E2 ATPases) have the ability to bind and hydrolyze ATP forming a phosphoenzyme intermediate dependent upon transport substrate. P-type ATPases are inhibited by vanadate and undergo conformational changes during ATP hydrolysis (18). Much experimentation has been done on the structure and function of mammalian P-type ATPase proteins. In particular, the Ca^{2+} transporter of the sarco(endo)plasmic reticulum continues to be extensively studied and is used as a general model for the function and structure of P-type ATPases. Studies performed on this and similar ATPases reveals extensive conservation of basic structure and mechanism among P-type ATPases proteins such as the Ca^{+}/H^{+}, Na^{+}/K^{+}, or H^{+}/K^{+} transporters (113). Copper-transporting P-type ATPases have been proposed to be part of a subgroup, designated CPx-type ATPases, based on conserved sequence features found among heavy metal transporters from many prokaryotic and eukaryotic species (114).

Transport by P-type ATPases has been studied in detail and is outlined briefly here, using the reaction cycle of the Ca^{2+} ATPase as an example (113). The resting intermediate of the protein is designated E1. Two calcium ions bind to the E1 protein, followed by binding of ATP. ATP is hydrolyzed, resulting in phosphorylation of a nearby aspartate residue forming the $E1P(Ca_2)$ intermediate. The hydrolysis of ATP and phosphorylation of E1 depend upon both calcium binding sites being occupied. The calcium ions become occluded within the pro-

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tein, no longer accessible to solvent from either side of the membrane. A rate-limiting conformational change occurs, forming the E2P intermediate. The affinity of this intermediate for calcium is reduced by three orders of magnitude, allowing calcium to dissociate from the enzyme and be transported through the membrane. Dissociation of the calcium ions triggers spontaneous hydrolysis of E2P, liberating inorganic phosphate, and resulting in conformational changes that return the protein to its E1 state to repeat the transport cycle. The phosphate bond of E1P can react with ADP, whereas E2P can only react with water. This fact has enabled the dissection of the P-type ATPase reaction mechanism and has allowed detailed biochemical analysis of the effect of site-directed mutations on protein function.

The transport mechanism just described can be used generally to describe P-type ATPases and is the likely mechanism by which the heavy metal transporters (CPx-type) function. The functional domains of the Ca^{2+} ATPases within this structure have been extensively mapped by biochemical and molecular means (113–118). These domains, as described for the copper transporter ATP7B above, are: the transduction domain, a conserved Asp-Lys-Thr-Gly-Thr (DKTGT) motif with the important aspartate (D) residue, ATP-binding domain with conserved Thr-Gly-Asp-Asn (TGDN) motif, ATP-hinge region. Although absolute sequence homology among the P-type ATPase family is not high, there is striking similarity in the hydrophobicity profiles among them, suggesting the formation of a common core membrane domain topology with six membrane-spanning segments (118,119). The overall hydrophobicity profile of CPx-type ATPases is consistent with a total of eight membrane-spanning segments. CPx-type ATPases have a large N-terminal domain, predicted to be a soluble heavy-metal-binding domain. This domain is joined to the core ATPase portion of the molecule by two additional transmembrane segments, making a total of four between the N-terminal domain and transduction domain of these proteins. There is no equivalent predicted N-terminal structure on non-heavy-metal-transporting ATPases, which begin immediately with the ATPase core preceded by a very short soluble sequence. Instead, non-heavy metal P-type ATPase transporters typically have four additional C-terminal transmembrane segments added to the ATPase core giving a predicted topology of 10 membrane-spanning helices, as supported by biochemical data (113,120,121).

4.3.2 The Heavy Metal P-Type ATPases

A striking feature distinguishing CPx-type ATPases from other P-type ATPases is the large N-terminal domain, originally predicted to be a heavy-metal-binding domain based on homology with the bacterial mercury binding protein MerP and bacterial cadmium efflux protein CadA (15–17,34,35). Each metal-binding motif contains a conserved Gly-Met-X-Cys-X-X-Cys (GMxCxxC) sequence, predicted to bind metal via the cysteine residues. ATP7A and ATP7B each contain six

copies of Cys-box copper-binding motifs within their heavy-metal-binding domains. Each Cys-box is likely part of an individual subdomain, which together form the entire copper-binding domain. Bacterial CPx-type ATPases typically contain only one or two Cys-box motifs within a metal-binding domain. A second type of putative copper-binding motif, designated the His-box, was also identified in bacterial CPx-type copper ATPases (117,119). The feature of CPx-type ATPases, including ATP7A and ATP7B, from which they were named, is the Cys-Pro-Cys/His (CPx) motif predicted within the sixth transmembrane domain of these proteins (114). The CPx motif is thought to be part of a copper-binding site, within the membrane domain of CPx-ATPases, to which copper is transiently bound during copper transport (117,119). The final sequence motif that distinguishes CPx-type ATPase from other P-type ATPases is the Ser-Glu-His-Pro-Leu (SEHPL) motif, C-terminal to the putative phosphorylated aspartate residue (35,119). The most common mutation in Wilson disease, His1069Gln, lies in this motif. This motif is not found in non-heavy metal P-type transporters.

4.3.3 Structure and Metal-Binding Properties of Copper-Binding Domains

Some facets of the structure and copper-binding properties of the copper-binding domains of ATP7B and ATP7A are known. The solution structure of the fourth copper-binding subdomain of ATP7A was solved by nuclear magnetic resonance (NMR), using silver as the metal ion (122). Met12 was hypothesized to be a metal-binding ligand, owing to its sulfur moiety and close proximity to the conserved cysteine residues. Metal binding by Met12 was not observed in the NMR structure of the ATP7A Cu4 motif.

The crystal structure of Atx1p, a small metallochaperone protein, has been solved by X-ray diffraction, using bound mercury instead of copper (123). Biophysical analysis of purified Atx1p with copper bound revealed one atom bound per protein molecule as Cu(I), with high affinity (124). There is considerable structural conservation between Cys-box copper-binding domains and subdomains. The structure of the fourth ATP7A copper-binding subdomain, solved by Gitschier et al. (122), likely represents the prototypical fold of copper-binding subdomains found in the copper-binding domains of CPx-type ATPases, although there may be subtle differences between this structure, solved with bound silver, and a copper-bound form of the protein. The individual Cys-box subdomains must then fold in relation to each other to form a complete copper-binding domain.

Metal binding to the entire copper-binding domains of ATP7B and ATP7A has been studied (125,126). The data suggest that at least six atoms of copper, in the Cu(I) form, can bind to the copper-binding domains of ATP7B and ATP7B, with one atom occupying each of the six copper-binding subdomains. Results have suggested selective and possibly cooperative copper binding to the copper-

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binding domain of ATP7B (127). Copper binding by the copper-binding domain of ATP7A also appears to be cooperative (128).

4.3.4 Copper Transport by CPx-Type ATPases

The first copper-transporting CPx-type ATPase shown to be capable of directly transporting copper was the CopB ATPase of *Escherichia hirae*, which contains two His-box copper-binding subdomains in its N-terminal copper-binding domain. The protein was able to transport copper into native membrane vesicles only under strongly reducing conditions, indicating that copper was transported in the Cu(I) form (129). Copper transport saturation kinetics were dependent upon copper concentration. Copper transport was also dependent upon ATP concentration and was inhibited by low, but not high, concentrations of vanadate, characteristic of P-type ATPases. Oxidation chemistry of vanadate in the reducing environment is a suggested cause for the lack of inhibition at high concentration. CopB transported silver as Ag(I) in a manner identical to copper. Purified CopB protein, reconstituted into proteoliposomes, was demonstrated to hydrolyze ATP and form a phosphorylated intermediate, another characteristic feature of P-type ATPases, when incubated with radioactive ATP in the presence of DTT (130). Neither phosphorylation nor ATP hydrolysis was strictly stimulated by copper addition; both occurred in the absence of added copper and did not increase upon copper addition. Phosphorylation and ATPase activity was reduced, but not eliminated, by addition of a Cu(I) chelator, suggesting a copper dependence of these activities. Vanadate showed the same effect as for copper transport.

ATP7A, expressed in stably transfected Chinese hamster ovary (CHO) cells, shows ATP-dependent copper transport into isolated plasma membrane vesicles. Saturation kinetics were dependent on copper concentration (131). Copper transport was dependent on the presence of a reducing agent, suggesting the transport of Cu(I), and was inhibited by vanadate. Thiol-reactive reagents inhibited copper transport consistent with a role for cysteine residues in copper transport in ATP7A. A phosphorylated ATP7A intermediate dependent on a reducing agent was demonstrated in membranes from stably transfected CHO cells (132). As with CopB, phosphorylation was not strictly stimulated by copper, but was reduced by a Cu(I) chelator.

ATP7B can replace Ccc2p in yeast, delivering copper to Fet3p, demonstrating that it too is a putative copper transporter (133–136). ATP-dependent copper uptake has been demonstrated in basolateral membranes of rat and human liver (137,138), and in Golgi membranes from rat hepatocytes (139). However, these activities were not specifically assigned to Atp7b. Recent reports have demonstrated that expression of ATP7B in Menkes patient fibroblast cell lines was able to reduce copper accumulation in these cells (140–142). In addition, infusion of an adenovirus expressing human ATP7B into the LEC rat, a rodent model of

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Wilson disease, restored ceruloplasmin biosynthesis and biliary copper efflux in the mutant rats (143,144). These observations, together with the impaired hepatic copper efflux found in patients with Wilson disease, all provide evidence that ATP7B is a copper transporter.

4.3.5 Intracellular Copper Trafficking

Copper efflux from the hepatocyte occurs mainly through biliary excretion, which provides regulation of body copper. Copper is incorporated into ceruloplasmin in the hepatocyte for circulation into plasma, but the main route of export of copper is via the bile. The ATP7B copper transporter is required for copper incorporation into ceruloplasmin, a function defective in patients with Wilson disease. The role of ATP7B has been shown directly in the LEC rat, where adenoviral-mediated reintroduction of human ATP7B directly into the liver of LEC rats in vivo results in the restoration of copper incorporation into apoceruloplasmin and the appearance of enzymatically active ceruloplasmin in the serum (145,146).

In the bile, copper is complexed to a variety of ligands including amino acids, peptides, proteins, and bile salts (reviewed in ref. 109). A glutathione transport system provides a minor route of copper efflux, as demonstrated through studies of the Groningen Yellow (GY) rat. This mutant rat has a defective transport of glutathione into the bile, because of a defective glutathione conjugate transporter protein cMOAT (138). The copper transporter ATP7B is responsible for the major excretion of copper from the hepatocyte. Normal biliary copper excretion is restored, with adenoviral-mediated reintroduction of human ATP7B directly into the liver of LEC rats (143).

The ATP7B protein was found to be localized to the trans Golgi network in HepG2 hepatoblastoma cells (133). An interesting change is observed when copper is added to the growth medium; ATP7B is redistributed from the trans Golgi network to a cytoplasmic vesicle compartment and returns to the trans Golgi location when copper is removed (133). This redistribution event is specific for copper, as zinc, cadmium, iron, and cobalt have no effect. In a study using polarized HepG2 cells, ATP7B was localized in the trans Golgi network only at a low copper concentration. With increased copper levels, ATP7B was shown to redistribute to vesicular structures, and then to apical vacuoles (147). The same copper-dependent trafficking event was previously demonstrated for the ATP7A copper transporter, with the exception that the movement upon copper stimulation was to the plasma membrane (148). The copper-induced redistribution that occurs is not dependent upon synthesis of new protein. This copper-dependent relocalization has also been shown in primary rat hepatocytes and in liver sections from copper-loaded rats, indicating the physiological relevance of this process (149). Importantly, direct immunohistochemistry on human liver has revealed intracellular punctate staining of hepatocytes, as well as canalicular membrane staining,

adjacent to bile canaliculae (150). This study furthermore indicated that ATP7B was found predominantly in canalicular-enriched membrane fractions isolated from liver.

The signals that facilitate this intracellular trafficking of the copper transporters are currently under investigation. A C-terminal dileucine motif is generally involved in recycling proteins from the plasma membrane to late endosomes (151). Molecular studies on ATP7A indicate that a C-terminal dileucine motif is required for localization of the protein to the trans Golgi network (152,153). Mutations in this motif resulted in ATP7A proteins that were localized entirely to the plasma membrane and could not recycle back to the trans Golgi network in response to copper depletion. The mutant proteins could still mediate copper efflux, suggesting that the dileucine motif is not involved in transport function, and that the plasma membrane is a site of ATP7A-dependent copper efflux from the cell. A similar leucine-containing motif is present in ATP7B, which may provide a similar function. A Golgi localization signal has also been reported in the third transmembrane segment of ATP7A (153). This may be due to a specific sequence-targeting signal, but could be the result of a change in conformation. Effective intracellular transport is a prerequisite for handling ingested copper.

The two functions of ATP7B, copper incorporation into ceruloplasmin and excretion of copper through the bile, can be assayed in separate systems. A yeast assay has been used to detect the ability of mutant ATP7B proteins to incorporate copper, reflecting the ability to incorporate copper into ceruloplasmin (135). Using this assay, in combination with direct observance of copper trafficking in CHO cells, several types of mutant ATP7B proteins have been identified (154). Some variants are unable to transport copper in the yeast system and appear to distribute in the endoplasmic reticulum. One particularly interesting mutant, glycine943serine, in the fifth transmembrane segment, showed an almost normal transport activity in yeast, but was completely unable to traffic from the trans Golgi network in response to copper (154). This type of analysis on other missense mutants occurring in patients with Menkes disease or Wilson disease should help shed light on the specific amino acid residues required for proper transport and trafficking functions.

Copper has been shown to accumulate in hepatic lysosomes in acutely or chronically copper-loaded rats (155). Lysosomes may be involved when the capacity for metallothionein is exceeded.

The normal excretion of bile appears to be a requirement for efficient elimination of hepatocyte copper. Patients with primary biliary cirrhosis, extrahepatic biliary atresia, or hereditary cholestatic diseases have high hepatic copper concentration. Two genes for hereditary cholestasis, *FIC1* and *PFCI2*, encoding P-type and ABC-cassette ATPase transport proteins, respectively, are involved in hepatic bile acid transport and can lead to an accumulation of hepatic copper.

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5. SELECTED IMPORTANT INTERACTIONS BETWEEN METALS

5.1 The Copper-Iron Connection

Transport of specific metals can be influenced by high concentrations of other metals. Copper provides several examples of this interaction, particularly with iron, zinc, and molbydate.

There has been evidence for the past several decades that ceruloplasmin might be involved in iron transport. Evidence was presented that ceruloplasmin acts as a ferroxidase, converting Fe^{2+} to Fe^{3+}, causing removal of iron from ferritin and binding of Fe3+ to transferrin for transport (156). From studies of copper-transporting genes in yeast, we have learned that Fet3p is of critical importance for the transfer of iron into yeast, and that the activity of Fet3p requires functional Ccc2p (equivalent to the Wilson disease copper transporter). It is particularly interesting that in the past, ceruloplasmin seemed to have no critical function and was even considered to be a remnant from important earlier functions in evolution. The recessively inherited disease, aceruloplasminemia, confirms the important role of ceruloplasmin in mammalian cells. Aceruloplasminemia is a rare condition in which homozygous individuals have a complete deficiency of ceruloplasmin, arising from lack of production of the apoprotein (157,158). Mutations have been identified in the ceruloplasmin gene (*CP*) on chromosome 3q24, which are all frame shift insertion/deletions or nonsense mutations predicted to result in premature truncation of the protein (159–161). Most patients are compound heterozygous. These data are consistent with the absence of plasma ceruloplasmin seen in patients.

Affected patients have iron deposition, in the form of hemosiderin, in liver and brain, low serum iron, and increased plasma ferritin. Affected individuals develop neurological disorders at an age of onset from about 38 to 65 years. The initial symptoms can be similar to those observed in Wilson disease. Patients may initially develop intellectual impairment, which does not occur in Wilson disease. The full-blown disease can be similar to Huntington disease or Parkinson disease. Patients also develop non-insulin-dependent diabetes mellitus from deposits of iron in the pancreas, and degeneration of the retinal pigment due to iron deposits in the retina. Although iron deposition in the liver can be as high as that seen in hemochromatosis, liver disease has not been reported, apparently because of difference in the intracellular deposition of iron. No abnormalities of copper are noted in these tissues, and specifically no signs of copper deficiency. While ceruloplasmin may be involved in copper transport, it clearly does not play an essential role. The key role of ceruloplasmin in iron homeostasis is also confirmed by the disruption of the ceruloplasmin gene in mice (162). Harris et al. have shown that the absence of circulating ceruloplasmin in $Cp^{-/-}$ mice results in

marked tissue iron storage, especially in the liver and spleen, as seen in patients with aceruloplasminemia. Initial iron uptake, tissue distribution, and plasma iron turnover is normal in the $Cp^{-/-}$ mice. However, iron efflux from the hepatocyte is impaired. This was further shown by the lack of recycling of damaged red blood cells out of the reticuloendothelial system and into new red blood cells, leading to progressive iron accumulation. As will be described subsequently, phlebotomy is the normal treatment for hemochromatosis, but is useless in aceruloplasminemia. In the mutant mice, and in individuals with aceruloplasminemia, there is no release of stored iron, and anemia results. The defect in ceruloplasmin production can be corrected by the infusion of holoceruloplasmin.

Iron storage has not been noted as a significant feature in Wilson disease. Although the plasma concentration of ceruloplasmin is frequently very low in Wilson disease patients, apoceruloplasmin is produced, and even trace amounts of holoceruloplasmin apparently are adequate for iron mobilization. Poor mobilization of iron has been described only in patients with less than 2% of the normal plasma ceruloplasmin (163). However, it is noteworthy that ceruloplasmin can become depressed to almost zero in patients on prolonged chelation therapy, and both anemia and iron storage in tissues could result.

5.2 Interaction of Copper and Zinc

Zinc in very high doses (up to 200 mg/day) interferes with absorption of copper from the gastrointestinal tract by an increase in metallothionein production in enterocytes with subsequent increased copper excretion in the stools (43,164). Zinc and metallothionein concentrations increase only with high or excessive zinc ingestion, except in the ileum, in which the response is also seen at low doses of zinc (165). Metallothioneins have been shown, in mice, to help protect against zinc deficiency and toxicity. Mice with a targeted metallothionein gene disruption (166) are not more sensitive than normal to copper, but are more sensitive to cadmium toxicity (167). Metallothionein has a greater affinity for copper than for zinc, so copper is then bound preferentially from the intestinal contents. Once bound, the copper would normally be excreted from the enterocyte, unless there is copper overload exceeding the rate of cellular efflux.

In Wilson disease, copper is not further absorbed but is lost in the feces as enterocytes are shed during normal turnover (43). The concentrations of metallothionein have been shown to be increased up to 150-fold after zinc treatment of patients with Wilson disease, and a significant correlation was found between metallothionein and duodenal zinc concentrations (164). In the same study, an increase in iron concentration was demonstrated in Wilson disease patients, whether on zinc or penicillamine treatment. Since iron has a particularly potent effect on the production of free oxygen radicals, the possibility of intestinal ma-

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lignancy should be investigated, although the presence of high zinc and metallothionein may be protective.

While high oral intake is usual only as treatment for copper overload, the use of high mineral supplements in excessive doses could trigger copper deficiency and has been reported associated with acne treatment (168). An abnormally high exposure to zinc from industrial pollutants could have the same effect.

5.3 Interaction of Copper with Molybdate

Molybdenum is a trace element essential for the activity of xanthine oxidase, sulfite oxidase, and aldehyde oxidase. Molybdates have a well-established effect in producing copper deficiency in ruminants (169). This effect does not occur in humans because they lack the ability to convert molybdate to the tetrathio derivative, a process that takes place through bacterial action in the rumen of ruminants. Tetrathiomolybdate (MoS_4), a potent chelator of copper, has been used to treat copper intoxication, which affects certain breeds of copper-sensitive sheep. Tetrathiomolybdate interferes with copper absorption from the intestine and binds to plasma copper with high affinity. Studies in LEC rats show that copper, accumulated in the liver, forms a complex (copper/thiomolybdate complex), which appears in the plasma, bound to albumin (61). Thiomolybdate, unlike penicillamine, has been found, in LEC rats, to remove copper from metallothionein at low doses; at higher doses, an insoluble copper complex is deposited in the liver (170). The highly acidic copper complex results in an enhanced uptake of molybdenum into the liver. Its chelating effect is so strong that copper is sequestered from copper chaperones, affecting the supply available for ceruloplasmin and superoxide dismutase (170). Molybdenum is then excreted into the feces, but not the urine.

These studies indicate that dose will need to be carefully considered for use in patients, and that high doses of copper and molybdenum can be mobilized into plasma. The fate of molybdenum needs to be more fully explored. Reports that molybdenum can deposit in the pituitary and affect pituitary function (171) need further evaluation. Although it is regarded as nontoxic, bone marrow suppression has been noted as an adverse side effect (46). Little is known about the sites where the mobilized copper and molybdate might be deposited. Dose and length of treatment and long-term side effects will require careful study. Such a potent copper-binding drug could produce copper deficiency, and further study will be needed before the use of thiomolybdate becomes widespread.

6. OVERVIEW OF NORMAL IRON METABOLISM IN HUMANS

Iron is another metal in the environment that can interact with genetic disorders to result in disease. Iron is an element that is indispensable for life because proteins

containing iron are essential for a number of important metabolic processes including oxygen transport, electron transfer, and DNA synthesis. As for copper, iron becomes highly toxic when present at levels in excess of physiological requirements, enabling the generation of reactive oxygen intermediates such as superoxide anion and hydroxyl radicals that result in peroxidative damage to vital cellular structures. Iron can exist in two readily convertible oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), and its ability to accept and donate electrons underlies both its biological importance and its potential for toxicity (172). Because of this essential but potentially toxic nature of iron, specialized molecules have evolved for its absorption, transport, storage, and utilization that enable it to be present in a soluble and nontoxic, yet bioavailable form. Defects in any of these specialized proteins may result in disruption of iron homeostasis, which can have significant clinical consequences. Environmental iron exposure can promote oxidative stress even in those who do not have inherited iron defects (173).

6.1 Iron Absorption

In a healthy adult male total body iron is approximately 3–5 g. The majority is contained in hemoglobin, myoglobin, and iron-containing enzymes, with smaller amounts present in storage (ferritin and hemosiderin) and transport (transferrin) proteins (174). There is no significant excretory pathway for iron in humans; losses are limited to small amounts lost from epithelial cell shredding and in females, during menstruation and pregnancy. Available dietary iron far exceeds physiological requirements, with an average intake of 6 mg/1000 calories. Since both excess and deficiency of iron are deleterious, body iron stores are normally tightly controlled at the point of absorption in the proximal small intestine (175).

The mechanisms by which dietary iron is absorbed are incompletely understood. However, the recent identification of two novel intestinal iron transporters in rodents is probably relevant in the study of human iron absorption. The natural-resistance-associated macrophage protein 2 (Nramp2) is mutated in mice with microcytic anemia (176) and the rat homolog of this gene, divalent cation transporter 1 (DCT1), carries the same missense mutation (Gly185Arg) in the anemic Belgrade rat (177,178). These rodent models exhibit severe microcytic, hypochromic anemia, and evidence suggests that this protein is important both for export of iron from the transferrin cycle endosomes in the bone marrow (179–181) and as an intestinal iron transporter involved in iron uptake from the intestinal lumen (182). A defect in hephaestin, a multicopper ferroxidase similar to ceruloplasmin, has been identified as the cause of iron deficiency in mice with x-linked anemia (183). These mice absorb iron from the intestinal lumen normally, but transport of iron from the intestinal enterocytes across the basolateral membrane into the circulation is diminished.

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6.2 Transferrin and the Transferrin Receptor

In vertebrates, iron is bound by circulating transferrin for transport between sites of absorption, storage, and use in the body. Transferrin is an 80-kDa polypeptide that is synthesized primarily in the liver and transports iron in the blood. Apotransferrin (iron free) can bind two Fe^{3+} ions tightly, but reversibly, in conjunction with an anion (carbonate or bicarbonate), to become diferric transferrin (184). Binding of iron to apotransferrin occurs at neutral pH, and most circulating transferrin is not fully complexed with iron; two monoferric transferrin species exist along with diferric and apotransferrin. Normal transferrin saturation in adults is approximately 30% (185). Genetic hypotransferrinemia and atransferrinemia are rare disorders characterized by hypochromic microcytic anemia with parenchymal iron overload (186).

Uptake of iron-bound transferrin by cells occurs via receptor-mediated endocytosis of the iron/transferrin complex (187). At neutral pH, diferric transferrin binds with high affinity to the transferrin receptor, which is present as 180-kDa homodimers in coated pits on the plasma membrane. Each 90-kDa transferrin receptor subunit is capable of binding one molecule of transferrin. The differic transferrin/transferrin receptor complex is then endocytosed with the coated pits, becoming coated vesicles and then endosomes. Subsequent acidification of the endosome (to pH about 5.5), possibly in conjunction with a conformational change in the transferrin receptor, leads to the release of iron from transferrin (187). The apotransferrin/transferrin receptor complex is returned to the plasma membrane when the endosome recycles and apotransferrin dissociates from the transferrin receptor at the neutral pH of the cell surface (184).

6.3 Iron Storage

Iron in excess of immediate metabolic requirements is incorporated into the storage protein ferritin, where the iron is maintained in a soluble, inert, and bioavailable form. Mammalian ferritin is a soluble protein shell of approximately 460 kDa, which consists of 24 subunits of two types, H-ferritin (heavy/heart, 21 kDa) and/or L-ferritin (light/liver, 19 kDa). The protein shell can contain up to 4500 atoms of iron in the form of ferric-oxyhydroxide phosphate (187). The configuration of the ferritin subunits allows iron to pass through the apoferritin shell and into the hollow center of the protein. H-ferritin subunits have ferroxidase activity that catalyzes the conversion of iron in the ferrous (Fe^{2+}) form into Fe^{3+}. L-ferritin subunits have a nucleation site that is involved in forming the central iron core from ferric (Fe^{3+}) iron (188). The subunit composition of ferritin depends mostly on multiple transcriptional regulations and varies between specific tissues (187). All tissue sources of ferritin contain at least some H-ferritin subunits, whereas serum ferritin is a homopolymer of L-ferritin subunits (188). The

genes encoding the H and L subunits of human ferritin are on chromosomes 11 and 19, respectively (189).

Ferritin is normally the major iron storage protein present in cells; however, in iron-overloaded cells another storage form, hemosiderin, is found. Hemosiderin is thought to be a degradation product of ferritin (188).

6.4 Iron Regulation Proteins

Iron-dependent regulation of both the transferrin receptor and ferritin occurs post-transcriptionally. If the amount of intracellular chelatable iron is high, translation of transferrin receptor mRNA is downregulated and that of ferritin mRNA is upregulated. In cases of iron deficiency, the converse is true. This regulation is mediated via the iron regulatory proteins, IRP1 and IRP2. When cellular iron levels are low, IRPs are iron deficient and are able to bind to highly conserved, 28-bp stem-loop structures in the mRNA called iron-responsive elements (IREs). The 3′ UTR of the transferrin receptor mRNA contains five potential IREs (190,191), and one IRE is located in the 5′ UTR of the ferritin mRNA (192,193). In iron-depleted states IRPs bind to these IREs, preventing translation of the ferritin mRNA and obscuring a degradation signal in the 3′ UTR of the transferrin receptor mRNA, increasing stability of the mRNA and thus increasing the receptor levels. If intracellular iron levels are high, iron binds to the IRPs and prevents their binding to IREs, allowing translation of ferritin to proceed. Also, transferrin receptor mRNA is no longer protected from degradation. In the presence of iron IRP1 binds an iron sulfur cluster (4Fe-4S) and acquires cytoplasmic aconitase activity while IRP2 undergoes proteolysis when cellular iron is high (194). An IRE has also been identified in the 3′ UTR of Nramp2 mRNA, suggesting that IRPs may be involved in iron-dependent regulation of this protein (177).

7. HEREDITARY HEMOCHROMATOSIS

Our overview of the iron transport system is helpful in understanding the effects of genetic transport disorders. Hereditary hemochromatosis is an autosomal recessive disorder of iron metabolism that affects approximately 1 in 300 in Caucasian populations (185,195). In hemochromatosis, the ability to regulate iron absorption is perturbed and iron uptake occurs in excess of physiological requirements, even in the presence of adequate dietary iron. Hemochromatosis is a progressive, adult-onset disorder. Gradual storage of iron in the parenchymal cells of a variety of organs, primarily the liver, heart, pancreas, pituitary, joints, and skin, eventually leads to tissue damage. Excess iron from environmental sources can exacerbate iron accumulation. Clinical consequences include liver cirrhosis, hepatocellular carcinoma, cardiomyopathy, diabetes mellitus, hypogonadism, arthritis, skin pigmentation, and ultimately premature death if the disorder is not

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treated (196). Treatment for hemochromatosis most commonly involves repeated venesection to remove the accumulated iron (197).

7.1 Mutations of the Hemochromatosis Gene (*HFE*)

The hemochromatosis gene, *HFE*, which encodes a nonclassic MHC class I molecule, was identified by positional cloning on the short arm of chromosome 6 approximately 4 Mb telomeric of HLA-A (198). In this initial study 83% of hemochromatosis patients were found to be homozygous for one missense mutation. This mutation resulted in an amino acid substitution of cysteine to tyrosine at residue 282 (Cys282Tyr, C282Y) (198). Subsequent studies have found at least 80% of hemochromatosis patients to be homozygous for the C282Y mutation (199–201).

A second missense mutation, resulting in a substitution of histidine to aspartic acid at codon 63 (His63Asp, H63D), was also identified (198). Many studies have reported hemochromatosis patients who are homozygous for either H63D or C282Y/H63D compound heterozygotes suggesting that H63D may result in clinically significant hemochromatosis when present in combination with C282Y, albeit with a very low (approximately 1–2%) penetrance (202–206). A third missense mutation (Ser64Cys, S65C) has also been identified in *HFE* and is implicated in a milder form of hemochromatosis (207).

It is noteworthy that excessive iron intake over several years can lead to hemochromatosis in both C282Y homozygotes and those negative for the mutation (208).

7.2 Function of the HFE Protein

HFE is a 343-amino-acid nonclassic MHC class I molecule (198). In contrast to true MHC class I proteins, however, HFE is not capable of binding antigen peptides because the region of the HFE protein that is similar to the peptide-binding groove in class I molecules has been narrowed (209). Since the identification of HFE, results of several functional studies have contributed to our understanding of how C282Y and H63D disrupt normal functioning of this molecule (198,210,211). However, the precise role of HFE in the regulation of iron absorption remains obscure.

MHC class I proteins are presented on the cell surface as a heterodimer in conjunction with β_2-microglobulin. A characteristic feature of both classic and nonclassic MHC class I molecules is four conserved cysteine residues that form intramolecular disulfide bridges essential to maintain the correct secondary and tertiary structure of the protein (212). These four cysteine residues are present in HFE and one is mutated by C282Y, disrupting one of the disulfide bridges and preventing the binding of HFE to β_2-microglobulin. As a result of its failure to complex with β_2-microglobulin, intracellular trafficking and transport of C282Y

mutant HFE to the plasma membrane is disrupted and the protein is not expressed on the cell surface (210). C282Y mutant HFE fails to undergo late Golgi processing, is retained in the endoplasmic reticulum and mid-Golgi compartment, and is subject to accelerated degradation (211,213).

The H63D mutation in HFE is located in the analog of the peptide-binding domain. This mutation does not appear to disrupt the binding of HFE to β_2-microglobulin; the intracellular trafficking and expression of H63D mutant protein on the cell surface does not differ from that of the wild-type molecules (211). The first indication that H63D may be functionally relevant was provided by a study that demonstrated a link between HFE and the transferrin receptor (213). This study showed that wild-type HFE could form a stable complex with the transferrin receptor, resulting in an approximately 10-fold reduction in the affinity of the receptor for iron-bound transferrin. In addition, this study demonstrated that H63D mutant HFE could complex with the transferrin receptor but did not reduce the affinity of the receptor for iron-bound transferrin. This interaction between wild-type HFE and the transferrin receptor, with the subsequent decrease in its affinity for transferrin, is proposed to be responsible for modulating iron uptake into cells (213).

A further study using soluble forms of HFE and the transferrin receptor confirmed the association between these two molecules and used immunoprecipitation to show that a complex of HFE, the transferrin receptor, and transferrin could form (209). The interaction between HFE and the transferrin receptor was also shown to be pH dependent, with the measured affinity being high at pH 7.5, similar to the pH of the cell surface, but not at pH 6.0, which is similar to the pH of the transferrin cycle endosomes. This implies that HFE enters the cell along with the transferrin receptor/transferrin complex and subsequently dissociates at the lower pH of the vesicles (209).

Salter-Cid et al. suggested that rather than reducing the affinity of the transferrin receptor for iron-bound transferrin, wild-type HFE modulates uptake of transferrin-bound iron by reducing the number of competent transferrin receptor molecules on the cell surface and by inhibiting the internalization of the transferrin receptor/transferrin complex (214). Roy et al. propose that HFE acts to block release of the iron from the transferrin receptor/transferrin complex in the endosome (215). These studies have demonstrated a link between HFE and molecules involved in iron metabolism and suggest that the functional importance of HFE may lie in its interaction with the transferrin receptor. Further studies are required to clarify the mechanisms by which HFE regulates dietary iron absorption.

Some evidence suggests that patients with hemochromatosis have increased iron transfer at the basolateral surface of the enterocyte in comparison with normal subjects. A greater portion of an oral dose of radiolabeled iron is transferred to the plasma in these patients, rather than being stored as ferritin in the enterocyte and lost when the cell is sloughed at the end of its life span (216,217). These

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TABLE 1 Allele Frequencies of the Two Common HFE Mutations in Various Populations

Population (no. of chromosomes)	C282Y		H63D		Ref.
	Allele frequency	Homozygote frequency[a]	Allele frequency	Homozygote frequency[a]	
Britain (826)	6.4%	4.1 in 1000	12.8%	16.4 in 1000	Merryweather-Clarke et al. (219)
Germany (230)	3.9%	1.5 in 1000	14.8%	21.9 in 1000	Merryweather-Clarke et al. (219)
France (278)	2.9%	0.84 in 1000	14.7%	21.6 in 1000	Jouanelle et al. (200)
Asia (484)	0%	0	1.9%	0.36 in 1000	Merryweather-Clarke et al. (219)
Africa (1042)	0%	0	2.6%	0.68 in 1000	Merryweather-Clarke et al. (219)
Australasia (644)	0%	0	0.2%	0.004 in 1000	Merryweather-Clarke et al. (219)

[a] Homozygote frequency as calculated from allele frequency.

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findings indicate that the lack of functional HFE somehow leads to increased transfer of iron across the basolateral membrane of the enterocytes. Possibly HFE may play a role in regulating the transfer of iron from the enterocytes across the basolateral membrane into the plasma. In support of this hypothesis, a recent study has demonstrated that expression of NRAMP2 and HFE is oppositely regulated by iron status in a human intestinal cell line. HFE expression may be increased and NRAMP2 expression decreased with iron treatment, consistent with opposing roles for these molecules in iron absorption (218).

Since the identification of the hemochromatosis gene, many studies have been done to determine the prevalence of the two common HFE mutations in different populations as compared with the prevalence of the disease. One comprehensive study showed the C282Y mutation was common among populations of Northern European origin, with the highest frequency being 10% in Irish chromosomes, and was absent from African, Asian, and Australian populations (Table 1). This observation is consistent with the distribution of clinical hemochromatosis. The H63D mutation was found to be both more common than C282Y, with frequencies of up to 30%, and more widely distributed being found in almost all groups examined (219).

8. CONCLUSION

Some of the genes involved in the transport of copper and of iron have been identified. However, even for these metals, knowledge of the transport pathway is incomplete. There are many other metals for which the transport pathways are less well known. We have strong evidence that metals interact, and that the concentration of one metal can have a profound effect on the absorption or excretion of another.

Metals are present in our environment, both as a result of environmental pollution and because of intentional ingestion of nutritional supplements. The exposure to metal that is harmless to some may be destructive to others with specific genetic changes. Understanding the interaction between genes and environment will be of critical importance for avoiding metal toxicity not only for humans, but for a wide variety of other animal species. Study of response to metals presents an exciting new frontier.

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Selenium in Nutrition and Toxicology

Seiichiro Himeno and Nobumasa Imura
Kitasato University, Tokyo, Japan

1. INTRODUCTION

Selenium (Se), which is named after the Greek goddess of the moon (Selene), is located in the same group of VIb as oxygen and sulfur in the periodic table. Together with arsenic, Se is categorized as a metalloid, an intermediate of metals and nonmetals. In the scene of trace element research, Se first appeared as a poisonous element causing ''blind staggers'' and ''alkali disease'' in stock animals in the 1930s. Since 1957, however, when the role of Se for preventing hepatic necrosis caused by vitamin E deficiency was reported (1), physiological functions of Se have been investigated extensively. The findings by two groups in 1973 (2,3) that Se constitutes an active site of glutathione peroxidase (GPx) have provided a logical basis for the role of Se as an antioxidative essential element. Furthermore, the findings that the mortality and morbidity of Keshan disease, an endemic cardiomyopathy in China, were dramatically reduced by supplementation with sodium selenite have promoted the studies on nutritional roles of Se in human health and disease.

Until now, more than 10 selenoproteins other than classical GPx have been isolated, though some of them have not been fully characterized (4). In addition, both epidemiological and experimental data have indicated that Se compounds act as anticarcinogenic agents (5). Recently, application of Se compounds for

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cancer chemoprevention has been tried in the United States. However, Se added to a diet exerts its anticarcinogenic effects in experimental animals at supranutritional levels (10 times higher than that necessary for achieving maximal GPx activity), and the underlying mechanism for anticarcinogenic effects of Se compounds still remains to be elucidated. Since the range of Se concentrations between requirement and toxicity is narrow, the application of Se compounds to humans may require further critical examination on its efficacy and toxicity. In fact, a case of human Se poisoning occurred in a woman who took Se tablets containing 200 times higher Se than indicated (6). In the field of metal toxicology, interactions of Se with mercury, cadmium, or other metals have been well documented. The nutritional status of Se may modulate the toxicity of metals either by forming inert complexes with these metals or by changing the antioxidant potential of the animals through composing selenoproteins. Thus, Se has multiple biological significances as an essential trace element, a modifier of other toxicants, an anticarcinogenic agent, and a toxicant per se. This chapter will overview the whole profile of biological actions of Se in nutrition and toxicology.

2. NUTRITIONAL IMPORTANCE OF SELENIUM

2.1 Source of Selenium

In human nutrition, Se is supplied from both plant and animal foods. Generally, Se concentrations in plants depend on soil Se contents. The major chemical form of Se in plants is selenomethionine (SeMet), while that in animal tissues is selenocysteine (SeCys). Seafood appears to be a good source of Se, but the bioavailability of Se in fish as assessed by the ability to raise GPx activity was reported to be lower than that in wheat (7). Inorganic forms of Se such as selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) are supplied as additives in the table salt or fertilizer in Se-deficient areas. Se-enriched yeast is frequently used for commercially available Se tablets, but it is a mixture of mostly unidentified forms of Se. SeMet content in Se yeast was reported to be only 20% (8).

As shown in Figure 1, average levels of dietary intake of Se and blood Se concentrations in various nations and areas exhibit a linear relationship in a log-scale plotting. China has both a Se-deficient area where Keshan disease occurred and a Se-excess area where human selenosis occurred. New Zealand and Finland are also categorized as Se-deficient areas where Se compounds are supplied to the fertilizer.

2.2 Metabolism of Selenium

Intestinal absorption rates of Se compounds in both humans and animals are generally good (more than 80%) irrelevant to its chemical form (9). The absorption of SeMet is reduced by high concentrations of methionine in the diet, sug-

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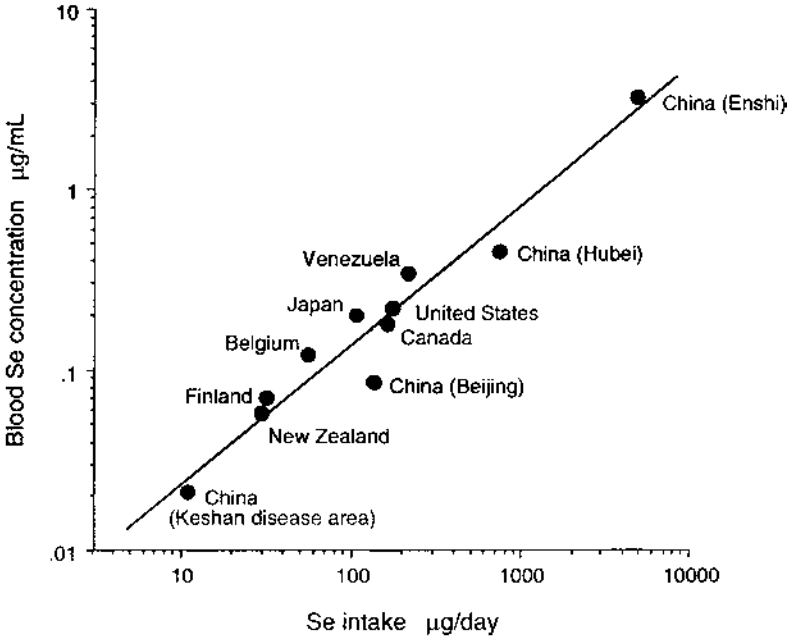

FIGURE 1 Relationship between the average levels of dietary selenium intake and blood selenium among various nations and areas.

gesting that SeMet is taken up from the intestine via the same mechanism as that for methionine (10). SeMet is metabolized in at least two ways (Fig. 2). First, SeMet is converted to SeCys through the pathway similar to the cystathionine pathway for methionine to cysteine, then to selenide by SeCys β-lyase. The second pathway for SeMet is a nonspecific incorporation into general proteins as a substitute for methionine since the tRNA for methionine cannot distinguish methionine from SeMet (11). The ratio of SeMet incorporated into general proteins to that incorporated into SeCys-containing proteins depends on the nutritional status of methionine. When animals are given SeMet in a diet deficient in methionine, the efficacy of SeMet incorporation into SeCys-containing proteins as judged by the increase in GPx activity is lower compared with animals fed a methionine-adequate diet (12). Usually, the proteins in which SeMet is incorporated as a substitute for methionine are not included in functional ''selenoproteins,'' but after the degradation of these proteins, at least a part of SeMet may be used for the synthesis of SeCys-containing selenoproteins. Thus, in the long term, SeMet existing as a methionine substitute in general proteins also serves as a Se pool in the body.

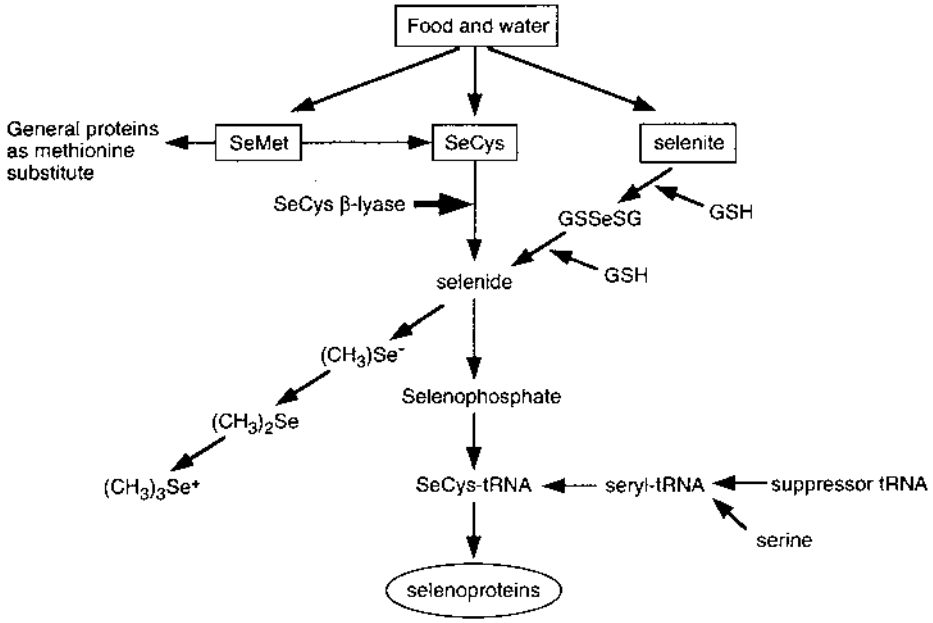

FIGURE 2 Metabolic pathways of selenium in the body.

SeCys in the diet cannot be directly converted to the SeCys moiety of selenoproteins. It needs to be converted to selenide by SeCys β lyase (13), and then is incorporated into selenoproteins. Inorganic Se compounds such as selenite and selenate are reduced by glutathione (GSH) to selenide. Selenide is an important intermediate of Se metabolism not only for synthesis of selenoproteins but also for interactions with other metals.

The major excretory pathway of Se is urine. Fecal excretion of Se is negligible both because intestinal absorption of Se is high and because biliary excretion of Se is low. When excess amounts of Se are given to animals, a part of Se is eliminated from the body by exhalation in a volatile form as dimethylselenide known for its garlic odor. As shown in Figure 2, selenide is sequentially methylated to methylselenol (CH_3Se^-), dimethylselenide [$(CH_3)_2Se$], and trimethylselenonium ion [$(CH_3)_3Se^+$]. Trimethylselenonium ion is detected in urine, although other unidentified forms of Se are also detected in urine. Methylation of Se metabolites is important not only for Se excretion but also for its anticarcinogenic actions (see below).

The whole-body retention of Se varies with nutritional status of Se (9). In Se deficiency, Se excretion becomes minimal to retain the ingested Se in func-

tional selenoproteins. On the other hand, the rate of Se excretion into urine increases with the dose of Se ingested. The percent of Se expired to the breath also increases with the dose of Se, and the exhalation of Se as dimethylselenide is an important pathway of Se excretion especially when a toxic amount of Se is administered to animals.

2.3 Structure and Functions of Selenoproteins

2.3.1 Synthesis of Selenoproteins

Se is contained in the polypeptide chain of selenoproteins in a form of SeCys, the twenty-first amino acid (14). This is a distinguishing characteristic of selenoproteins compared with other metal-binding proteins in which metal ions are bound to the protein as a ligand and the synthesis of an apoprotein precedes the incorporation of metals into the protein. Se is incorporated into selenoproteins at the time of translation of selenoproteins.

The mechanism of SeCys incorporation into the polypeptide chain of selenoproteins has been extensively studied since 1983 when Chambers et al. (15) found the corresponding codon for SeCys to be one of the stop codon UGA. Figure 3 illustrates the so-far-elucidated mechanism of SeCys incorporation into mammalian selenoproteins (16–19). A specific suppressor tRNA that recognizes the UGA codon and is charged with serine plays a key role in the synthesis of selenoproteins. The OH of serine bound to this suppressor tRNA is converted to SeH, thus forming SeCys-tRNA. In a sense, ''selenoserine'' could have been the proper name of this uncommon amino acid. The enzyme responsible for this reaction has been isolated and characterized in bacteria (20), but not yet in mammals (21).

The second key role player in the synthesis of mammalian selenoproteins is a unique stem-loop structure located in the 3′-untranslated region (3′-UTR) of mRNA. This motif is designated SeCys insertion sequence (SECIS) (16). In bacteria, a similar stem-loop structure exists in mRNA but is located just behind the UGA codon for SeCys (22), whereas motifs of SECIS in mammalian selenoprotein mRNAs are located far downstream of the real stop codon (Fig. 3). The protein that binds to SECIS in mRNA may serve as a specific elongation factor for selenoproteins. Several candidate proteins that bind to SECIS have been proposed (23–25), but further evidence may be necessary to fully understand the mechanism of SeCys incorporation via the SECIS motif.

2.3.2 Glutathione Peroxidase

Cellular Glutathione Peroxidase. The biochemical basis for the essentiality of Se was first conferred by the finding that Se constitutes the active site of GPx (2,3). GPx had been the only mammalian selenoprotein with known enzymatic function until the 1990s. Now, a family of GPx comprises four isoforms

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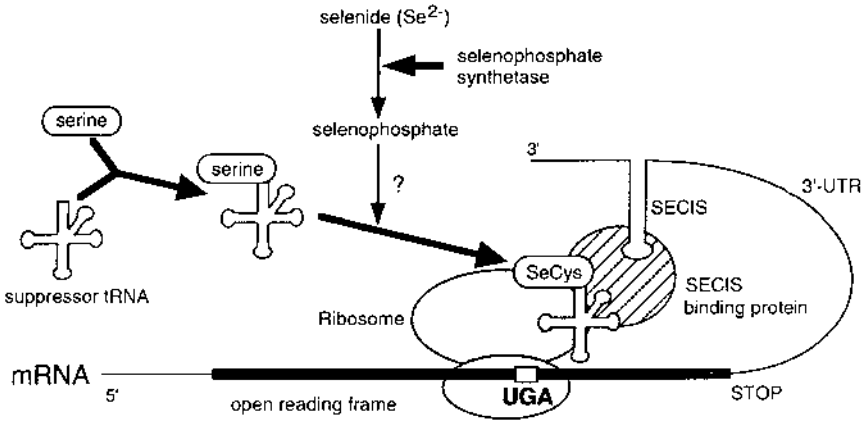

FIGURE 3 Possible mechanism of selenocysteine incorporation into mammalian proteins. SECIS, selenocysteine insertion sequence; 3′-UTR, 3′ untranslated region.

having different localization and substrate specificity (26). The first identified GPx is now called classical or cellular GPx (cGPx or GPX1) owing to its intracellular localization. Extracellular GPx (eGPx or GPX3) is found in the blood plasma (27), milk (28), and bronchoalveolar lavage fluid (29), while GPx-GI (GPX2) is expressed exclusively in gastrointestinal tracts (30). These three isoforms of GPx have a common tetrameric structure. On the other hand, phospholipid hydroperoxide GPx (PHGPx or GPX4) exists as a monomer (31). This isoform can use phospholipid hydroperoxide as a substrate, whereas cGPx or GPx-GI cannot.

The structure and enzymatic properties of cGPx have been most extensively characterized (32,33). Each subunit of cGPx contains one Se atom as a SeCys moiety that constitutes the active center for the reduction of hydroperoxides. Cellular GPx catalyzes the reduction of H_2O_2 and fatty acid hydroperoxide at the expense of oxidation of GSH to GSSG. Since catalase is primarily located in peroxisomes, cGPx is considered to function as a major scavenger for H_2O_2 in the cytosol. However, owing to its inability to catalyze the reduction of phospholipid hydroperoxides (34), it has been argued whether cGPx plays a significant role in the protection of biomembranes against peroxidation. Nevertheless, cGPx is the most abundant selenoprotein in various tissues and cells. Based on the observation that cGPx activity responds to the change in dietary Se intake more sensitively than other selenoproteins, Sunde proposed that cGPx is functioning not only as an antioxidant enzyme but also as a cellular Se pool for other more important selenoproteins (35).

Evidence showing less importance of cGPx has also been provided by the development of cGPx knockout mice, which exhibited no apparent health defects or impairment in growth and reproduction (36). Thus, cGPx may not be essential for survival of animals at least under physiological conditions. Although there was no difference in sensitivity to hyperoxia between cGPx knockout and control mice (36), cGPx knockout mice exhibited greater sensitivities to diquat and paraquat toxicity (37,38). Another model for examining the role of cGPx is guinea pigs, which have extremely low cGPx activity in the liver and kidney (39,40). In guinea pigs, catalase is expressed highly in the cytosol of the liver and kidney, suggesting a compensatory role of catalase for removing H_2O_2 in the absence of cGPx (40). This, in turn, suggests that cGPx is functioning as an important scavenger for H_2O_2 in the cytosol in other animal species. Recently, Beck et al., who had found that avirulent coxsackievirus can be converted to virulent type in Se-deficient mice (41), reported that the same phenomenon was observed in cGPx knockout mice (42), suggesting that cGPx has an important role in protecting viral genome against oxidation-induced mutational conversion that produces the virulent form.

Extracellular Glutathione Peroxidase. Extracellular GPx is synthesized in both the liver and kidney in humans (43) and mainly in the kidney in rodents (44), and is secreted into the blood plasma (45). The K_m and V_{max} values of eGPx for GSH and *tert*-butylhydroperoxide are almost similar to those of cGPx (46). It was shown that eGPx has an ability to use phosphatidylcholine hydroperoxides as a substrate (47). The activity of eGPx in blood plasma has been frequently determined in various human populations in epidemiological studies since it reflects nutritional status of Se well. However, because GSH concentration in plasma is very low (10–50 μM), it remains to be clarified whether eGPx is actually functioning with such a low level of GSH.

Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx). PHGPx was isolated by Ursini et al. (31) as a member of GPx that can directly convert phospholipid hydroperoxide into its alcoholic form. Apart from other tetrameric isozymes of GPx, PHGPx has several unique characteristics, as follows (48–51): (1) it is located in both membrane and cytosol fractions of cells, (2) it has a broad substrate range covering phospholipid hydroperoxides, fatty acid hydroperoxides, cholesterol hydroperoxides, and H_2O_2, (3) its activity is little affected by the change in dietary Se intake, and (4) it is abundantly expressed in the testis under the control of gonadotropin. Because of its broad substrate specificity and localization in the lipophilic milieu, an important role of PHGPx in the protection of biomembranes against peroxidation has been postulated, although less evidence has been provided until recently.

Sequence analyses of cDNAs of PHGPx have demonstrated that rat PHGPx has two translation initiation sites in its mRNA, thereby leading to the synthesis

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of long (23 kDa) and short (20 kDa) forms of PHGPx from the same gene (52). The long form of PHGPx contains a signal peptide for transport into mitochondria, and is cleaved out to form a 20-kDa mitochondrial PHGPx (53). On the other hand, the short form without a signal peptide is located in the cytosol or associated with intracellular membranes. Imai et al. established several transformants of rat basophilic leukemia cells (RBL-2H3) in which either mitochondrial or nonmitochondrial PHGPx was overexpressed, and analyzed the role of each form of PHGPx (54–56). Mitochondrial PHGPx was found to suppress the apoptotic cell death via the mitochondrial death pathway that is caused by ultraviolet irradiation or glucose deprivation, whereas nonmitochondrial PHGPx was ineffective in suppressing the apoptosis mediated by the mitochondrial lesion (56). On the other hand, overexpression of nonmitochondrial PHGPx suppressed the activity of 5-lipoxygenase by reducing the levels of fatty acid hydroperoxides (55). Interestingly, after the transfection of nonmitochondrial PHGPx, it is abundantly associated with the nuclear fraction, where 5-lipoxygenase is also translocated upon its activation. Because the suppression of 5-lipoxygenase results in a decrease in the formation of leukotrienes, PHGPx may have a function as a modulator of leukotriene levels in addition to its role as a protector against oxidative stress.

Se deficiency is known to cause impaired motility and structural disorder of sperm, leading to male infertility in animals (57–59). Since a high concentration of Se is detected in the midpiece of sperm where mitochondria are abundant, Se-containing protein in the midpiece has been considered to be associated with the maintenance of structure and/or function of sperm (60). Recently, Se-containing protein in mitochondrial capsules of midpiece in rat sperm was identified as PHGPx, which accounted for 50% of the total material in the capsule (61). However, PHGPx protein in the mitochondrial capsule was recovered primarily from the insoluble fraction as a polymerized form and was inactive. On the other hand, active PHGPx was detected in round spermatids derived from testicular tubules. This is consistent with the observation that the mature spermatozoa prepared from the epididymis have less PHGPx activity, while the activity of PHGPx in testis is highly elevated especially at the time of pubertal maturation of rodents. These results suggest that PHGPx functions differently at each step of sperm maturation, i.e., as an active soluble enzyme in spermatid but as an insoluble structure protein of the mitochondrial capsule after the maturation into spermatozoa.

Gastrointestinal Glutathione Peroxidase. Gastrointestinal GPx (GPx-GI) was identified as a new gene having homology to Gpx (30), and it was shown to be expressed exclusively in the epithelial cells of gastrointestinal tracts (62,63). The structure and enzymatic properties of GPx-GI are close to those of cGPx, but the specific role of GPx-GI remains obscure.

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2.3.3 Other Selenoproteins

Selenoprotein P. Approximately half of Se in the blood plasma in humans and rats is associated with selenoprotein P, while eGPx accounts for only 20–30% of plasma Se (64–66). Selenoprotein P is synthesized in many organs and secreted into the blood plasma as a 55–60-kDa glycoprotein. The sequence analyses of rat and human selenoprotein P cDNA revealed that selenoprotein P in both species contains 10 in-frame UGA codons encoding SeCys and two SECIS motifs in 3′-UTR (67,68). However, peptide analysis of the mature protein isolated from rat plasma showed only 7–8 SeCys residues per molecule (69,70). This discrepancy has not yet been elucidated. Recently, the second UGA codon in rat selenoprotein P was shown to have dual functions for both SeCys insertion and translation termination (70). Since four out of 10 SeCys residues are located in the C-terminal 15 amino acids, it seems likely that selenoprotein P is actually a mixture of approximately similar-molecular-weight proteins terminated at either of the four UGA codons located in the C-terminal region, which may explain why the number of SeCys residues is less than 10 per molecule on the average.

When ^{75}Se-labeled selenite is administered to rats, the maximum level of ^{75}Se-labeled selenoprotein P can be detected in the plasma within a few hours, while the ^{75}Se incorporation into eGPx reaches the maximum level in a few days (65,66). This quick response of selenoprotein P to Se supply compared with other selenoproteins suggests that selenoprotein P is synthesized and secreted into the blood immediately after Se supply so as to exert a critical role for animals. To date, either possibility that selenoprotein P has an antioxidant function or is involved in Se transport has been envisaged. Burk et al. suggested an antioxidant role of selenoprotein P based on the observation that only the enhanced level of selenoprotein P can explain the protection by selenite administration against diquat-induced hepatotoxicity in Se-deficient rats (71). They showed that pretreatment with selenite just 8 h prior to diquat injection reversed the level of selenoprotein P close to the normal level and prevented diquat-induced hepatic injury in Se-deficient rats, whereas little change in eGPx activity was observed at that time. Also, Burk et al. added another piece of evidence on how this extracellular protein is involved in the protection of hepatic tissues (72). Selenoprotein P contains a histidine-rich region that gives selenoprotein P an affinity to heparin (73). Histochemical examination revealed that selenoprotein P is located at the surface of endothelial cells, indicating that selenoprotein P is anchored to heparan sulfate moieties of proteoglycans at the plasma membrane of endothelial cells via a histidine-rich stretch of this protein (72). Thus, selenoprotein P may function as an extracellular protectant against oxidant injury in a similar way as does extracellular superoxide dismutase, which also has heparin-binding ability and is associated with endothelial cells of blood vessels (74). However, the catalytic activity of selenoprotein P as an antioxidant enzyme remains unexplained. Recently, Saito

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et al. demonstrated that selenoprotein P purified from human plasma has weak PHGPx activity (75), although this activity may not account for whole functions of selenoprotein P.

The other possible explanation for the function of selenoprotein P is that it is involved in Se transport, although little evidence have been provided (76). Recently, a serum factor necessary for promoting growth of cultured neuronal cells, which never grow in the absence of serum in the medium, was purified and identified as selenoprotein P (77). The addition of antibody against selenoprotein P in the serum abolished the growth of neuronal cells. Interestingly, in an in vivo experiment with Se-deficient and Se-sufficient rats, enhanced Se uptake by the tissues of Se-deficient rats was observed only in the brain when purified selenoprotein P was intravenously injected into the rats of both groups (78). One possibility suggested by this evidence is that selenoprotein P is used for Se transport into neuronal cells by a yet-unknown mechanism. However, compared with other metal transporters such as transferrin to which metals are coordinated as ligands, utilization of selenoprotein P as a Se carrier appears to be rather inefficient because it requires synthesis, secretion, and degradation of a protein for the transfer of an element. Whether this inefficient pathway of Se transfer is actually functioning in the body requires further critical verification.

Selenoprotein W. Selenoprotein W is a low-molecular-weight (9.5–10.0 kDa) muscle selenoprotein with unknown function (79,80). Sequence analysis of selenoprotein W cDNA showed that SeCys encoded by UGA exists in the N-terminal region (81–83). Four isoforms of selenoprotein W have been identified by mass spectrometry (84,85). These isoforms were found to arise from a single protein conjugated with GSH or other unknown small molecules as adducts. In rodents, selenoprotein W is found not only in skeletal muscle, but also in brain, spleen, and testis, but not in the heart, while the levels of selenoprotein W in the heart and muscle increased well in response to selenite supplementation in sheep (86). Since both white muscle disease in domestic animals (87) and Keshan disease in humans (88) are related to muscle dysfunction, elucidation of the biochemical function of selenoprotein W in humans and domestic animals is of importance to explore the etiology of these diseases.

Thyroid Hormone Deiodinase. Thyroid hormones consist of thyroxine (T_4) and triiodothyronine (T_3), the latter being derived from the deiodination of T_4 at the 5′-position in the outer phenolic ring. Type I deiodinase that is responsible for the 5′-deiodination was identified as selenoprotein by two groups independently in 1990 (89,90). In the next year, the cDNA of type I deiodinase was cloned, and the deduced sequence confirmed the existence of SeCys encoded by UGA (91). The Cys-for-SeCys mutant of this protein exhibited less than 1% deiodination activity compared with the wild-type protein, indicating that SeCys residue in type I deiodinase serves as an active site for the reductive cleavage

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of C-I bond in thyroid hormones (92). Furthermore, homology sequencing has identified both type II and type III deiodinases also as selenoproteins (93–97). Type I deiodinase catalyzes deiodination at both the 5′- and 5-positions for activation of T_4 to T_3 and for inactivation of T_4 to rT_3, respectively, and is expressed highly in the liver, kidney, and thyroid glands in humans and animals. Type II deiodinase can catalyze deiodination only at the 5′-position with low K_m, and is expressed in the central nervous system, pituitary gland, and brown adipose tissues in rodents, but is expressed rather widely in human tissues including skeletal muscle, heart, and placenta. Type III deiodinase catalyzes 5-deiodination alone and is expressed primarily in the placenta, suggesting a function as a suppressor for transfer of T_4 and T_3 to the fetus. The effects of Se deficiency on thyroid hormone levels are rather complicated owing to dual functions of deiodination enzymes for both activation and inactivation of T_4 and T_3, and the existence of feedback systems for maintaining levels of T_4 and T_3 (98,99). In addition, Se concentration in the thyroid gland is conserved well and hardly affected by dietary Se deficiency (100).

Thioredoxin Reductase. Thioredoxin reductase (TRx reductase) catalyzes NADPH-dependent reduction of thioredoxin (TRx) (101). TRx is a low-molecular-weight protein that can reduce protein disulfide, and thereby is involved in redox regulation of numerous cellular events such as activation of transcription factors, DNA-protein interaction, and cellular proliferation as well as protection against oxidative injury (102,103). Although rat liver TRx reductase was first isolated and characterized in 1982 (104), it was not recognized as a selenoprotein until 1996 (105). In 1995, the full-length cDNA of human TRx reductase was cloned, and the deduced amino acid sequence showed only 24% identity to bacterial TRx reductase but 44% identity to human GSSG reductase (106). The major difference between bacterial and human TRx reductase arises from the additional 150 amino acid residues in the C-terminal domain of human TRx reductase (Fig. 4). In 1996, Tamura and Stadtman purified the ^{75}Se-labeled 57-kDa protein expressed in human lung adenocarcinoma cells, and found that this protein has TRx reductase activity (105). Determination of the C-terminal amino acid sequence of purified protein revealed that the C-terminus (Gly-Cys) predicted from the cDNA sequence was actually followed by two more amino acid residues including SeCys (Fig. 4). Thus, the UGA codon in TRx reductase, previously considered a stop codon, encodes SeCys (107). A motif of SECIS in 3′-UTR of TRx reductase mRNA was also identified in rat TRx reductase (108). Recently, isoforms of TRx reductase located in mitochondria have also been identified from human (109), mouse (110), rat (111), and bovine (112) tissues.

A distinct property of mammalian TRx reductase is its location of SeCys in the penultimate position at the C-terminus. Several pieces of evidence have indicated a crucial role of C-terminal SeCys residue as the second active center

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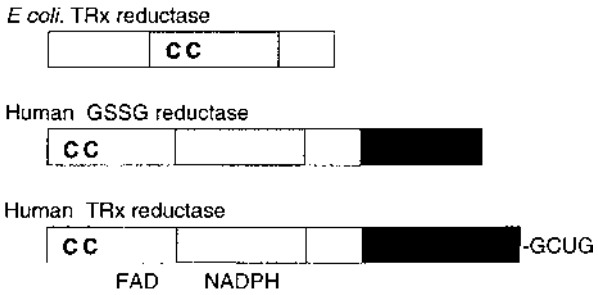

FIGURE 4 Schematic structure of *Escherichia coli* and human thioredoxin reductase. C C, active center with two cysteine residues; FAD, flavin adenine denucleotide (FAD)-binding domain; NADPH, NADPH-binding domain; GCUG, Gly-Cys-SeCys-Gly.

of mammalian TRx reductase. Hill et al. reported that rats fed a Se-deficient diet exhibited a decrease in TRx reductase activity (113). They also showed that aurothioglucose, which can react with SeCys moiety, inhibited TRx reductase activity at a much lower concentration than that necessary for the inhibition of cGPx. Furthermore, partial digestion of the C-terminal domain of purified TRx reductase by carboxypeptidase resulted in a loss of TRx reductase activity (108).

Existence of C-terminal SeCys residue may also contribute to the broad substrate specificity of mammalian TRx reductase toward lipoic acid, vitamin K_3, alloxan, dehydroascorbic acid, and ascorbyl free radical, whereas bacterial TRx reductase can reduce oxidized TRx alone (114). In fact, rats fed a Se-deficient diet exhibited a decrease in ability to reduce dehydroascorbic acid to ascorbic acid (115). In addition, C-terminal SeCys appears to be responsible for greater sensitivity of TRx reductase to inhibition by aurothioglucose, imidazoles, quinones, and alkylating agents at the magnitudes of three orders compared with that of cGPx (114). Ganther suggested a possibility that intermediates of Se metabolism such as methylselenol may also react with SeCys in TRx reductase to form a diselenide bond (114). Recently, Sun et al. demonstrated that SeCys residue in TRx reductase can be oxidized by reactive oxygen species (ROS), which in turn induces enhanced expression of TRx reductase itself (116). This suggests that TRx reductase is acting as a sensor for the increase in cellular ROS using its C-terminal SeCys and subsequently modulates the redox status of cells by its own activation.

Other Newly Identified Selenoproteins. Selenophosphate synthetase (SPS) is an enzyme that produces selenophosphate from selenide in an ATP-

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dependent manner (117). Selenophosphate is used as a donor for the synthesis of SeCys tRNA from seryl tRNA (Fig. 3). Human SPS1 and SPS2 have been found as homologs of the product of bacterial *SelD* gene, which catalyzes the production of selenophosphate in bacteria. The putative catalytic center of SPS2 contains SeCys (118), while threonine in SPS1 (119) and cysteine in *SelD* product (120) constitute the active site. However, biochemical evidence that SPS1 or SPS2 contributes to the production of selenophosphate in mammals has not yet been provided.

Another selenoprotein with molecular size of 15 kDa was isolated from human Jurkat T cells by labeling proteins with ^{75}Se selenite (121). The cDNA sequence of this protein confirmed an inframe UGA codon encoding SeCys in the middle of the coding region and a SECIS motif in 3′-UTR. This 15-kDa protein is expressed highly in the prostate, thyroid, and parathyroid glands. The amino acid sequence of this protein exhibited little homology to other known selenoproteins and biological functions remain unknown.

By using in vivo labeling of tissue or cellular proteins with ^{75}Se and subsequent resolution of each protein or subunit by SDS-PAGE analysis, Behne et al. predicted the existence of about 30 selenoproteins in mammals (122). Only 14 of them have so far been characterized in terms of gene and function (Table 1). The ^{75}Se-labeling technique appears to be a productive approach in identifying novel selenoproteins as in the case of the 15-kDa protein (121). However, isolation of selenoproteins by this method encounters difficulty when the proteins are expressed at low levels. Recently, two groups independently developed a screening method to find a gene containing a SECIS motif in its 3′-UTR using a computer protocol for searching the gene database (123,124). Gladyshev's group identified two novel human genes, *SelT* and *SelR*, which contained inframe UGA codons and a SECIS motifs in 3′-UTR (123). The products of these genes were detected as Se-containing proteins in various human tissues. Krol's group also identified several novel human genes containing functional SECIS motifs in their 3′-UTR, and designated them *SelN*, *SelX*, *SelZfl*, and *SelZf2* (124). *SelX* appears to be identical to *SelR* reported by Gladyshev's group, while *SelZf1* and *SelZf2* seem to be homologs of mitochondrial TRx reductase gene but with different N-terminal structures. Although biological functions of these novel selenoproteins have not yet been established, ''digital cloning'' from the gene database may further permit the detection of important but not highly expressed selenoproteins.

In 1997, Bösl et al. demonstrated that the disruption of the gene for SeCys tRNA in the genome of mice led to embryonic lethality (125). This finding indicates that mice cannot survive if biosynthesis of all SeCys-containing proteins is hampered. This is the first evidence based on molecular mechanism that the micronutrient Se is essential for survival of mammalian species. However, the disruption of the cGPx gene alone did not result in any apparent disorder in mice

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TABLE 1 Selenocysteine-Containing Proteins in Mammals

Selenoprotein	Function	Location
Cellular glutathione peroxidase (cGPx or GPX1)	Removal of hydroperoxides	Ubiquitous
Gastrointestinal glutathione peroxidase (GPx-GI or GPX2)	Removal of hydroperoxides	Gastrointestinal tract
Extracellular glutathione peroxidase (eGPx or GPX3)	Removal of hydroperoxides	Plasma
Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPX4)	Removal of phospholipid hydroperoxides	Ubiquitous, testis
Selenoprotein P	Antioxidant? Se transport?	Plasma
Selenoprotein W	?	Ubiquitous muscle
Type 1 triiodothyronine deiodinase	Conversion of T4 to T3, T4 to rT3	Thyroid gland, liver, kidney
Type 2 triiodothyronine deiodinase	Conversion of T4 to T3	Pituitary gland, central nervous system, brown adipose tissue
Type 3 triiodothyronine deiodinase	Conversion of T4 to rT3	Placenta
Thioredoxin reductase (TRxR1)	Reduction of thioredoxin	Ubiquitous (cytosol)
Thioredoxin reductase (TRxR2)	Reduction of thioredoxin	Ubiquitous (mitochondria)
Thioredoxin reductase (TRxR3)	Reduction of thioredoxin	Testis
Selenophosphate synthetase 2 (SPS2)	Synthesis of selenophosphate	Ubiquitous
15-kDa selenoprotein	?	Ubiquitous
SelT	?	?
SelR/SelX	?	?
SelN	?	?

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under physiological conditions (36). Thus, identification of further selenoproteins and disruption of each gene may be required for fully understanding the essential role of Se in mammals.

2.4 Diseases Associated with Selenium Deficiency

2.4.1 Keshan Disease

Keshan disease is an endemic cardiomyopathy, which was prevalent in a belt-like zone stretching from northeast to southwest China where Se contents in the soil and crops are extremely low (88,126–129). The pathological features of this disease include multifocal myocardial degeneration, marked ventricular dilation, and fibrous replacement of myocardium (128). Se concentrations in hair and blood samples and blood cGPx activity of residents in endemic areas were markedly lower than those in nonaffected areas. A large-scale intervention study with sodium selenite among several thousand residents living in a Se-deficient area revealed that Keshan disease is Se-responsive. The death rates of Keshan disease patients in a Se-supplemented group exhibited a significant decrease compared with those in a placebo group. Thus, Se deficiency is considered to be the primary cause of Keshan disease.

However, the incidence of Keshan disease is greatly affected by various factors including area, season, and residents' age, sex, and occupation (126). It also varies greatly from year to year. For example, in the southwest area, the incidence of Keshan disease is high in children 2–10 years old, while women of childbearing age are mainly affected in the northeast area. Also, the peak of the incidence is observed in summer in the southwest area but in winter in the northeast area. Therefore, it has been postulated that additional biological factor(s) may be involved in the etiology of Keshan disease, although Se deficiency figures predominantly in the origin of this disease.

Recently, Beck et al. suggested an involvement of coxsackievirus B, which belongs to enterovirus and produces myocarditis in both humans and mice, in the development of Keshan disease (41,130). Another study in China has already demonstrated that pathological changes of mouse heart caused by coxsackievirus B are enhanced by Se deficiency (131). Beck et al. used an avirulent strain of coxsackievirus (CVB3/0) that does not cause heart damage in normal mice (41). When the mice fed a Se-deficient diet were inoculated with CVB3/0, moderate heart damage was observed, while no change was seen in Se-adequate mice. Furthermore, Beck et al. harvested the virus from the heart of Se-deficient mice challenged with CVB3/0, and then inoculated them into the mice fed a Se-adequate diet. Interestingly, these mice exhibited heart damage, suggesting that the benign CVB3/0 strain was changed to a virulent strain by replication in the host where Se is deficient. Sequence analysis of the gene of CVB3/0 strain recovered from Se-deficient mice revealed base changes in six out of seven sites that are

considered to be important determinants for virulence. The nucleotide sequence of the mutated CVB3/0 gene resembled that of the wild-type virulent strain (CVB3/20).

Thus, Se deficiency in hosts allowed a benign virus to acquire virulence (cardiotoxicity) owing to base changes in the viral genome. As mentioned previously, the same phenomenon was observed also in cGPx knockout mice inoculated with the CVB3/0 strain, indicating that cGPx is involved in the protection of viral genome mutagenesis possibly triggered by oxidative stress (42). Although the pathological features of virus-induced myocarditis and those of Keshan disease are not exactly identical, infection with coxsackievirus B appears to be a candidate for a factor superimposed on Se deficiency in the etiology of Keshan disease. Moreover, these studies suggest a novel role of antioxidant nutrient Se as a modulator of the virulence of infectious virus (131).

2.4.2 Other Cardiovascular Disease

Numerous epidemiological studies have been performed to examine whether Se deficiency in humans also enhances the risk of other heart diseases (132). Most studies have focused on the association between Se deficiency and coronary heart disease since the development of atherosclerosis is known to involve excess formation of ROS and oxidation of low-density lipoproteins.

An early study conducted by Salonen et al. in Finland demonstrated a significantly increased risk of cardiovascular deaths and incidence of myocardial infarction in the low-Se group with serum Se concentration less than 45 μg/L at the time of prediagnosis (133). However, other studies have produced conflicting results (134). In the European Antioxidant Myocardial Infarction and Breast Cancer Study (EURAMIC study) covering nine countries in Europe, no apparent association between toenail Se concentration, which reflects long-term Se intake, and the risk of myocardial infarction was observed (135). In a 3-year follow-up study of over 3000 males in Denmark, men with serum Se concentration less than 1 μmol/L (79 μg/L) exhibited a slightly increased relative risk (1.70) for ischemic heart disease, but it was reduced to 1.55 after the adjustment for cholesterol, social class, smoking, and age (136). A nested case-control study among physicians in the United States demonstrated no evidence of association between plasma Se levels and risk of myocardial infarction (137).

One possible factor causing inconsistency among these epidemiological studies may be a regional variation of Se intake. In the Finnish study, the serum concentration of Se in the low-Se group was set as <45 μg/L (133), whereas the lowest quintile of plasma Se concentration was <92.5 μg/L in the U.S. physicians' study (137). In the EURAMIC study, a significant contribution of toenail Se concentration to the odds ratio of myocardial infarction was observed only in Germany (Berlin) where the average toenail Se concentration was the lowest among nine countries (135). Thus, positive evidence showing a relationship between indi-

cators for Se nutrition and the risk of coronary heart disease has been observed only in markedly low-Se areas. Huttunen suggested that there is a threshold of Se intake for enhancing the risk of cardiovascular disease (134). Among the people whose Se intake is over the threshold level, the risk of cardiovascular disease may not be altered by further Se supplementation. In support of this hypothesis, no significant change in the incidence of coronary heart disease was observed in a large-scale intervention trial for cancer chemoprevention, in which 200 μg Se was daily supplemented to the participants for several years (138).

Generally, development of atherosclerosis in coronary arteries is associated with a high-fat diet and resultant high-cholesterol plasma, which is prevalent in industrialized countries. On the other hand, Keshan disease is a cardiomyopathy that involves no pathological changes in coronary arteries (128). Therefore, although Se deficiency is a predisposing factor for both types of heart disease, different mechanisms may be operating between coronary heart disease and endemic cardiomyopathy like Keshan disease. Similarly, it seems unlikely that Se-responsive heart failures occasionally observed in patients receiving long-term total parenteral nutrition (TPN) involve atherosclerotic events caused by a high-fat diet.

In the development of atherosclerosis, alterations in arachidonic acid metabolism by dietary Se intake may be involved. It has been reported by in vivo and in vitro studies that Se deficiency results in a decrease in the release of prostacyclin by endothelial cells (139,140) and it is reversed by Se supplementation (141,142). Se supplementation also reduces the production of thromboxane A_2 by platelets (143,144). Since both events are known to be regulated by ''peroxide tone'' in plasma, changes in the level of fatty acid hydroperoxide caused by Se deficiency may induce decreased release of prostacyclin and increased production of thromboxane A_2, both of which are known to promote the platelet aggregation and then the formation of atherosclerosis.

2.4.3 HIV Infection and Selenium

Increasing amounts of evidence have indicated that Se deficiency is involved in pathogenesis and progression of acquired immune deficiency syndrome (AIDS) caused by infection with human immunodeficiency virus (HIV) (145). Several studies have documented that AIDS patients show moderate Se deficiency (146–148). On the other hand, follow-up studies on HIV-positive patients have indicated that a low level of plasma Se detected in the initial phase is a predictive factor for greater progression of the disease and higher mortality (149,150). Thus, it seems likely that HIV infection causes Se deficiency in patients and the resultant Se deficiency then enhances the replication of HIV and progression of the disease.

The reason for the disturbance in Se metabolism in HIV-positive patients is unclear. Recently, the effect of in vitro HIV infection on the synthesis of seleno-

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proteins was examined in human T lymphocytes by determining the incorporation of ^{75}Se into each selenoprotein by SDS-PAGE analysis (151). The HIV-infected T lymphocytes exhibited an overall reduction of ^{75}Se incorporation into known selenoproteins and a characteristic increase of unidentified ^{75}Se-containing protein migrating at around 6–7 kDa, which was not detected in uninfected cells. This suggests that a portion of Se was trapped by this low-molecular-weight protein, leading to the reduction of Se incorporation into other selenoproteins in HIV-infected cells.

Computer analyses of HIV genome have provided an intriguing explanation for the disturbance in Se metabolism in AIDS patients, though not yet verified by biological evidence. Taylor et al. identified potential sequences encoding GPx-like modules with UGA codons in the -1 shifted frame of the HIV genome (152). They suggested that the expression of these genes encoding selenoproteins by ribosomal frame shifting, which is not uncommon in the gene expression of HIV, results in the deprivation of Se from host cells, thereby leading to a suppression of Se-dependent antioxidative protection against virus infection in host cells (152,153). However, it remains unclear whether selenoproteins are actually synthesized from these modules in viral genome, and whether the 6–7 kDa selenoprotein observed in the HIV-infected human T lymphocytes is related to this viral gene.

As to the mechanism by which Se deficiency promotes the replication of HIV, both the suppression of immunological responses and the activation of NF-κB caused by enhanced oxidative stress may be involved. The activation of NF-κB is known to play a key role in HIV gene expression and viral replication (154). Since a variety of oxidative stimuli enhance the activation of NF-κB, reduced ability of scavenging ROS may enhance the replication of HIV. In fact, the addition of Se in the medium of HIV-infected monocytes suppressed the HIV replication activated by tumor necrosis factor-α (155). In support of this notion, Se supplementation was shown to suppress the activation of NF-κB by H_2O_2 or tumor necrosis factor-α in cultured T lymphocytes (156). However, the mechanism underlying the modulation of HIV replication by Se requires further critical study because there is a contrasting report showing enhanced replication of HIV in cGPx-overexpressed cells (157).

3. SELENIUM AND CANCER

3.1 Epidemiological Evidence

Over the last 30 years, a number of epidemiological studies have been conducted to examine the relationship between dietary Se intake and the risk of cancer. Strategies of these studies can be categorized as follows: (1) geographical studies that compare cancer death rates and average Se intake among each area, (2) case-

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control studies that compare the levels of Se intake between cancer patients and controls retrospectively, (3) prospective studies including nested case-control studies that determine the levels of Se intake before the onset of cancer and follow the subsequent occurrence of cancer, and (4) intervention trials that examine the preventive effect of supplementation with Se compounds.

An early study by Shamberger et al. demonstrated an inverse relationship between cancer death rates and average blood Se concentrations in 19 cities of the United States (158). Schrauzer et al. also reported inverse correlations between the mortality rates for cancers of colon, breast, and other organs and the estimated Se intake in 27 countries (159). In China, where there is a large regional variation in Se intake, an inverse relationship between blood Se levels and total cancer death rates in eight provinces was reported (160). However, these geographical studies suffer greatly from other confounding factors such as intake of other nutrients, exposure to different carcinogens, and demographic characteristics of the subject population.

Most of the case-control studies conducted in various countries have demonstrated lower blood Se levels in patients with breast, ovary, skin, lung, and gastrointestinal cancer than in controls (161,162). However, owing to the shortcomings of case-control study, one cannot conclude whether the reduction of blood Se levels is the cause or the consequence of cancer. Progression of cancer itself may compromise the metabolism of Se by trapping greater amount of Se into rapidly growing cancer tissues.

To investigate the causal relationship between Se and cancer, prospective studies may be a better approach. A nested case-control study is a sophisticated and inexpensive way of conducting a prospective study in which cases and controls are selected from individuals whose blood samples have already been collected and stored for other large-scale cohort studies. Using a large cohort of a hypertension study in the United States, Willett et al. compared Se levels in the serum samples that were collected at the time of prediagnosis among cancer patients and matched controls (163). They found that the subjects in the lowest quintile of serum Se had excess risk for cancer compared with those in the highest three quintiles. In Finland, Salonen et al. conducted nested case-control studies using two independent cohorts (164,165). Both studies demonstrated that the lowest Se group exhibited excess risk for cancer. However, most of the other prospective studies conducted thereafter in various countries have yielded no evidence for the relationship between Se levels and cancer risk (161). Recently, additional evidence showing a positive role of Se in reducing cancer risk was provided by a nested case-control study on prostate cancer (166). Toenail samples were collected from more than 30,000 men in the United States in 1987, and 181 cases of advanced prostate cancer were reported from 1989 to 1994. The odds ratio for the highest to lowest quintile of toenail Se was 0.49, suggesting that higher Se intake is associated with lower incidence of prostate cancer.

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Preventive effects of Se on the occurrence of cancer can be more directly evaluated by intervention studies. Large-scale intervention trials have been conducted in China and the United States. In Linxian in east China, where the prevalence and mortality ratio of esophageal cancer are among the highest in the world, approximately 30,000 residents were supplemented with vitamins and minerals including Se daily for 5.25 years (167,168). The rates of mortality of total cancer and stomach cancer were significantly reduced in the group receiving the combination of β-carotene, vitamin E, and Se (50 μg as Se yeast), while the other three groups, receiving the combinations retinol and zinc, riboflavin and niacin, or vitamin C and molybdenum, did not show any changes in cancer mortality.

Recently, a randomized and placebo-controlled intervention study was conducted in the United States (138). Patients ($n = 1312$) with a history of basal cell or squamous cell carcinomas of the skin were supplemented with tablets of Se yeast that contains 200 μg Se or placebo tablets for 4.5 years and were followed for 6 years. Se supplementation did not reduce the recurrence of skin cancer, as was the primary goal of this study. Unexpectedly, however, the Se-supplemented group exhibited significant reductions in relative risks of total cancer mortality (0.50), total cancer incidence (0.55), and incidences of lung (0.54), colorectal (0.42), and prostate (0.37) cancers. The results of this study need to be evaluated with caution because the subject population has a bias compared with the general population; i.e., they had already suffered from a nonmelanoma skin cancer prior to the trial. In addition, since the forms of Se in Se-enriched yeast remain unidentified, the reproducibility of this study should be carefully evaluated. Nevertheless, the reduction of cancer death rate and incidences of leading cancers by about 50% by Se supplementation has had a strong impact and raised the possibility that Se compounds can be used as agents for cancer chemoprevention in humans.

3.2 Experimental Evidence

The anticarcinogenic effects of Se compounds have been extensively investigated using various carcinogenesis models in experimental animals. Overall, evidence so far obtained has indicated that several Se compounds can act as anticarcinogenic agents, but positive effects are usually observed when supranutritional levels of Se are given to animals. Earlier studies had used selenite or SeMet as a source of Se, but numerous synthetic Se compounds have been developed and tested for their actions during the last 15 years. Recent findings with novel Se compounds have provided new insight into the dose-response relationship and the mechanism of anticarcinogenic effects of Se (5).

It has been shown that the addition of selenite in the diet significantly reduced the rate of tumor formation evoked by chemical carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA) (169,170), *N*-methyl-*N*-nitrosourea (171),

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and 1,2-dimethylhydrazine (172) in rats. The effects of dose and chemical forms of Se and the duration and timing of Se supplementation have been extensively examined by the group of Ip using a model of DMBA-induced mammary gland tumor formation in rats (173–179). Their findings include: (1) addition of selenite in the diet at levels of 2–5 ppm Se is most effective, (2) SeMet is less effective than selenite, (3) Se is effective at both initiation and postinitiation of cancer, (4) vitamin E can modulate the effective dose of Se but cannot compensate for Se actions, and (5) the increase in selenoenzymes such as cGPx is not associated with anticarcinogenic actions of Se.

The most important point of their findings is that Se exerts its anticarcinogenic effect at ''pharmacological'' or ''supranutritional'' doses. The minimum essential level of dietary Se for animals is known to be 0.1 ppm, and the activity of selenoenzymes such as cGPx reaches the plateau level at around 0.4 ppm Se in the diet. Thus, an approximately 10 times higher dose of Se than the nutritionally necessary level is required for suppressing carcinogenesis in a DMBA model. Furthermore, the rats given 3–5 ppm Se as selenite for a long time exhibited a decrease in body weight gain, suggesting that the effective dose of Se for anticarcinogenic action is close to that for toxic effects.

To elucidate the role of the metabolic pathway in cancer chemoprevention by supranutritional levels of Se, Ganther and his co-workers synthesized a variety of precursors for methylated Se metabolites, and tested their effects on DMBA-induced carcinogenesis in collaboration with Ip (180,181). Selenobetaine and Se methylselenocysteine are precursors for methylselenol and are metabolized in a distinct way compared with selenite (Fig. 5). After conversion to methylselenol, the greater part of them enters the methylation pathway to form dimethylselenide and trimethylselenonium, and little is used for the formation of selenide at least under Se-adequate conditions (182–184). When rats were given selenite, selenobetaine, or Se methylselenocysteine in the diet at the same Se levels, selenite and selenobetaine exhibited similar anticarcinogenic effects and Se methylselenocysteine exhibited greater effect in a DMBA-induced mammary tumor model (180,181). In contrast, dimethylselenoxide, which undergoes rapid conversion to dimethylselenide and is expired to the breath, exhibited low activity of cancer chemoprevention (181). Trimethylselenonium, the form of Se found in urine, exhibited no chemopreventive effect (185). Thus, the monomethyl form of Se may be responsible for chemoprevention by selenobetaine and Se methylselenocysteine. The efficient anticarcinogenic effect of Se methylselenocysteine is noteworthy, since this form of Se was found to be the major component of Se-enriched garlic (186), which also has a potent anticarcinogenic effect (187).

In the search for Se compounds having higher efficacy in cancer chemoprevention and lower toxicity, a variety of synthetic Se compounds have been developed (Fig. 6). When a series of aliphatic selenocyanates were added in the diet at 2 ppm Se, heptyl and pentyl selenocyanate reduced the total yield of

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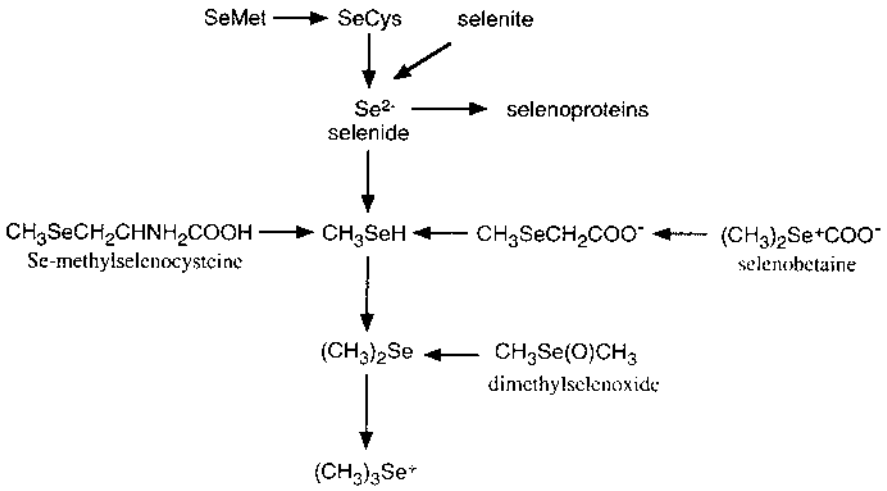

FIGURE 5 Formation of methylselenol from Se methylselenocysteine and selenobetaine, and dimethylselenide from dimethylselenoxide. (Adapted from ref. 5.)

DMBA-induced mammary tumors by about 60% (188). El-Bayoumy and his coworkers synthesized a series of aromatic selenocyanate compounds, as shown in Figure 6, and found that 1,4-phenylenebis(methylene)selenocyanate, commonly called *p*-xylylselenocyanate (*p*XSC), is a potent anticarcinogenic agent with low toxicity (189–191). Feeding of rats with a diet containing *p*XSC at 80 ppm (40 ppm Se) reduced DMBA-induced formation of mammary gland tumors at both initiation and postinitiation (189,190). *p*XSC was also effective in reducing lung cancer induced by 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), a specific carcinogen contained in cigarette smoke, at a level higher than 10 ppm (5 ppm Se) in the diet, while selenite at 5 ppm Se did not have any protective effects on NNK-induced lung cancer (191). Ip et al. showed that triphenylselenonium chloride is another promising chemopreventive agent (192). When triphenylselenonium chloride was added to the diet at the level of 30 ppm Se, the total yield of DMBA-induced mammary gland tumors was reduced by 60–70%, while tissue Se concentrations were not substantially increased. No apparent toxicity was observed in rats fed a diet containing triphenylselenonium chloride even at the level of 200 ppm Se.

Thus, several synthetic Se compounds were shown to be as effective as, or more effective than, sodium selenite in cancer chemoprevention in animal models, while the toxicity of these compounds was generally lower than that of selenite.

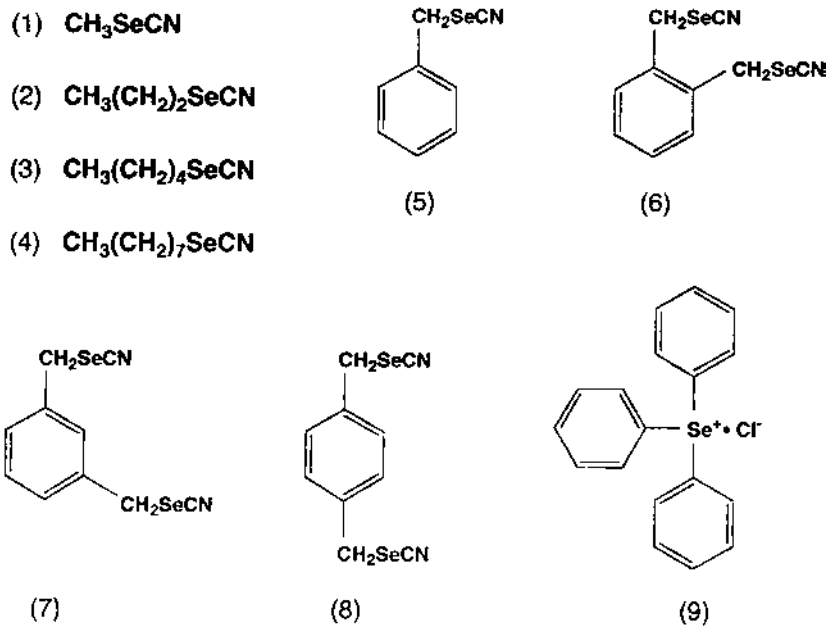

FIGURE 6 Structures of synthetic selenium compounds used for prevention of carcinogenesis. (1) Methyl selenocyanate, (2) propyl selenocyanate, (3) pentyl selenocyanate, (4) heptyl selenocyanate, (5) benzene selenocyanate, (6) *o*-xylylselenocyanate (*o*XSC), (7) *m*-xylylselenocyanate (*m*XSC), (8) *p*-xylylselenocyanate (*p*XSC), (9) trimethylselenonium chloride.

3.3 Possible Mechanisms of Cancer Chemoprevention

In animal experiments, Se compounds were shown to be able to suppress carcinogenesis at initiation phase, postinitiation phase, or both phases, suggesting that Se exerts its effects in multiple steps of carcinogenesis. Several in vitro studies have reported that Se reduces DNA-adduct formation and mutagenesis by carcinogens, thereby inhibiting the initiation process of cancer. The effects of Se compounds in the postinitiation phase may involve growth inhibition of cancer cells.

In early studies, the mutation induced by benzo(a)pyrene (193) or DMBA (194) in the Ames test was shown to be reduced to about 50% by the addition of selenite to the medium at 10–20 ppm. Se supplementation has been shown to reduce in vivo DNA-adduct formation by several carcinogens. Dietary supplementation with selenite (2 ppm as Se) (195), Se-enriched garlic (3 ppm as Se) (196), or pXSC (5 ppm as Se) (197) reduced the formation of DNA-DMBA adducts in the liver and/or mammary glands of rats. A dose-dependent reduction

of DNA-adduct formation by aflatoxin B1 was reported in rats fed selenite up to 8 ppm (198). Dietary supplementation with *p*XSC also reduced DNA methylation, i.e., the formation of O^6- and O^7-methylguanine, in the lung and liver of rats administered NNK, a tobacco-specific nitrosamine (199). The higher efficiency of *p*XSC in reducing NNK-induced DNA methylation compared with that of selenite paralleled the finding that *p*XSC, but not selenite, suppressed the lung tumor caused by NNK (191). The lowered mutagenesis and DNA-adduct formation by supranutritional levels of Se may be a result of enhanced detoxification of carcinogens or reduced conversion of procarcinogens to active forms. It has been well documented that Se deficiency causes enhanced activity of GSH S-transferase, a phase II detoxification enzyme (200). Interestingly, however, several isoforms of GSH S-transferase were shown to be upregulated also by supranutritional levels of Se, suggesting that enhanced detoxification of carcinogens is involved, at least in part, in the reduction of DNA lesions, thereby inhibiting the initiation of carcinogenesis (200).

A number of studies have demonstrated that addition of selenite at μM order to cell culture medium causes inhibition of cell growth (201). Se compounds may either retard the proliferation or induce the apoptosis of premalignant and malignant cells. It has been shown that selenite (202), selenodiglutathione (an intermediate metabolite of selenite reduction by GSH) (203), *p*XSC (202), triphenylselenonium chloride (204), and Se methylselenocysteine (205) are able to induce apoptosis in cancer cell lines. The profile of cell death caused by Se methylselenocysteine, the precursor for methylselenol (Fig. 5), appears to be distinct from that of selenite (205). Selenite causes changes in cell morphology, DNA single-strand breaks, cell cycle block at S/G2-M phase, and both apoptotic and necrotic cell death. On the other hand, Se methylselenocysteine does not induce changes in cell morphology or DNA single-strand breaks, and induces cell cycle arrest at G1 phase, resulting primarily in apoptotic cell death. Thus, each form of Se compound seems to exert its growth-inhibitory effect via a different mode of action.

In the 1980s, Medina and his co-workers reported that the level of a 58-kDa Se-containing protein is increased specifically by the addition of selenite to the medium at cell-growth-inhibitory concentrations (206,207). However, the sequence of the 58-kDa protein was found to be identical to that of protein disulfide isomerase, which does not contain SeCys (208). Recently, another selenoprotein having similar molecular size (57 kDa) was identified as TRx reductase (105). Considering the highly reactive property of the C-terminal SeCys moiety of TRx reductase, Ganther suggested the possibility that an apparent increase of the 58-kDa selenoprotein in selenite-treated cells is a result of increased formation of diselenide bond at the C-terminal SeCys moiety of TRx reductase with a small molecule of Se metabolite such as methylselenol, since the diselenide bond is resistant to the cleavage by the reducing agent such as mercaptoethanol or dithiothreitol used in SDS-PAGE (114).

The growth of cancer cells may also be modulated by immune functions of host animals. Numerous studies have reported the suppression of immune functions by Se deficiency and activation by Se supplementation (209). Recent studies have demonstrated that cytotoxic activities toward tumor cells of activated lymphocytes, macrophages, and natural killer cells were enhanced by dietary supplementation with selenite (2 ppm Se) in mice (210). It is noteworthy that similar effects were observed in human volunteers supplemented with 200 μg Se/g for 8 weeks (211). Cytotoxic T lymphocytes derived from peripheral blood mononuclear cells of Se-supplemented volunteers exhibited a higher cytotoxic activity toward tumor cells in a mixed culture system of lymphocytes and tumor cells. In both mouse and human studies, expression of IL-2 receptor in activated lymphocytes was enhanced, although the levels of IL-1, IL-2, or interferon-γ were unchanged (212,213).

Thus, multiple actions of Se may be involved in the prevention of carcinogenesis. Although the role of Se in maintaining the antioxidative functions of selenoenzymes cannot be excluded, other pharmacological actions of Se, including cytotoxic or growth inhibitory effects at supranutritional levels, may be more important in suppressing the carcinogenesis and cancer progression. Also, the effective dose and the mode of action appear to be greatly influenced by the form of Se.

4. INTERACTION OF SELENIUM WITH METALS

4.1 Interaction with Mercury

The ability of Se to interact with metal compounds and thereby to depress metal toxicity is another prominent characteristic of this trace element. Early studies by Parizek and Ostadalova have demonstrated that the administration of sodium selenite efficiently suppressed the toxic effects of inorganic mercury compounds in rats (214). Toxicity of methylmercury has also been shown to be reduced by dietary supplementation of Se (215). Interestingly, the toxicity of sodium selenite was also reduced by simultaneous administration of inorganic mercury in chicks (216). In marine mammals, which accumulate a substantial amount of mercury in tissues owing to their high rank in the hierarchy of the food chain, a positive correlation between mercury and Se concentrations with a molar ratio of 1:1 has been observed, suggesting a role of HgSe complex formation in the detoxification of mercury (217,218). A number of studies have been conducted to elucidate the mechanisms underlying detoxification of mercury by Se. In the case of inorganic mercury, Se does not enhance excretion of mercury, but rather induces increased accumulation of mercury in tissues by forming an inert complex with Se. On the other hand, it remains unclear how Se depresses the toxicity of methylmercury.

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Naganuma and Imura have extensively investigated the mechanism of mutual detoxification of inorganic mercury and Se in vivo and in vitro systems (219–222). When inorganic mercury ($HgCl_2$) and sodium selenite (Na_2SeO_3) were administered simultaneously to animals, higher concentrations of mercury and Se were detected in blood and tissues except for the kidney compared with animals given a single administration of each compound (219). Especially, the incorporation of mercury and Se into erythrocytes was drastically enhanced by simultaneous administration of $HgCl_2$ and Na_2SeO_3. Gel filtration analyses of stroma-free hemolysates obtained from the rabbit injected simultaneously or separately with radiolabeled $HgCl_2$ and/or Na_2SeO_3 revealed that a novel peak of mercury and Se appeared at the high-molecular-weight (HMW) fraction by coadministration of both compounds, and the HMW fraction contained equimolar amounts of mercury and Se (220). Since mercury and Se were released from the HMW fraction by tryptic digestion, it appears that mercury and Se formed a complex with a protein in erythrocytes. It was shown that the mercury and Se bound to this HMW complex were retained in the erythrocytes for a long time and hardly released into the plasma, thereby leading to a reduction of mercury accumulation in the target organs (221).

The formation of HMW HgSe complex was observed also in plasma after simultaneous administration of $HgCl_2$ and Na_2SeO_3 (220). The HMW HgSe complex in plasma was less stable than that in erythrocytes, and intravenous administration of the plasma containing HMW HgSe complex resulted in incorporation of both elements into the liver but not the kidney. When $HgCl_2$ and Na_2SeO_3 were subjected to in vitro reaction with plasma, the HMW HgSe complex was not formed unless GSH was added to the reaction mixture (221). Thus, selenite must be reduced to selenide by GSH in erythrocytes, which contains GSH at mM order, prior to the formation of the complex with mercury in plasma.

Recently, Yoneda and Suzuki have characterized the HMW HgSe complex in rat plasma by using stable isotopes of Se and high-performance liquid chromatography connected with inductively coupled plasma–mass spectrometry (ICP-MS) (223). Their analyses indicated that the protein part of the HMW HgSe complex in plasma is mainly composed of selenoprotein P, the major selenoprotein in the plasma (66). Although the exact structure of the HgSe complex formed with selenoprotein P remains to be elucidated, a novel function of selenoprotein P was added in the defense against toxicity of inorganic mercury.

After chronic administration of inorganic mercury and selenite, higher concentrations of mercury and Se were found in the liver but less toxicity was observed compared with animals given inorganic mercury alone, suggesting a formation of high-molecular-weight (HMW) HgSe complex also in the liver. Naganuma et al. demonstrated the existence of HMW complex with an equimolar amount of mercury and Se in both soluble and insoluble fractions of the liver of rabbits administered simultaneously with $HgCl_2$ and Na_2SeO_3 (222). The HMW

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HgSe complex in the liver was found in both SDS-soluble and insoluble fractions, and the percent of SDS-insoluble HMW HgSe complex increased with time, suggesting that the formation of this inert and stable form of complex with mercury and Se contributes to the detoxification of mercury deposited in the liver (220).

Chronic toxicity of organic mercury such as methylmercury is also known to be reduced by a concurrent exposure to Se compounds (215). However, a single dose of selenite cannot reduce the toxicity of methylmercury, and tissue distribution and existing forms of mercury and Se were not substantially changed by coadministration of methylmercury and selenite. Thus, the mechanism of Se-induced detoxification of methylmercury seems to be different from that for inorganic mercury. Naganuma and Imura isolated a benzene-extractable complex of mercury and Se as a reaction product of methylmercury, selenite, and rabbit blood, and identified it as bis(methylmercuric) selenide (BMS) (224). BMS can be easily formed from methylmercury and selenite in the presence of GSH, but BMS is readily degraded when it encounters proteins. Thus, it seems likely that BMS is repeatedly formed and degraded in the body. Yet, the role of BMS formation in vivo for the modulation of methylmercury toxicity remains unresolved.

The above-mentioned interactions of mercury and Se have been examined in an attempt mainly to elucidate how selenite can mutually reduce the toxicity of itself and the counterpart mercury compounds when both compounds are given to animals at toxic doses. The next question is whether the dietary level of Se also reduces the toxicity of mercurials. In animal experiments, dietary Se deficiency has been shown to enhance neurotoxicity (225) and fetotoxicity (226), but not the teratogenicity (227) of methylmercury, although the underlying mechanisms, including the involvement of antioxidative functions of selenoenzymes, have not been fully understood. As mentioned previously, marine mammals such as dolphins and seals exhibit a stoichiometric accumulation of mercury and Se in their tissues (217,218). However, the major form of mercury in fish consumed by these animals is reported to be methylmercury (228). Since it has been reported that there is an activity of demethylating methylmercury in the body, Se coming from the diet may form a complex with inorganic mercury produced by demethylation of methylmercury and then be deposited gradually in tissues of these animals (229,230). A positive correlation of mercury and Se concentrations has also been observed in tissues of humans with no occupational exposure to mercury (231).

4.2 Interactions with Other Metals

An early study by Parizek et al. has indicated that selenite injection can reduce the toxicity of cadmium (232). It has been shown that cadmium, like inorganic mercury, can form a HMW complex with Se in rat plasma after simultaneous injection with selenite (233). Also, the reduction of selenite to selenide is required for the interaction with cadmium. Sasakura and Suzuki recently demonstrated

that the HMW CdSe complex in rat plasma is also derived from selenoprotein P (234). Although the administration of selenite with cadmium was shown to efficiently reduce the acute toxicity of cadmium, especially the hemorrhagic lesion of the testis (235), inconsistent results have been obtained for the effects of Se on chronic toxicity of cadmium (236,237).

In addition to cadmium, several metals having affinity for sulfhydryls have been shown to be able to interact with Se. These metals include gold (Au), arsenic (As), platinum (Pt), copper (Cu), silver (Ag), lead (Pb), zinc (Zn), cobalt (Co), bismuth (Bi), tin (Sn), manganese (Mn), tellurium (Te), palladium (Pd), tungsten (W), molybdenum (Mo), thallium (Tl), chromium (Cr), nickel (Ni), and iron (Fe) (238). However, the efficacy of Se on the reduction of toxicity of these metals is far less than that on mercurials and in vivo studies have produced inconsistent results. Among the metals mentioned above, platinum is used as an anticancer drug, known as *cis*-diamminedichloroplatinum. In our laboratory, extensive studies have been performed in an attempt to utilize selenite for reducing the renal toxicity of as *cis*-diamminedichloroplatinum without affecting antitumor activity in animals (239–241). However, because of its toxicity, selenite is not currently used for cancer chemotherapy.

5. TOXICITY OF SELENIUM

5.1 Animal Studies

In the first half of the twentieth century, studies of Se had been exclusively focused on its toxicity, because ''blind staggers'' and ''alkali disease'' in farm animals observed in the midwestern part of the United States (mainly in South Dakota, Nebraska, and Wyoming) had been considered to be caused by ingestion of Se-accumulating (seleniferous) plants (242). Blind staggers is an acute syndrome in cattle and sheep characterized by wandering and stumbling of animals with impaired vision. Alkali disease is a chronic form of selenosis of cattle, horses, mules, and hogs characterized by emaciation, lameness, loss of hair, and disorganized forms of hooves. Since Se concentrations in the soil of these areas are extremely high, grains and native plants that accumulate Se have been considered to be responsible for selenosis in livestock. Although alkali disease is reproducible by feeding animals with Se compounds, the peculiar neurological symptoms like blind staggers were observed primarily in Wyoming and cannot be produced by feeding of animals with any Se compounds, suggesting the involvement of other toxins or microorganisms in blind staggers (243).

In experimental animals, the acute toxicity of Se varies with its chemical form. Overall, the magnitude of lethal toxicity of Se compounds is in the order as follows; selenite $>$ selenate $>$ selenomethionine $\gg$ methylated Se metabolites such as trimethylselenonium chloride and dimethylselenide (244). The reported

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LD_{50} values for orally administered sodium selenite fall in the range of 2.25–13.2 mg Se/kg in various animals, and these values are similar to or higher than that of methylmercury (245). Administration of sodium selenite in the early postnatal period causes cataract in rats (246) but not in hamsters (247).

When experimental animals are chronically exposed to Se compounds, growth retardation and hepatic lesions are commonly observed. Rats fed a diet containing 6.4 ppm Se as either sodium selenite or seleniferous wheat for 6 weeks exhibited liver cirrhosis, enlarged spleen, and decreased growth rate (248). In a long-term feeding study, rats fed a diet containing 16 ppm Se as selenite developed acute hepatitis and died at around 100 days (249). Rats fed a diet containing 4.0 ppm Se also exhibited a reduced survival time. Pathological changes observed in these rats include emaciation, hepatitis, hydrothorax, ascites, enlarged adrenals, edematous pancreas, and myocardial damage (250). Taken together with other studies, a diet containing 2–10 ppm Se as inorganic Se causes liver lesions and depression of growth. It is noteworthy that anticarcinogenic effects of sodium selenite were also observed in animals fed a diet containing 2–5 ppm Se. The chronic toxicity of SeMet is less than that of inorganic Se (244,251).

Feeding excess amounts of Se compounds appears to be harmful to the reproduction of both livestock and experimental animals. In high-Se area in the United States, reduced fertility and malformation of the hoof in offspring have been reported (242). However, inconsistent results have been obtained for the teratogenicity of Se among animal species tested. Female rats given sodium selenate from drinking water (7.5 mg Se/L) exhibited unsuccessful mating with normal males, while male rats given the same amount of selenate and mated with normal females showed no reduction in mating rate or survival of offspring (252). Schroeder and Mitchener conducted a multigeneration study in which rats were given sodium selenate from drinking water (3 mg Se/L) continuously for three generations (253). The male/female ratios of Se-treated rats were increased to 1.3–1.5 at every generation compared with controls (0.94–1.03). One report (254) shows that administration of subacute doses of sodium selenite to pregnant mice on day 12 or 16 of gestation induces premature detachment of placenta (abortion). More detailed studies are necessary to elucidate the mechanism of disturbance of reproduction by Se compounds.

5.2 Human Studies

Several cases of acute, sometimes fatal, Se poisoning in humans have been reported. Most of them are caused by accidental or suicidal ingestion of the gun-bluing solution containing inorganic Se compounds (255). Accidental chronic Se intoxication by taking commercially available Se tablets occurred in the United States (6). A 57-year-old woman who took Se tablets containing 200 times higher Se than indicated for about 3 months (about 32 g Se in total) developed symptoms

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such as vomiting, nausea, deformed nails, and loss of hair. Approximately 80% of the Se contained in the misformulated tablets was found to be selenite.

Chronic exposure to high levels of environmental Se have occurred in South Dakota in the United States, Venezuela (256), and Enshi County in Hubei Province of China (257), the last being best documented. Since the coal produced in the area around Enshi County contains high concentrations of Se (more than 300 ppm), the soil and water are contaminated by Se. The residents were exposed to Se by consuming high-Se corn, rice, vegetables, and contaminated water. The usage of coal for fuel inside the house also contributed to Se exposure from the air. The highest prevalence of human selenosis was recorded during the time of drought (1961–1964) when half of the residents in the affected villages were diagnosed as having selenosis. The reported symptoms include morphological changes in fingernails and toenails, skin lesions, tooth decay, loss of hair, garlic odor of the breath, and neurological symptoms such as paresthesia in severe cases. Average Se intake among individuals showing selenosis was estimated to be 5000 μg/day. The major source of Se is the contaminated plants that contain organic Se such as SeMet, but the contribution of inorganic Se from the contaminated water cannot be underestimated.

In the follow-up study on Enshi County residents, the relationship between Se intake and selenosis has been investigated (258). The level of Se intake was estimated by blood Se level, and selenosis was diagnosed by the presence of fingernail damage. In 1986, the average blood Se levels among subjects showing fingernail damages was 1550 ± 500 μg/L, corresponding to a daily Se intake of 1540 ± 653 μg. The lowest blood Se level of the patient showing persistent fingernail damage was 1054 μg/L, corresponding to a daily Se intake of about 900 μg. In 1992, the survey was again conducted in the same seleniferous area, and the average level of blood Se in individuals with no signs of selenosis was found to be 968 ± 115 μg/L, corresponding to a daily Se intake of 819 ± 126 μg. Based on these studies, Chinese scientists proposed the mean lowest adverse effect level (LOAEL) of Se to be 1540 μg/day, the individual LOAEL of Se to be 900 μg, and the no adverse effect level (NOAEL) of Se to be 800 μg/day.

In South Dakota, where alkali disease in livestock was prevalent, no signs of selenosis or biochemical changes indicative of hepatic or hematological damage were observed in residents whose daily Se intakes were estimated to be 200–724 μg (259). No overt toxicity of Se was observed also in the intervention trial for cancer chemoprevention conducted by Clark et al., in which 200 μg Se/day was given to the participants for several years (138).

5.3 Mechanism of Selenium Toxicity

The mechanism of Se toxicity in vivo has been poorly understood. One reason may be the lack of a specific target organ of Se toxicity.

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In in vitro experiments, it has been demonstrated that intracellular sulfhydryl compounds, especially GSH, are involved in the manifestation of selenite toxicity. Incubation of rat erythrocytes in vitro with sodium selenite resulted in a rapid loss of intracellular GSH followed by hemolysis, and the addition of GSH in the incubation medium enhanced the selenite-induced hemolysis (260). The inhibitory effect of sodium selenite on colony formation of HeLa cells was diminished by pretreatment with buthionine sulfoxide, an inhibitor of the synthesis of GSH (261). Treatment of several tumor cell lines with selenite was shown to inhibit both DNA and RNA synthesis, but the inhibitory effects of selenite were much greater in cell lines containing higher amount of intracellular sulfhydryls (262). When the cell lines containing high GSH were pretreated with diethylmaleate, a depletor of cellular GSH, the toxicity of selenite was attenuated (263). Thus, reaction with GSH may be necessary for the manifestation of selenite toxicity.

One possible metabolite of selenite produced by the reaction with GSH is selenodiglutathione (Fig. 7), but this compound is unstable under physiological conditions. Seko et al. proposed that active oxygen species are generated by the reaction of selenite with GSH, as shown in Figure 7 (264,265). Generation of active oxygen species was confirmed by measurement of chemiluminescence from luminol or lucigenine mixed with selenite and GSH in the presence of oxygen in a cell-free system. Also, in hepatocytes treated with sodium selenite, the depletion of GSH paralleled the consumption of oxygen, and subsequently the cells exhibited enhanced lipid peroxidation and lactate dehydrogenase leakage (266). These changes were inhibited by the addition of desferroxiamine Mn, a synthetic superoxide dismutase mimic, suggesting that the formation of active oxygen species generated by selenite in the presence of GSH and oxygen is the primary cause of selenite cytotoxicity.

Based on Seko's scheme, Yan and Spallholz demonstrated that superoxide anions and H_2O_2 were generated by the reaction of selenite with GSH, but not by SeMet, in tumor cells, and suggested that the difference in the ability to produce ROS between selenite and SeMet is the cause of the difference in

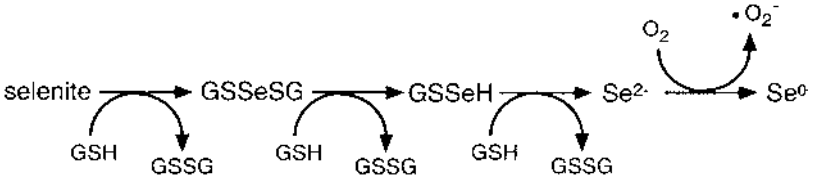

FIGURE 7 Possible pathway for generation of active oxygen species from selenite in the presence of glutathione and oxygen.

cytotoxicity of these Se compounds toward tumor cells (267). They also demonstrated that selenite treatment of keratinocytes resulted in the production of 8-hydroxydeoxyguanosine in DNA and caused apoptosis of cells, while SeMet did not produce 8-hydroxydeoxyguanosine or apoptosis (268). Thus, the prooxidant nature of selenite in the presence of GSH may account for its cytotoxicity.

Since the solution used for TPN patients contains GSH and cysteine, the addition of sodium selenite in the TPN solution may cause enhanced generation of active oxygen species. Terada et al. demonstrated that selenite, but not selenate or SeMet, produced active oxygen species in the presence of clinical concentrations of GSH and cysteine, and suggested the use of selenate or SeMet, but not selenite, in the TPN solution (269).

6. RECOMMENDATION FOR SELENIUM INTAKE

In 1980, the National Research Council recommended a dietary Se intake of 50–200 μg as the estimated safe and adequate daily dietary intake (ESADDI) based on animal experiments (270). In 1989, values of recommended dietary allowance (RDA) for Se were established based on the data of an intervention study conducted in China (271). In a Se-deficient area in China, the residents were given SeMet at various concentrations and eGPx activity in the plasma was monitored (272). It was found that the plasma eGPx activity reached a plateau level after 5 months' supplementation with 30 μg/day Se as SeMet. In addition to the basal level of Se intake (about 10 μg), 40 μg was considered to be the minimal required level of daily Se intake. After the application of a safety factor of 1.3 and adjustment by body weight, 70 μg/day and 55 μg/day were recommended as RDA values for men and women, respectively, in the United States.

To determine the safety level for Se intake, a reference dose (RfD) of Se was established by the Workshop on Selenium Compounds in Cancer Chemoprevention Trials (273) based on the data obtained in Enshi (258). RfD is ''an estimate (with an uncertainty spanning perhaps an order of magnitude) of a lifetime daily dose to the human population (including sensitive subpopulation) that is likely to be without an appreciable risk of deleterious effects during a lifetime'' (273). As previously described, the NOAEL of Se for selenosis as judged by the occurrence of fingernail damage was estimated to be 800–850 μg/day. Considering the variability in interspecies and individual sensitivities, the uncertainty factor of 10 is usually applied to determine the safety levels for toxic substances. However, this rule may not be suitable for an element like Se that is toxic but essential as well. The Workshop used an uncertainty factor of 3 (half of 10 in log-scale) for Se and recommended RfD as 350 μg/day for a 70-kg man (5 μg/kg/day) in the United States. Relying on the similar data obtained by the Enshi study, the maximum safety level of Se intake was set as 450 μg/day in the United

Kingdom (273), and the upper limit of Se intake was set as 300 μg/day by Nordic Nutrition Recommendations in 1996 (274).

7. CONCLUSION

As numerous review papers on Se have already pointed out, Se has dual faces as an essential nutrient and as a toxic substance. In this review, the multiple faces of biological actions of Se are described. In concluding this chapter, the following points should be emphasized again.

1. Multiplicity of functions of selenoproteins. Although biochemical studies on functions of Se have started from the finding that Se is an integral constituent of cGPx, more than 10 mammalian selenoproteins have been identified until now and the relative importance of cGPx under physiological conditions is considered to be lower than expected. As described in this chapter, the range of functions of selenoproteins is broad and not restricted to protection against oxidative lesions.

2. Importance of the chemical form of Se. Most studies on Se had used only commercially available Se compounds such as sodium selenite, sodium selenate, selenocystine, and SeMet. However, a number of novel Se compounds have been synthesized in attempts to produce less toxic and more potent anticarcinogenic agents. In addition, possible roles of methylated metabolites of Se such as methylselenol in the prevention of carcinogenesis have been implicated. However, the biochemical basis for the actions of these forms of Se is still unclear. Elucidation of the metabolic pathways and functions of these Se compounds or metabolites may provide new insight into the ''pharmacological actions'' of Se.

3. Dose-response consideration. The relationship between dietary Se intake and multiple biological actions of Se can be expressed as three different dose-response curves for physiological, pharmacological, and toxicological responses, as shown in Figure 8. Physiological responses are evaluated by the elevation of selenoenzymes such as cGPx. The ''nutritional supplementation'' of Se to humans or animals whose Se intake is at low levels is aimed at achieving recovery of the physiological functions of Se. This type of Se supplementation includes the addition of sodium selenite in table salts for residents in the Keshan disease endemic area, the use of Se-supplemented solution for TPN patients, and the fortification of fertilizer with Se in low-Se areas such as Finland and New Zealand. On the other hand, ''supranutritional supplementation'' of Se to humans and animals whose Se intake is in a normal range is intended to achieve pharmacological functions of Se. Trials for cancer chemoprevention by Se supplementation are a typical example of supranutritional supplementation. In this case, strict monitoring of Se toxicity is necessary since the effective dose and toxic dose of Se are too close. Similarly, nutritional supplementation and supranutritional supplementation of Se should be strictly discriminated in the evaluation of Se actions on other diseases such as cardiovascular disease and AIDS. Further eluci-

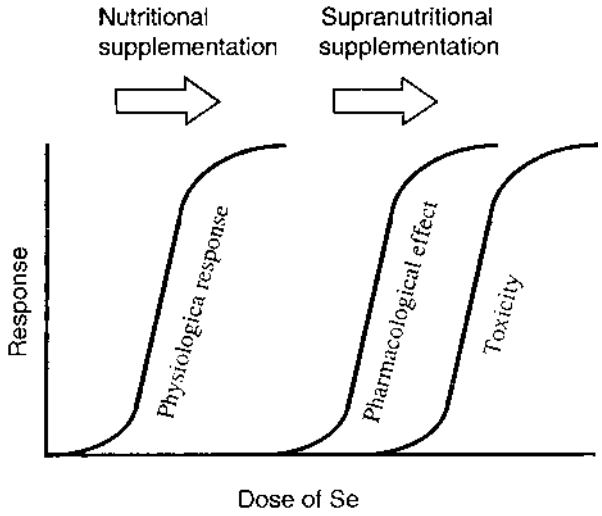

FIGURE 8 Dose-response relationship between dietary selenium intake and its biological responses.

dation of the mechanism of action and quantitative examination of the effects are required for safe utilization of supranutritional Se.

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18

Semiconductors

Bruce A. Fowler and Mary J. Sexton
University of Maryland, Baltimore, Maryland

1. INTRODUCTION

The use of elements such as arsenic, antimony, gallium, and indium has long been featured, in the manufacture of semiconductors for computer chips, cellular telephones, and light emitting diodes (LEDs). Over the last 30 years, tens of tons of these elements have been incorporated into these devices either as dopants (1,2) for silicon-based computer chips or in the manufacture of the higher-speed III–V semiconductors such as gallium arsenide or indium arsenide (2). As the demand for higher-speed devices has increased, older devices with slower electronic speeds have been discarded, in the absence of well-established recycling programs, generating a large stockpile of electronic devices containing these elements collectively known as ''e-waste.'' The magnitude of this growing problem has only recently been appreciated in California and Europe (3) but much about the biological properties of these high-technology materials is not yet known. Experimental animal studies (4–9) have demonstrated that particles of GaAs or InAs are broken down in vivo resulting in the release of both constitutive elements (4–9). This creates a binary chemical mixture situation raising the issue of interactive effects. The situation in the semiconductor manufacturing plants is even more complex since a number of solvents are also present and semiconductor workers are exposed to a number of toxic agents in the clean room environ-

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ments (10). Epidemiological studies of these workers have shown an increased incidence of miscarriages and there are case reports of brain and testicular cancers among workers employed in a gallium arsenide plant.

This chapter will review the known biological effects of arsenic, including arsine gas (11–16), antimony (17), gallium (18), and indium (19), from the perspective of experimental systems and attempt to relate these data to the findings of epidemiological studies in semiconductor workers. It is hoped that this review will provide a contemporary assessment of the present state of knowledge regarding the current uses and biological effects of metals/metalloids utilized in the semiconductor industry. The potential human health and environment effects of these elements either alone or as mixtures will be discussed in relation to needed areas of research.

2. EXPERIMENTAL STUDIES

The toxic properties of the elements arsenic and antimony have been appreciated for many years but the use of these elements in the production of semiconductors has occurred only during the last 30 years. The utilization of these elements in increasing quantities has given rise to concern about toxic effects in workers and long-term environmental effects as devices containing these materials are discarded. It is important to note that exposures to these elements via the III–V semiconductors will be as binary mixtures of gallium, indium, and arsenic or antimony as well as arsine gas (AsH_3). As discussed below, the combination of these elements is generally more toxic than each of them on an individual basis owing to metal-metal interactions. Exposure of workers is usually via inhalation of particles of GaAs or InAs or via arsine gas. Each of these compounds has its own distinctive toxic properties as discussed below.

2.1 Chemical Disposition and In Vivo Metabolism

It has been shown by a number of investigators that particles of GaAs and InAs undergo biological attack in vivo resulting in the dissolution of these particles and the release of both GA/In and As components. The Ga and In components are concentrated in organs such as the kidney while the As component undergoes a metabolism similar to that of As^{3+} resulting in the urinary elimination of monomethyl arsonic and dimethyl arsinic acids (4–9). Arsenic internalized from arsine gas exposures seems to follow a similar metabolic pathway.

2.2 Arsenic

The toxic properties and metabolism of arsenic via methylation have been recently reviewed by the National Research Council (20). Inorganic arsenic is a general cellular poison that produces a variety of effects in different organ sys-

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tems (20). These include liver toxicity, renal toxicity, skin dermatoses, peripheral neuropathy, and cancers in a variety of organ systems (20). Urinary excretion of methylated metabolites such as monomethyl arsonic acid and dimethyl arsinic acid is the main route for elimination of arsenic in vivo for both man and a majority of animal species (20). The mitochondrion is a major target organelle for arsenicals (21,22) and the production of reactive oxygen species (ROS) secondary to inhibition of mitochondrial respiratory function is a likely mechanism for initiation of cell injury and arsenical-induced carcinogenesis (20). Arsenical induction of the stress protein response has been observed by a number of investigators (23–26) in a variety of cellular systems following both in vivo (23) and in vitro (24–26) exposures to this agent. Prolonged in vivo exposure of laboratory animals (27–29) and humans (30,31) to arsenicals has also been shown to produce a characteristic porphyrinuria pattern characterized by increased excretion of uroporphyrin with lesser amounts of coproporphyrin. Similar porphyrin excretion patterns characteristic of arsenic have been observed in rodents exposed to arsine gas (32), GaAs (12), and InAs (23,29,33). These findings are consistent with the observations noted above regarding the dissolution of GaAs and InAs particles following in vivo exposure releasing the Ga, In, and As moieties.

2.3 Antimony

Antimony compounds and stibine gas (SbH_3) have received less attention than arsenicals since they are generally used in smaller quantities and have lower toxicities than inorganic arsenic and arsine gas (17).

2.4 Gallium

Gallium compounds have been used as antitumor agents and in the manufacture of III–V semiconductor gallium arsenide (GaAs) (2). The kidney is the chief target for gallium toxicity (4,18) after acute administration and is the limiting factor in its usage in cancer chemotherapy. Nephrotoxic effects have also been reported in rodents following intratracheal instillation of GaAs particles (4) or subcutaneous administration of GaAs particles (7–9). GaAs administration has also been shown to markedly inhibit blood ALAD resulting in the increased excretion of ALA in the urine (4). Administration of GaAs particles to rodents in vivo has been shown to cause the induction of a characteristic stress protein response pattern in kidney proximal tubule cells and development of renal tubular proteinuria pattern (34,35).

2.5 Indium

Indium is also nephrotoxic (19) and has been shown to be a potent inhibitor of renal ALAD (36). Administration of InAs particles has been shown to produce

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a porphyrinuria pattern (29) that has elements of both the In and As moities. Administration of InAs particles to rodents also produces characteristic alterations in gene expression patterns in rodent renal proximal tubule cells and a more marked tubular proteinuria pattern than that of GaAs administered at equivalent dosage levels (35). These data indicate that the InAs compound is more nephrotoxic than GaAs at equivalent dose levels. The underlying difference seems to be related to the potent inhibitory effects of In on the protective stress protein induction response in these cells.

2.6 Gender Differences in Responsiveness

The semiconductor industry has a diversified workforce with a high percentage of women employed in the manufacture of semiconductor chips (37). For this reason, concern has been focused on women of childbearing age and the potential reproductive health effects associated with exposure to chemicals used in this industry as discussed below. On a cellular basis, gender differences in the responsiveness of renal proximal tubule cells from both hamsters and humans exposed to combinations of Ga, In, or As in vitro have shown marked differences in the responsiveness of alterations in gene expression patterns between cells derived from males and females (34). Since these exposures were conducted in vitro in the absence of hormones, it is suggested that cellular imprinting of the gene regulatory mechanisms may be the most likely explanation for the observed differences. The potential importance of such findings rests with the fact that gender-based differences in the stress protein response may account for differences in susceptibility to toxicity from these agents.

3. ORGAN SYSTEM EFFECTS

3.1 Immune System

Administration of GaAs particles to rodents in vivo or rodent splenocytes in vitro has been shown to produce inhibitory effects on the humoral antibody reponse (38–43). Similar inhibitory effects on both humoral and cellular immune responses have also been observed in rodents following prolonged exposure to tolerated concentrations of arsine gas (16). Data from these studies suggest that the immune system is a target organ system for these commonly used metals/metalloid compounds and are consistent with finding of increased semiconductor worker absenteeism (44) due to illness relative to other manufacturing sectors.

3.2 Liver

Acute administration of indium (44,45) and acute and chronic administration of arsenicals (20) have been shown to produce hepatoxicity. For indium, this is

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highly dependent upon the chemical form with ionic indium producing hepatocellular toxicity (44,45) and colloidal indium compounds producing marked toxicity to cells of the reticular endothelial system. The endoplasmic reticulum and its attendant metabolic biochemical system in hepatocytes is a major site of intracellular toxicity (45). Prolonged exposure to arsenicals produces hepatocellular toxicity with the mitochondria as a major site of intracellular action.

As noted previously, arsenicals including arsine produce a specific porphyrinuria pattern of apparently primarily hepatic origin in both rodents and humans (20) following prolonged exposures. The potential hepatotoxic effects of gallium have not been studied in detail.

3.3 Kidney

The group III elements Ga and In have each been shown to produce toxicity in renal proximal tubule cells (4,18,44). Arsenicals such as arsenate have also been demonstrated to produce toxicity in this renal cell population (46). As in the liver, the mitochondrion appears to be a primary target organelle with inhibition of respiratory function as a primary underlying mechanism of toxicity (20). Alterations in renal gene expression patterns and attendant tubular proteinuria are important and marked biochemical and physiological responses of this organ system following exposure to GaAs or InAs (35).

4. INHALATION TOXICOLOGY STUDIES

The inhalation toxicology of GaAs particles in rats and mice has been extensively studied by the National Toxicology Program (NTP) and the results published as a peer-reviewed report (47). Data from the 14-week studies confirmed findings from previous intratracheal instillation studies (4) with regard to lung toxicity and demonstrated the testicular toxicity of GaAs in both rats and mice. This latter effect is undoubtedly due to the known toxicity of Ga to the testes (47). In addition, GaAs exposure also produced a microcytic anemia with increased zinc protoporphyrin/heme ratios also confirming biochemical effects on the heme biosynthetic pathway. In the 2-year exposure study, a significant increase in the incidence of lung tumors was observed in female but not male rats leading to the conclusion that there was clear evidence of carcinogenic activity in female but not male rats following chronic GaAs inhalation exposure.

5. EPIDEMIOLOGICAL STUDIES

Uncertainty regarding whether workers in the semiconductor industry experience adverse health effects has led to three kinds of investigations: (a) assessments of frequencies of occupational illnesses and injuries, (b) comparisons of rates of

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specific illnesses, such as respiratory functioning, and (c) examinations of reproductive health, in particular, spontaneous abortions among female workers. To date, studies have addressed short-term health effects, and none has addressed more chronic conditions, such as cancer.

5.1 Assessments of Incidence of Injury and Illness

Annual reports from the Bureau of Labor Statistics (BLS) provide data on occupational injuries and illnesses based on workers' compensation information that are categorized by the Standard Industrial Classification (SIC) codes for industries in the United States. The information available is limited in scope and can give only suggestive indications of whether semiconductor workers have any excess of occupational injuries or illnesses because the database is not a research one. Robbins et al. (37) compared workers in SIC 367, the code for companies that manufacture all electronic components and accessories, SIC 3674, the code for those that more narrowly manufacture semiconductors and related devices, all manufacturing industries, and all private industries on incidences of injuries and illnesses for the period of 1980–85. The incidence of illness was about the same for workers in SIC 3674 and in SIC 367. The incidence of illness, however, was higher for workers in the 3674 industry when compared with those in private industries for each of the 6 years and higher than that of workers in manufacturing industries overall for 3 of the years and about equal in incidence of illness in the other 3 years. When specific types of illnesses were examined, the semiconductor workers had notably higher rates of respiratory conditions due to toxic agents when compared with workers in each of the other three industries. As the authors indicated, there was no way to establish the probability that these differences arose by chance given the limitations of the available data. However, an examination of the 1998 data indicate that the incidence rate per 10,000 full-time workers for respiratory conditions was 5.9 compared to 3.5 and 2.0 for all manufacturing industries and private industries, respectively (48).

Similarly, LaDou (49) more recently reported higher rates of illnesses among semiconductor workers (SIC 3674) based on California BLS data for 1987–91. He found that as a percentage of workloss cases, occupational illnesses were two to three times higher for semiconductor workers than for those in all manufacturing industries and was somewhat higher than for workers in SIC 367. Of the occupational illnesses, about one-third to one-half were categorized as systemic poisoning resulting from exposure to toxic materials. The percentages of systemic poisoning were comparable to those for SIC 367 but were about twice as high as for workers in all manufacturing industries.

As LaDou (49) pointed out, changes in the manufacturing process of semiconductors occur rapidly making it difficult to pinpoint clearly the etiology of any excess health problems that might be present. Despite this difficulty, the

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inherent variation of year-to-year rates based on BLS data, and the limitations of the data collected, it appears that injuries are not higher but that the illness rates for workers in SIC 3674 may be higher than for workers in other manufacturing jobs and were higher in the earlier years of the eighties than for the later years.

5.2 Specific Health Conditions

5.2.1 Nonreproductive Health Conditions

There is suggestive evidence that semiconductors have increased respiratory and other symptoms of compromised health. Pastides et al. (50) found that a number of symptoms were reported by manufacturing workers at a higher frequency than reported by nonmanufacturing workers in the semiconductor industry. McCurdy et al. (51) surveyed over 3000 employees in both large and small semiconductor sites. They found a higher prevalence of upper respiratory symptoms among fabrication workers than among nonfabrication workers. Other symptoms, such as dermatitis and headaches, were reported in subsets of the work groups. Luo et al. (52) conducted a similar study of self-reported health symptoms and of measured pulmonary functioning among workers in a semiconductor plant in Taiwan in 1995. They found that compared to controls and after adjustments were made for smoking and age, males working in either the photolithographic process or the ion-implantation process had about a fourfold risk of having restrictive lung functioning indicated by spirometric measurements, although this was not a statistically significant difference. Significant differences were found between female controls and photolithographic or diffusion workers in airway irritation and eye irritation as well as in other self-reported symptoms. Both groups of investigators acknowledge that the sites studied had concentrations of chemicals well below the legally acceptable levels when monitored measurements were reviewed. However, neither study obtained individual exposure measurements that could be linked to symptoms. The question remains of whether some workers are exposed at levels exceeding acceptable limits or whether there is a group of workers sensitive enough to experience symptoms at very low levels. Further, given the degree of cooperation needed from plant management to support such studies, it is almost surely the case that any adverse health effects will be underestimated since the more cooperative plants are expected to have the cleanest work conditions. Despite these issues, the results from very different studies, conducted under quite varied conditions, are consistent in finding elevated respiratory symptoms in semiconductor workers.

5.2.2 Reproductive Outcomes

A higher risk of adverse pregnancy outcomes, in particular spontaneous abortions and congenital malformations, from exposure to semiconductor chemicals has

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been examined in a handful of studies. No one chemical or metal has been singled out in the semiconductor industry as a more possible causative agent than another. Furthermore, the difficulty of isolating one chemical has been raised along with the realization that the more realistic work environment is one with multiple exposures (53).

Most human studies though have addressed solvent exposure because animal studies have established glycol ether as a teratogen (54–57) and because a occupational solvent exposure has been linked to adverse pregnancy outcome (58).

Spontaneous Abortions. All but one published study on the association of exposure related to the semiconductor industry and reproductive outcomes have focused on exposure at the workplace, and most of these have focused on female workers. However, Wrensch et al. (59) examined pregnancy outcomes in women in two communities in which the drinking water was contaminated in 1980–81 from solvents that leaked from an underground storage tank of a semiconductor firm in California. An earlier examination (60) of pregnancy outcomes for women in one of these communities had found a statistically significant excess of spontaneous abortions (SABs) compared to a control community, but Wrensch et al. (59), in the follow-up investigation, failed to find an association with a long period, 1980–85, and with changes in the study design. The later study included a postcontamination period and estimates of exposure based on hydrogeological modeling that took into account the amount of contaminated water delivered to individual households as well as a second exposed community. Comparisons were made with two control communities. The authors concluded that ''the contaminants probably did not have a measurable impact on adverse pregnancy outcomes in this community given the doses received and the numbers of women exposed'' (p. 299).

Table 1 provides a summary of three key cohort studies that assessed the risk of SABs in female employees with potential exposure from their semiconductor jobs. Most investigators adjusted for other risk factors known to relate to SAB, but the adjusted risks were not substantially different from the crude estimates. In the 1988 Pastides et al. (50) study a statistically significant elevation of about a twofold risk in diffusion workers compared with workers, such as clerks and administrators, who were judged to have minimal or no exposure was reported. Other comparisons were not statistically significant. Because of the small numbers in the Pastides et al. investigation, an investigation, known as the Semiconductor Health Study (SHS), with several components, was conducted. It included a larger number of workers and was designed to examine several types of health outcomes, with one component being an historical cohort study. The results have appeared been published (61–64). A statistically significant finding of an elevated risk of 1.45 for SABs among fabrication workers was reported (65). In the historical cohort a dose-response relationship was shown between level of exposure to solvents and SABs, which provided stronger evi-

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TABLE 1 Features and Results of Historical Cohort Studies to Assess Risk of Spontaneous Abortion in Female Semiconductor Workers

Authors	Site	Measure of Exposure	Measure of SAB	Comparison Group (n)	Exposed Group (n)	Rel Risk	95% CI
Pastides, et al., 1988	1 Mass facility	work hx by interview and plant records/observations of use of chemicals	self-reports of pregnancies	clerical, admin, engineers (398) clerical, admin, engineers (398) non-fab (288)	photolithographic (16) diffusion (18) fabrication (15)	1.75[c] 2.18[c] .87[a]	0.77, 3.25 1.11, 3.60 0.45, 1.60
Beaumont, et al., 1995	14 U.S. facilities	work hx by company records & interview	self-reports of pregnancies	non-fab (444)	fabrication (447)	1.45[c]	1.02, 2.05
Correa, et al., 1996	2 Eastern U.S. facilities	work hx by interview and plant records	self-reports of pregnancies (some confirmed by medical records)	no exposure (332)	low (125) medium (74) high (30)	1.0[a] 1.4[a] 2.8[a]	0.60, 1.70 0.80, 2.60 1.40, 5.60
Pinney and Lemasters, 1996	1 Southeastern U.S. facility	work hx by interview exposure categories developed by industrial hygienists	self-reports of pregnancies	non-fab, no exposure (191)	fab (189) non-fab, exposure (74)	1.62[a] 2.00[a]	

a = adjusted.
c = crude.

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dence of a causal relationship (64,66). Correa et al. (53) focused on one specific type of chemical, ethylene glycol ethers, and quantified the risk into no-, low-, medium-, and high-exposure categories. They found elevated risks of SABs for female workers in the medium- and in the high-exposure categories, with the latter having a 2.8-fold increased risk of an SAB. They also found a statistically significant trend of a higher risk of SAB related to a higher exposure, thus suggesting a dose-response relationship and thereby lending more biological credibility to a causal association.

The results from the historical cohort studies, conducted under different conditions and times, are consistent in suggesting an elevated risk of SABs. In three of the investigations a statistically significant increase in risk was observed either overall for women in process jobs that entailed potential exposure to chemicals or for women in jobs in a more narrowly defined part of the process. Pastides et al. (50) and Pinney and LeMasters (67) found an increase in SABs for fabrication workers but the difference was not statistically significant. The findings from these cohort studies provide evidence of an increase of at least 50% in the risk that a pregnancy will end in an SAB for process workers in semiconductor jobs. We agree with Correa et al. (53) that the point estimates for the magnitude of risk are almost surely underestimated. These investigators found a smaller proportion of women at each level of increased risk. On the one hand, investigations that examine only one overall risk estimate will tend to find it weighted toward a smaller risk since the level of exposure for the largest proportion of the employees is low. On the other hand, the partitioning of the workers into specific work or exposure units will decrease the sample size and thus the power of the study unless these small numbers have been carefully considered in the design of the study. Thus the confidence in the reported findings will be weakened because of the broad statistical confidence limits around the point estimate, but these large confidence intervals are a direct result of a sample under study that was far too small to detect elevated risks of 50–100%. Nevertheless, the cohort studies, to date, indicate an elevation in the risk of SAB in female semiconductor workers.

A particular difficulty in studying SABs is the fact that some are not clinically recognized. Eskenazi et al. (63), as a component of the SHS, addressed this problem with a study of women who worked in semiconductor production at seven sites. Eligible women gave daily urine samples and kept a diary over a 6-month period. The urine samples were analyzed for concentrations of human chorionic gonadotropin for determination of pregnancy. This prospective cohort was then followed to assess the outcomes of the pregnancies. These investigators reported a relative risk of 1.39 (confidence intervals of 0.84, 2.31) for an SAB in women working in fabrication jobs that put them at higher risk of exposure ($n = 152$) compared to females working in nonfabrication jobs ($n = 251$). Though not statistically significant, the risk estimate is comparable to estimates from his-

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torical cohort studies. The analysis was based on only 19 pregnancies in the fabrication workers and 33 in the nonfabrication workers. The results of this small study are consistent with those reported by Gray et al. (68). It is worth noting that about three-fourths of the pregnancies detected by the assay that ended in SABs were not clinically recognized nor recognized by the woman herself. Since it is well established that a substantial proportion of these early fetal losses are chromosomal anomolies (69), it raises the question of whether these unrecognized pregnancy losses occur more frequently in women in semiconductor jobs. The findings from this investigation again point up the underestimation of the magnitude of SAB risk when unrecognized pregnancies are not included.

In general, a case/control study is considered to be a weaker study design than a cohort study because of the greater potential for the presence of bias (19). Nevertheless, findings from two small case/control studies should be recognized since both have failed to find a positive association between SABs and work in the production of semiconductors. Schusterman et al. (70) reported an odds ratio of 0.94 (confidence intervals of 0.58, 1.5) for SAB cases and any electronics production work, and Elliott et al. (71) reported an odds ratio of 0.65 (confidence intervals of 0.30, 1.40) for fabrication workers. A number of limitations in these case/control studies could account for the lack of consistency in the findings with those from the cohort studies (72,73). Even so, their results underscore the lack of establishing definitively a causal link between health problems among semiconductor workers and specific occupational exposures, and the need for studies with strong designs and methodologies to address the issue.

Other Reproductive Outcomes. A few studies have focused on whether the reproductive health of males because of exposure to chemicals in their semiconductor jobs is compromised. Investigators have examined fertility in male workers and SABs in women married to men working in semiconductor jobs. Findings from other studies support the relationship of compromise in reproduction health among men exposed to gycol ether (47,74–76). Pastides et al. (50) found an elevated risk of SAB among spouses of male employees, but the numbers of pregnancies on which the relative risk were based were extremely small. Correa et al. (53) reported that spouses of male workers had no increased risk of SAB but that there was a nonsignificant increased risk of subfertility. Schenker et al. (64) summarized the inconsistency of the findings on fertility between the cross-sectional SHS study and the SHS cohort studies. In sum, the human data assembled to date are inconclusive regarding whether there is any risk to the reproductive health of men because the findings have been based on such small numbers, and the results are inconsistent.

Some investigators have commented on adverse pregnancy outcomes other than SABs, such as low birth weight, short gestational age, or congenital malfor-

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mations (50,59), but have had few data to report. Thus, there is no understanding of the long-term health status of liveborn children whose mothers or fathers are exposed to chemicals because of their occupations in the semiconductor industry.

The studies to date, taken as a whole, are not reassuring with regard to the protection of the semiconductor workers' health. With the exception of two case/control studies, the epidemiological studies are consistent in finding increased risks in respiratory symptoms and in adverse reproductive outcomes. The confidence in these findings is increased because of variation in the study designs, in the study populations, and in the methodologies applied (66). Moreover, laboratory and animal studies have produced findings consistent with the human health studies that provide insights into the biological mechanisms (47). While the processes, specifically in the United States with the use of ethylene glycol ethers, have changed since the earliest investigations, associations have continued to be found that suggest adverse health effects.

6. CHEMICAL MIXTURE INTERACTIONS

It is clear that binary compounds such as GaAs and InAs produce biological effects that reflect the individual and combined effects of each of the constituent elements. These interactive effects appear in a number of different organ/biochemical systems including the lung, liver, kidney, hematopoietic, and reproductive systems. Taken in combination with the other chemicals known to be present in work environments found in the semiconductor industry, it is not unreasonable to expect the health effects findings of epidemiological studies published to date on the semiconductor workforce.

The best method of understanding the current relationship of occupational exposure from the manufacturing of semiconductors with the workers' health is to conduct well-designed studies with sufficient numbers of subjects to insure adequate statistical power that can detect even modest health effects. While the assembled evidence provides guidance in the design features, the past studies have contained major limitations. Particularly notable has been the weakness in measuring exposure because of a lack of any personal monitoring, by objective means, of exposure of an individual level. What has been relied upon has been recall of work through interview, sometimes with some confirmation through job records, and then an assignment, sometimes by industrial hygienists, to an exposure category. This method is far too imprecise because workers are not confined to one job or one area. Of particular concern, is that some workers thought not to be exposed—and thus used as controls for comparative persons—may have some exposure. Exposure measurements on an individual level have regrettably not been done. Furthermore, the measurement of SABs has been primarily by recall over a long period and, more importantly, relies on the woman's knowledge of the SAB. Since a large percentage of SABs go undetected (63) it again indi-

cates that the risk is quite probably underestimated. The omission of former workers—whether at lower or higher risk—means that the estimators of risk remain uncertain. Without the generation of a well-founded base of evidence, the current controversy regarding the degree of safety for this large number of workers will not be resolved and will remain as a debate that has not yet been informed with evidence from a strong database. Until that database is generated, decisions about semiconductor workers' health will continue to be made and justified on the basis of weak and uncertain information.

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19

Bacterial Metal-Responsive Elements and Their Use in Biosensors for Monitoring of Heavy Metals

Ibolya Bontidean and Elisabeth Csöregi
Lund University, Lund, Sweden

Philippe Corbisier
Institute for Reference Materials and Measurements, Geel, Belgium

Jonathan R. Lloyd and Nigel L. Brown
The University of Birmingham, Edgbaston, Birmingham, United Kingdom

1. INTRODUCTION

Society is learning to adapt to pollution by heavy metals in the environment, and is now attempting to remediate, control, and minimize such pollution wherever possible. To do this, there is a need for methods of assessing the amount of heavy metal pollution in the natural and industrial environments. Although it is relatively straightforward to use the techniques of analytical chemistry to detect total amounts of heavy metal in a given location, this rarely tells you how much

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of this metal is a *biological* hazard. To achieve this, biological methods may offer distinct advantages over chemical methods.

It is really only since the industrial revolution that large numbers of people have been exposed to significant levels of toxic metals, although at least since Roman times heavy metals have been used in medicine and cosmetics (1). In contrast, microorganisms have always lived with ''pollution'' by heavy metals, as they have evolved to occupy ecological niches in which these toxic metals naturally occur and in which they may be released by geochemical processes. Consequently, bacteria in particular, but also yeasts, fungi, and many plants, have developed specific mechanisms to tolerate or detoxify heavy metals. This chapter describes some of the ways in which we and others have begun to exploit these biological mechanisms to determine the amount of ''bioavailable'' heavy metal in natural and industrial environments. These methods are still in their infancy compared with the techniques of analytical chemistry, but may offer some advantages in ease of use as well as in biological relevance. In particular, we are attempting to couple the high specificity of biological systems with the high sensitivity of modern microelectronics in the development of biosensors.

Elsewhere in this book, you will find information on the occurrence of heavy metals in the natural environment, and we will not repeat it here. However, knowledge of the occurrence and amount of heavy metal ions is important in many fields, such as environmental monitoring, clinical toxicology, wastewater treatment, and industrial process monitoring. Therefore, many spectroscopic methods, including atomic absorption and emission spectroscopy (2), flame atomic absorption spectrometry (3), and inductively coupled plasma mass spectroscopy (2,4), have been developed and are commercially available. These methods exhibit good sensitivity, selectivity, reliability, and accuracy, but they often require sophisticated instrumentation and trained personnel. Electrochemical methods like ion selective electrodes, polarography, and other voltammetric methods (5) are much simpler and require less complex instrumentation, but are often unable to monitor at very low concentrations. None of these techniques can define or quantify the amount of heavy metal that is bioavailable and therefore likely to be a risk to living organisms. To achieve that, one needs a measurement that is biologically relevant, and the development of biosensors offers considerable promise in this respect.

A biosensor is a combination of a highly selective biological recognition element, responsible for the selectivity of the device, and a detection system (the transducer) for quantifying the reaction between the biological component and the target substance (analyte) to be monitored. In this chapter, we describe the background of bacterial interactions with heavy metals, and illustrate how that information is being used in the development of biosensors for heavy metals.

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2. BACTERIAL RESISTANCES TO HEAVY METALS

Heavy metals interact with living organisms in a variety of ways. A number of metals (e.g., Cu, Fe, Zn, V, Ni) are essential components of metalloenzymes (6). Others (e.g., Hg, Pb, Cd) are highly toxic with no known beneficial function. Metal-binding proteins are synthesized by many cell types in response to the presence of specific metals (7). Both prokaryotic and eukaryotic cells have mechanisms to transport essential metals to the sites of synthesis of metalloproteins, and many bacterial cells have specific systems for conferring resistance to heavy metals. These transport or resistance systems may be inducible by the metal, and therefore gene regulatory systems may be required that recognize the metal (8). The best understood of these systems at present are those responsible for conferring resistance to heavy metals in bacterial systems.

A list of some of the determinants of resistance to heavy metals found in bacteria is given in Table 1. These include resistances to cations and to oxyanions of metals in their most common physiological forms, and many of these resistance determinants have been described in recent reviews (9–12). These resistance determinants confer specificity to one or a few related metal ions, unlike most eukaryotic systems, where resistance is due to sequestration by relatively broad-range determinants, such as metallothioneins or phytochelatins. Metallothioneins have rarely been identified in bacterial systems (13,14). For all the resistance determinants in Table 1, the genes have been sequenced and the identities of the proteins conferring resistance have been predicted.

The mechanisms of metal tolerance and resistance vary, but the majority are due to efflux of the toxic metal from the cell (15). Some of these efflux systems are part of the normal metal homeostasis systems of the bacterial cell and the efflux pumps are encoded on the bacterial chromosome. These can be considered as proteins that confer the normal metal tolerance of the bacterial cells in which they occur. Examples of such proteins are the ZntA zinc transporter (16) and the CopA copper transporter (17) in *Escherichia coli*, or the CopA and CopB copper transporters in *Enterococcus hirae* (18). These proteins and some of the metal resistance proteins [e.g., CadA from *Staphylococcus aureus*, which confers Cd(II) resistance (19), or PbrA from *Ralstonia metallidurans*, which is part of the lead resistance determinant (20)] have similar structures. They are P-type ATPases, with eight transmembrane helices, one of which contains the amino acid sequence Cys-Pro-(Cys/His/Ser), and are known as CPx-ATPases (21). The N-terminus of about 100 amino acids shows some sequence similarity to the periplasmic mercury-resistance protein, MerP (see below), and contains a Cys-X-X-Cys motif associated with heavy metal binding (where X is any amino acid) (21). This N-terminal MerP-like region may be repeated and was thought to confer metal specificity on the transporter.

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TABLE 1 Some Heavy Metal Resistance Determinants in Bacteria

Metal	Organism	Mechanism	Location	Ref.
Hg	*Ps. aeruginosa* (and large number of other gram-negative and gram-positive genera)	Uptake of Hg^{II} and reduction to Hg^{0} by mercuric reductase	Plasmids and transposons	25
Cd	*Staph. aureus*	Efflux CPx-ATPase	Chromosome	19
	Ralstonia sp.	Efflux pumps (*czc*, Cd, Zn, and Co; *cnr*, Cd and Ni)	Plasmid	70
	Ps. aeruginosa CMG103	Efflux pumps (*czr*, Cd, Zn)	Plasmid	71
Zn	*Ralstonia* sp.	See Cd, *czc* system	Plasmid	70
	E. coli	Efflux CPx-ATPase	Chromosome	16
	Ps. aeruginosa CMG103	See Cd, *czr* system	Plasmid	71
Cu	*Ps. syringae*	Surface sequestration (*cop* system)	Plasmid	72
	E. coli	? Surface sequestration/efflux (*pco* system);	Plasmid	73
		efflux CPx-ATPase (*copA*)	Chromosome	17
	E. hirae	Efflux CPx-ATPase (*copA/B*)	Chromosome	74
	Ralstonia sp.	Efflux CPx-ATPase	Plasmid	
Co	*Ralstonia* sp.	Efflux pumps (*czc* Cd, Zn, and Co)	Plasmid	70
	Synechocystis	Efflux pump (*coaT*)	Chromosome	75
Ni	*Ralstonia* sp.	Efflux pump (*cnr* Cd and Ni)	Plasmid	76
Pb	*Ralstonia* sp.	Possible efflux and sequestration	Plasmid	20
As	*Staphylococcus aureus*	Arsenate reductase and arsenite efflux	Plasmid	77
Cr	*Ralstonia* sp.	Efflux	Plasmid	78
	Ps. aeruginosa	Efflux	Plasmid	79
	Bacillus sp.	Efflux	?	80
Ag	*Salmonella*	Sequestration and efflux	Plasmid	81

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Expression of the metal homeostasis proteins is usually regulated as part of the mechanisms whereby the bacterial cell adjusts the intracellular concentration of the individual metal. ZntA and CopA are regulated by activator proteins (ZntR and CueR), which respond to the specific metal (22; 22a), and the *E. hirae* system involves a complex interaction of the regulatory proteins CopY and CopZ (23).

Resistance determinants per se are frequently plasmid-borne and may interact with the chromosomally encoded systems for metal homeostasis if the metal is also an essential nutrient. For example, the copper resistance determinant of *E. coli* appears to require proteins that are part of the normal homeostasis system of *E. coli* (24) and are therefore encoded on the chromosome. Determinants of mercuric ion resistance, on the other hand, appear to require only those genes carried on the mercury-resistance plasmid (25).

The proteins required for metal homeostasis or metal resistance are often expressed by the bacteria in response to metal ion concentration (26). For example, many resistance determinants are expressed only in the presence of the specific metal ions at high subtoxic concentration (8). This involves specific regulatory proteins, either repressors or activators, that bind the metal ion and alter transcription of the structural genes responsible for metal sequestration, transport, or modification. Metal-resistance determinants and the chromosomal determinants of metal homeostasis contain metal-responsive genetic elements responsible for expression of structural gene products that bind and/or transport the metal ions. These regulatory elements, the regulatory proteins, or the products of the structural genes could be used in the construction of metal-specific biosensors.

Probably the best understood of all metal resistances is the widespread group of mercury-resistance (*mer*) determinants (25,27). Mercury is not an essential nutrient and the resistance determinants are often found in plasmids or transposons. Among the simplest of these *mer* determinants is that of transposon Tn*501* from *Pseudomonas aeruginosa*. This is shown in Figure 1. Three structural genes, encoding (a) a small periplasmic protein, MerP, (b) an inner membrane transport protein, MerT, and (c) the enzyme mercuric reductase, are expressed under the regulation of the activator protein, MerR, which binds Hg(II) and activates gene expression (25). Possibly because of our detailed knowledge of this system, several different components of the *mer* system have been used in the design of biosensors. These include the NADPH-dependent mercuric reductase in an enzyme-linked biosensor (28), the *mer* regulatory region in a whole cell biosensor (29), and the MerR protein in a capacitance biosensor (30).

We believe that, as a general principle, we can use many of the bacterial resistance determinants for other metals in the development of biosensors. Some examples of the creation of such biosensors are given below.

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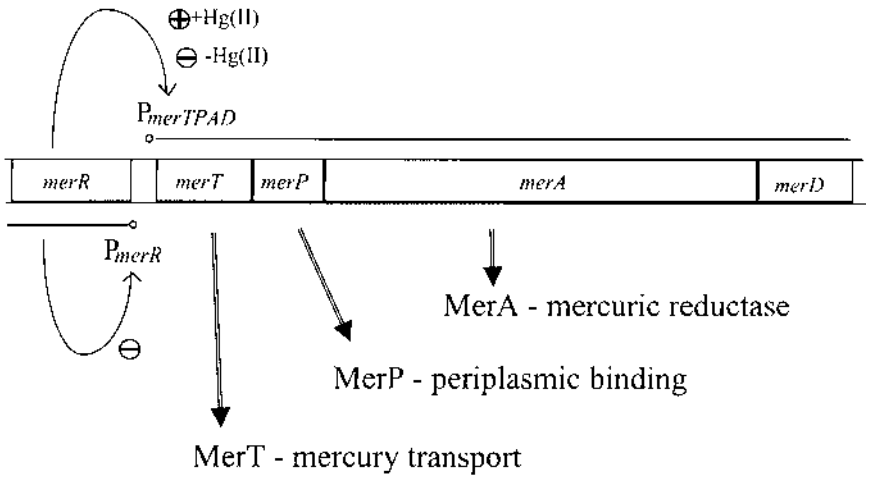

FIGURE 1 Diagram of the mercuric ion resistance (*mer*) operon of transposon Tn*501* showing the genes, gene products, and regulatory sequences. The regulatory region, the mercuric reductase enzyme, and the MerR protein have all been used in the development of biosensors for Hg(II).

3. OTHER METAL-REGULATED SYSTEMS

A number of heavy-metal-regulated bacterial systems not directly related to heavy-metal-resistance mechanisms have been described. Those systems are mainly involved in the intracellular regulation of essential transition metals ions such as iron, nickel, molybdenum, and magnesium.

The transport and regulation of iron concentration in bacteria has been studied in detail. This essential metal for cellular metabolism is needed as a cofactor for a large number of enzymes, but is not easily available to microorganisms in aerobic environments. Therefore, most aerobic bacteria produce and secrete low-molecular-weight compounds termed siderophores to capture Fe^{3+} from the extracellular medium. The iron uptake has to be very well regulated to maintain the intracellular concentration of the metal between desirable limits, since too high an intracellular concentration of iron can catalyze Fenton reactions and generate toxic species of oxygen. An understanding of how bacteria regulate iron transport (31) through the Fur protein (for ferric uptake regulation) was gained by mapping, cloning, and eventually sequencing the *fur* gene (32). The Fur protein has been purified (33), and recently the abundance of the Fur protein, the form of interaction with target DNA sequences, and the involvement of Fur in many cell func-

tions indicate that the Fur protein performs more like a general regulator than a specific repressor (34). The cooperative binding of the Fur protein in extended promoter regions would explain how a relatively simple protein controls a complex regulon in a gradual fashion.

The type of regulation described for Fur appears to be very similar to that of other metal-dependent repressors. Zinc is also an essential element that, depending on the concentration, becomes a potent toxin. In addition to the regulation of zinc efflux by ZntR (22), the regulation of zinc uptake by the Zur protein has been described in *E. coli* (35). The genes involved were named *znuACB* (for zinc uptake) and localized at 42 min on the genetic map of *E. coli*. A *znuA-lacZ* operon fusion was repressed by 5 μM zinc and showed a more than 20-fold increase in β-galactosidase activity when zinc was bound to a zinc chelator; this was under the control of the *zur* (zinc uptake regulator) gene. High-affinity ^{65}Zn transport of the constitutive *zur* mutant was 10-fold higher than that of the uninduced parental strain. An in vivo titration assay suggested that Zur binds to the bidirectional promoter region of *znuA* and *znuCB*. The Zur protein showed 27% sequence identity with the iron regulator Fur and is very similar to the *Bacillus subtilis* (36) and *Listeria monocytogenes* (37) homologs.

The Zur and Fur proteins have significant sequence identities (24% in *B. subtilis* and 27% in *E. coli*), and Zur-binding sequences have been described for promoters of genes related to zinc uptake that are similar to the Fur box. Moreover, the fact that Fur has recently been defined as a zinc metalloprotein containing one structural ion of zinc per polypeptide (38) makes the relation between these proteins even more complex.

Another example, in addition to Fur and Zur, is SirR—a novel iron-dependent repressor in *Staphylococcus epidermidis* with homology to the DtxR family of metal-dependent repressor proteins (39). SirR functions as a divalent metal-cation-dependent transcriptional repressor and is widespread among the staphylococci. In *B. subtilis* the PerR regulon (40) has been shown to respond to iron as well as the genes involved in the response to oxidative stress such as *katA* (encoding catalase A) and *aphC* (alkyl hydroperoxide reductase). However, the Per boxes are associated with oxidative stress genes in several gram-positive bacteria rather than with iron transport. The Fur, Zur, SirR, and PerR are all proteins that can be used as potential biological components of biosensor devices.

Other nonspecific metal-regulated genes related to global stress responses have also been described and used as biological components of biosensor devices (41). Heat shock gene expression is induced by a variety of environmental stresses, including the presence of metal ions. *Escherichia coli* heat shock promoters for *dnaK* and *grpE* were fused to the *lux* genes of *Vibrio fischeri*, and it has been suggested that biosensors constructed in this manner have potential for environmental monitoring (42).

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4. BIOSENSORS FOR HEAVY METALS

Biosensors are often cheap analytical devices in which a simple biological event is transduced into an electronic signal in a quantitative fashion, and ideally these should show high sensitivity and high specificity, and should work robustly in complex matrices, such as soil, water, and biological material. The selectivity of a biosensor depends on the biological component, and its sensitivity depends on the response of that component and the ease with which this can be transduced into a measurable signal. A large variety of biological components and transducers that can be used for heavy metal sensing are summarized in Table 2, showing their main analytical characteristics, such as limit of detection (LOD), dynamic range (DR), and selectivity. As can be seen, these characteristics are highly dependent on the type of biological molecule and the transducer used for biosensor design and construction. The various biosensors also display different stability, and those based on immobilized enzymes are characterized by a low operating period.

Recently we have been involved in the development of two different types of biosensor (43). In one, bacterial cells are genetically modified to respond to the presence of a heavy metal by the emission of light (44). These *whole-cell biosensors* (or in vivo biosensors) are now commercially available. The other biosensor uses immobilized bacterial proteins that bind heavy metals and alter the surface properties of an electrode in response to metal binding (30). Such *capacitance electrodes* show high sensitivity and some selectivity, but are at an early stage of development.

Some publications use ''biosensor'' in the context of detection of toxic compounds by viability assays, of varying types, on whole cells. We eschew such a definition, and use ''biosensor'' in the context of detection of a *specific analyte* or a small *range of chemically related compounds*.

4.1 Whole-Cell Biosensors

A significant area of research in bacterial molecular genetics has been the study of the control of gene expression (8,26). As part of these studies, many ''reporter systems'' have been developed that allow a transcriptional regulatory element to be placed such that it regulates expression of a gene that has a quantifiable product. Two of the most commonly used systems are the *lacZ* gene of *E. coli* and the *lux* genes of *V. fischeri* (for example, see ref. 45). The former encodes β-galactosidase, the production of which can be determined in a simple enzyme assay using the chromogenic substrate *o*-nitrophenol-β-D-galactose (46). This is of some use in the laboratory, but of little use in making a biosensor. The *lux* genes of *V. fischeri* are much more useful in the construction of biosensors, as they produce and oxidize long-chain aldehydes and generate photons as part of the reaction (47). The light that is emitted can be measured.

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TABLE 2 Heavy Metal Biosensors and Their Properties

Biological molecule			Transducer type	Operating conditions	M^{2+}	LOD	DR	Ref
Whole cell	Mosses	*Sphagnum* sp.	Stripping differential pulse voltammetry	Acetate pH 6.0, IS 0.7, 10% moss, carbon paste electrodes	Pb^{2+}	2 ng/ml	5–125 ng/ml	82
		E. coli + *mer* promoter + lux genes from *V. fischeri*	Optical detection	Bioluminescence is measured at 28°C, 30 min response time under aeration	Hg^{2+}	0.1 μM	20 nM–4 μM	83
		E. coli + *lux* genes from *V. fischeri*	Optical detection		Hg^{2+} Cu^{2+}	0.1 μM 0.1μM		84
		R. silverii + *lux* operon from *V. fischeri*	Optical detection	23°C, 0.2% acetate, 20 mM MOPS, pH 7.0, 20 μg/ml tetracycline	Cu^{2+} Zn^{2+} Cd^{2+} Cr^{6+} Pb^{2+} Tl^{+} Ni^{2+}	2 μM 5 μM 5 μM 1 μM 1 μM	2–40 μM 5–250 μM 5–200 μM 1 μM–40 μM 1 μM–40 μM	43,50, 55,85
	Bacteria	*R. silverii* + *lux* operon from *V. fischeri*	Optical detection	Microorganisms immobilized in polymer matrices, 25°C	Cu^{2+}	1 μM	1	86
		E. coli + *lux* operon	Optical detection	30°C, M9 medium	Hg^{2+} Cu^{2+}	10 nM 1 μM		52
		E. coli + firefly luciferase gene	Optical detection	Luminescence is measured in microtiter plate after 60 min at 30°C	Hg^{2+}	0.1 fM	0.1 fM–0.1 μM	87

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TABLE 2 Continued

Biological molecule			Transducer type	Operating conditions	M^{2+}	LOD	DR	Ref
		Staph. aureus + firefly luciferase gene	Optical detection	Luminescence is measured in scintillation counter after 60 min	AsO^{43} Cd^{2+}	1 μM 1 μM	1–5 μM 1–20 μM	49
		Staphy. aureus + firefly luciferase gene	Optical detection	Luminescence is measured in microtiter plates, 30°C	Cd^{2+} Pb^{2+} Hg^{2+}		10 nM–1 μM 33 nM–330 μM 33–100 nM	88
		B. subtilis + firefly luciferase	Optical detection	Luminescence is measured in microtiter plates, 30°C	Cd^{2+} Zn^{2+}		3.3 nM–1 μm 1–33 μM	88
Enzyme	Urease		ISFET	Inhibition of urease immobilized on different membranes, 0.02 M HEPES, 25°C, batch mode	Cu^{2+} Hg^{2+} Cd^{2+} Pb^{2+}		1–10 mg/L 0.25–5 mg/L 3–10 mg/L 2–10 mg/L	89
			ISFET	Inhibition of urease immobilized in a Nafion film, 20°C	Hg^{2+} Cu^{2+}	1 μM 3 μM		90
			Ammonia sensor	Inhibition of urease, cuvette test with ammonia-sensitive coating on the wall, 0.1 *N* maleate buffer pH 6	Cu^{2+} Hg^{2+} Zn^{2+} Pb^{2+}	0.25 ppm 0.07 ppm 50 ppm 100 ppm	0.4–0.7 ppm 0.07–1 ppm 50–70 ppm 100–350 ppm	91

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Ammonia sensor	Enzyme reactor with urease inhibited by mercury, enzyme immobilized on glass beads	Hg^{2+}		0–15 nM	92
pH sensor	Inhibition of urease immobilized with thymol blue covalently bound to aminopropyl glass at the tip of an optical fiber	Cu^{2+} Hg^{2+}	 2 ppb		93
Conductometric detection	Enzyme on interdigitated gold electrodes, residual activity of urease is measured, 5 mM Tris-HNO_3 pH 7.4, 50 mM urea	Hg^{2+} Cu^{2+} Cd^{2+} Pb^{2+} Co^{2+}		1–50 µM 2–100 µM 5–200 µM 0.02–5 mM 10–500 µM	94
Conductometric detection	Inhibition of urease is monitored with a standing acoustic wave device	Hg^{2+}	20 ppb		95
Fluorimetric detection at 340/485 nm	Flow system, enzyme immobilized on controlled pore glass, 0.005 M phosphate buffer pH 6.5	Hg^{2+}		0.5–100 ng/ml	96

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TABLE 2 Continued

Biological molecule		Transducer type	Operating conditions	M^{2+}	LOD	DR	Ref
		Fluorescence detection at 340/455 nm	Flow system, inhibition of urease detected using *o*-phthaladehyde	Hg^{2+}	2 ppb		97
	Urease	IrTMOS	Ammonia detection by IrTMOS, 0.05 M Tris-HCl, pH 8.3	Hg^{2+}	0.005 μM		98
	Carbonic anhydrase	Fluorescence anisotropy detection	Enzyme labeled with derivatives of benzoxadiazole sulfonamide	Cu^{2+} Co^{2+} Zn^{2+}	pM pM pM		99
	L-Lactate dehydrogenase	Amperometric detection	Enzyme coimmobilized with L-lactate oxidase on the top of an oxygen electrode	Hg^{2+} Cu^{2+} Zn^{2+}	1 μM 10 μM 25 μM		100
	Glycerophosphate oxidase	Oxygen electrode	Inactivation of enzyme by metal ions, enzyme immobilized by reticulation in gelatin film or covalent binding on a membrane	Hg^{2+}	μmolar	20–500 μM	101

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Pyruvate oxidase	Oxygen electrode		Hg^{2+}	10 nM		101
Cholinesterase	Voltammetric detection	Flow system, enzyme immobilized on nitrocellulose film with glutaraldehyde	Pb^{2+} Cu^{2+} Cd^{2+}	5 µM 50 nM 5 µM		102
Alkaline phosphatase	Spectrophotometric detection	Chemiluminescence from enzyme-catalyzed hydrolysis of a phosphate derivative of 1,2-dioxetane is measured	Zn^{2+}	0.17 ppm		103
Horseradish peroxidase	Spectrophotometric detection	Inhibition of enzyme immobilized on solid supports is measured	Hg^{2+}	0.1 pptr	4 orders of magnitude	104
Invertase	Amperometric detection	Inhibition of enzyme immobilized on a membrane is measured	Hg^{2+}	1 ng/ml		105
Acetylcholinesterase	Amperometric detection	Inhibition of enzyme by metal ions is measured	Cu^{2+} Cd^{2+} Fe^{2+} Mn^{2+}	0.01 pM 1 pM 10 pM 100 pM		106
Apoenzyme Alkaline phosphatase	Calorimetric detection	Enzyme immobilized on epoxide acrylic beads, 100 mM TRIS-HCl pH 8.0	Zn^{2+} Co^{2+}		0.01–1.0 mM 0.04–1.0 mM	107

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TABLE 2 Continued

Biological molecule	Transducer type	Operating conditions	M^{2+}	LOD	DR	Ref
	Spectrophotometric detection	Flow injection system, change in absorbance at 405 nm is measured	Zn^{2+} Co^{2+}	sub-μ	0.1–10 μM 1–200 μM	108, 109
	Potentiometric detection	Flowthrough ISFET, pH shift detected	Zn^{2+}		0.01–1.0 mM	110
	Optical detection	Chemiluminescence from enzyme-catalyzed hydrolysis of a phosphate derivative of 1,2-dioxetane is measured	Zn^{2+}	0.5 ppb	0.5–50 ppb	103
Ascorbate oxidase	Calorimetric detection	Flow system, enzyme immobilized on porous glass beads	Cu^{2+}		1–50 μM	111
	Spectrophotometric detection	Absorbance at 265 nm is measured	Cu^{2+}		0.1–10 μM	112
	Amperometric detection	Polarographic oxygen electrode is used	Cu^{2+}		0.5–2 μM	113

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	Carbonic anhydrase	Calorimetric detection	Flow system, enzyme immobilized on porous glass beads	Zn^{2+} Co^{2+}	25–250 μM 50–200 μM	114, 115
		Optical detection at 326/460 and 560 nm	Recognition of metal ion by apoenzyme transduced by the dansylamide fluorescent probe	Zn^{2+}	40–1000 nM	99,116
	Galactose oxidase	Calorimetric detection		Cu^{2+}	5–20 mM	117
		Amperometric detection	Detection with oxygen electrode	Cu^{2+}	0.1–10 mM	113
	Alkaline phosphatase + ascorbate oxidase	Amperometric detection	Enzymes coimmobilized on a polymer membrane attached to a polarographic oxygen electrode	Cu^{2+} Zn^{2+}	2–100 μM 2–200 μM	118
	Tyrosinase	Amperometric detection	Flow system with oxygen electrode		Up to 0.05 mM	119
Protein	Apophytochelatin	UV-spectrophotometric detection at 215 nm	270 mM apophytochelatin is used	Cd^{2+}	1–6 ppm	120

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TABLE 2 Continued

Biological molecule		Transducer type	Operating conditions	M^{2+}	LOD	DR	Ref
	MerR-LacZα:M15 complex	Spectrophotometric detection	Microtiter plates coated with BSA-divinylsulfone- glutathion were treated with Hg^{2+} concentrations and after washing the protein was bound to it	Hg^{2+}	ppb level		121
	Glutathione	UV-spectrophotometric detection at 215 nm	160 mM glutathione is used	Cd^{2+}		1–8 ppm	120
		pH measurement	Protein cross-linked with glutaraldehyde and entrapped behind a dialysis membrane	Cd^{2+}		10–80 ppm	120
Antibody	Antibody against Cd-EDTA complex	Spectrophotometric detection	Microtiter plates were coated with Cd-EDTA-BSA conjugate and then the antibody was added, HEPES buffer pH 7.0–7.2	Cd^{2+}	7 ppb	10–2000 ppb	122

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The principle of whole-cell biosensors is simple (43,44). The biological component is a viable bacterial cell that has been modified to contain, say, the *lux* genes under the control of a metal-responsive promoter, together with the regulatory proteins required to express that promoter in the presence of metal. A stylized system is shown in Figure 2. The *lux* ''reporter gene'' is only expressed, and therefore, the *lux* gene products are only produced, in the presence of metal. Therefore, a calibrated system can detect the presence of metal by the emission of light. As virtually all metal homeostasis genes and all metal resistance

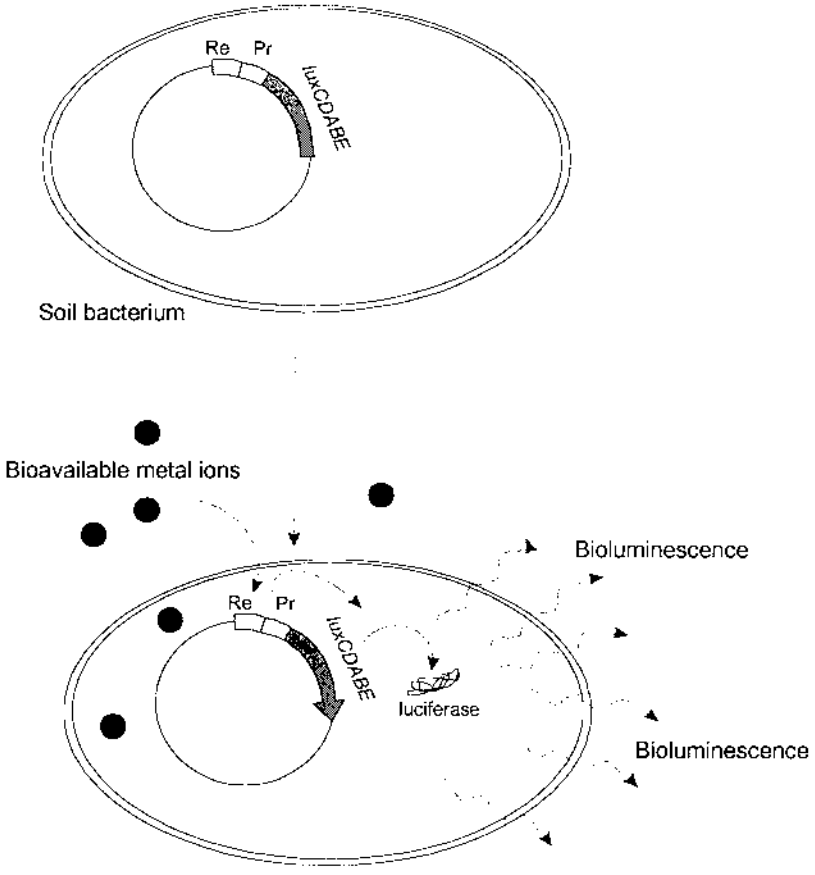

FIGURE 2 Diagram of the *lux* transposon in whole-cell biosensors for heavy metals. Luciferase is expressed from the gene fusion with a metal-regulated promoter (Pr) under the control of the regulatory gene (Re).

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proteins appear to be expressed only in the presence of the cognate metal, a wide variety of metals could be detected by variants of this simple system.

A patent has been published on an early biosensor constructed in such a fashion (29,48) in which the mercury resistance operon of *Serratia marcescens* was coupled to the *lux* genes of *Vibrio* to detect in quantitative fashion the presence of mercuric salts in aqueous solution. However, as pointed out by Rouch et al. (45), the regulatory region of mercury resistance determinants is not a good system for quantitative estimates of Hg(II), as it responds in a hypersensitive manner across a very narrow concentration range. Other systems, such as the copper resistance (*pco*) determinant of *E. coli*, respond across a wide range of metal ion concentration (45) and may be better suited to the development of quantitative biosensors. Corbisier and co-workers (49) have linked the regulatory regions of the cadmium (*cad*) and arsenate (*ars*) resistance determinants of *Staphylococcus aureus* to the *lux* genes of *Vibrio harveyi* in a shuttle vector that allows expression in *Staph. aureus* and *E. coli*.

More recent developments exploit the unusual properties of *Ralstonia metallidurans* (formerly *Alcaligenes eutrophus*) CH34. This organism was isolated from the soil downwind of a zinc-smelting plant in Belgium. This area is so heavily polluted with heavy metals from the smelting plant that little grows. *Ralstonia metallidurans* CH34 contains two large plasmids, which between them encode a large number of metal-resistance determinants conferring resistance to copper, cadmium, cobalt, copper, lead, mercury, thallium, and zinc, among others (50). A transposable element has been constructed that places the *lux* genes of *V. harveyi* under the control of adjacent promoters outside the transposon. This allows the selection by genetic techniques of different strains in which light is emitted in response to different metals. A panel of such strains is now commercially available under the name BIOMET.

The use of these bacterial sensors to detect the biologically available metal fraction in polluted environmental samples has also been demonstrated (51). A bacterial copper sensor, AE1239 (based on *Ralstonia silverii* DS185), and a zinc, cadmium sensor, AE1433 (based on *R. metallidurans* CH34), have been used to assess the quality of incinerator fly ashes (52) contaminated by heavy metals and high concentrations of inorganic salts. The analysis of this type of sample was made possible only because the sensor was inserted into a soil bacterium able to grow even in the presence of a high concentration of salts. Those sensors have also been very efficient for quick evaluation of the efficacy of bioremediation techniques such as in situ metal inactivation in contaminated soils (53,54). Vangronsveld et al. have combined the use of sequential extraction procedures, the BIOMET microbial heavy metal biosensors, phytotoxicity tests, and a zootoxicity test as a test system for the evaluation and monitoring of the efficacy and durability of in situ immobilization of metals in contaminated soils (53). Good agreement was found between the different evaluation criteria, and the BIOMET sensors

were recommended by the main competent authority, the Public Waste Agency of Flanders (OVAM), as a relevant tool to detect bioavailable heavy metals in polluted soils.

A *R. metallidurans* nickel sensor, AE2515, was also used to quantify nickel bioavailability in various nickel-enriched soils that had been treated with additives for in situ metal immobilization (55). The nickel bioavailability measured with the biosensor correlated linearly with data on the biological accumulation of nickel in specific parts of important agricultural crops. Therefore, the biosensor could be used to assess risk associated with the potential transfer of nickel to organisms in the food chain, in this case maize and potato plants grown on nickel-enriched soils (55).

The analysis of lead-polluted soils with the bacterial sensor responsive to lead ions is given below as an example. The lead bacterial biosensor was based on a genetically engineered *R. metallidurans*, AE2450. This bacterium bears a plasmid with a *pbrRpbrA::luxCDABE* fusion (43), and a bioluminescent signal is produced when the bacteria are in contact with lead ions, as illustrated in Figure 2. Those ions activate the lead-sensitive promoter within the cell, which controls the production of luciferase enzyme responsible for the bioluminescence signal. The bioluminescence was recorded with a luminometer and was proportional to the biologically available fraction of lead present in the sample.

4.2 Enzyme-Based Biosensors

Enzyme-based biosensors are by far the most common class of biosensor yet constructed and constitute the majority of biosensors in commercial use. These are commonly used for detecting metabolites in clinical chemistry, owing to the specificity and variety of enzymes available that act on biochemical compounds (56). However, heavy metals participate in relatively fewer specific biochemical reactions and any such reaction may be relatively nonspecific. Mercuric reductase is a member of the dithiol oxidoreductase class of proteins and its enzymology is well understood (57–59). The enzyme is a flavoprotein that catalyzes the two-electron reduction of Hg(II) to Hg(0) in an NADPH-dependent manner. The likely physiological substrate is the dimercaptan rather than the divalent cation (Equation 1).

$$\mathrm{RS\text{-}Hg^{II}\text{-}SR' + NADPH + H^+ \rightarrow Hg^0 + NADP^+ + RSH + R'SH} \qquad (1)$$

As was pointed out some years ago (60), this reaction can be followed using an enzyme electrode to follow the consumption of H^+. Alternatively, the NADPH can be regenerated in a linked second reaction, which can be followed. Recently a biosensor has been patented (28) in which the NADPH regeneration is linked to the production of a long-chain aldehyde, such as octanal or decanal, which are substrates for luciferase. As the aldehyde is produced it is oxidized

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by luciferase to the corresponding long-chain acid with the concomitant production of light, which is detected. This biosensor has not yet been described in the peer-reviewed literature, but the patent also covers the use of organomercury lyase to convert organomercurials to Hg(II), thus allowing the detection of organomercurials, and the patent extends to cover other metal ions that may be reduced in biochemical reactions. Hg, Cr, As, Tc, Cu, Ag, Se, V, Mo, and U are specifically mentioned, although suitable enzymes for reduction of all of these have yet to be purified.

For sake of completeness, we should mention that apoenzymes are sometime used to detect the presence of the metal ion cofactor normally found in the corresponding holoenzyme (61). The metal ion can be detected in quantitative fashion owing to gain of enzyme activity on binding the metal ion.

4.3 Capacitance Biosensors

Recently, a new heavy metal biosensor was designed, based on heavy-metal-binding proteins, as the biorecognition element, and a highly sensitive capacitive transducer (30,62–64). The characteristics of two classes of metal-binding proteins used for biosensor design are presented below.

The biosensor uses a capacitive transduction principle that can be briefly outlined as follows. There is a double layer between a metal electrode (gold) and the solution, resulting in a double-layer capacitance as well as in a Faradaic current giving rise to a Faradaic background current in electrochemical measure-

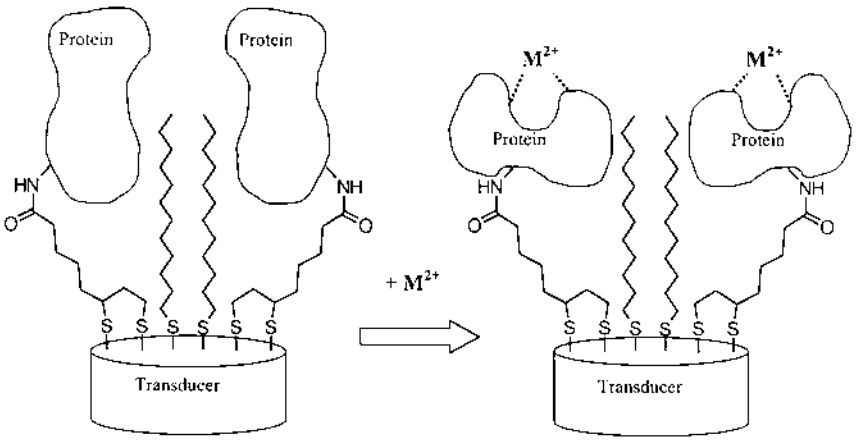
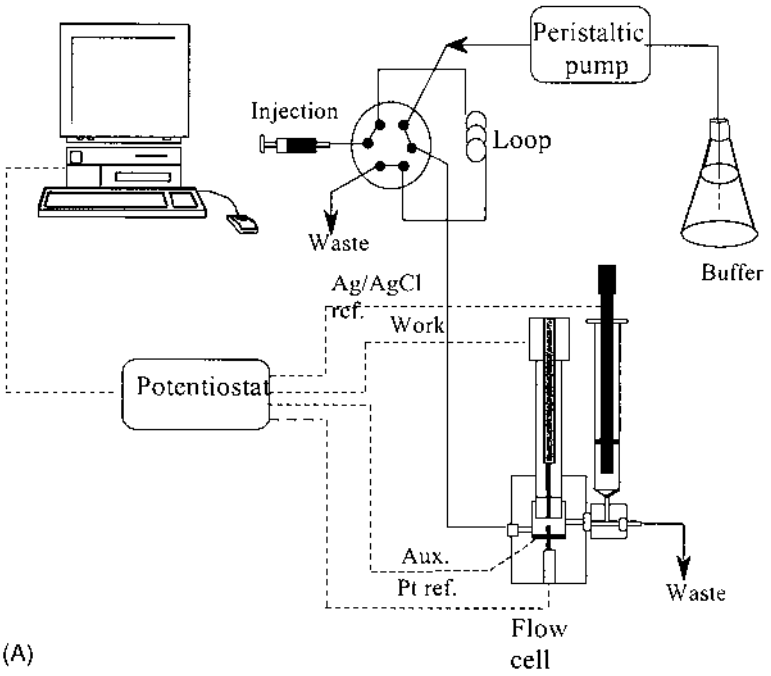


FIGURE 3 Principle of the capacitance biosensor, showing the basic construction and the proposed effect of metal binding.



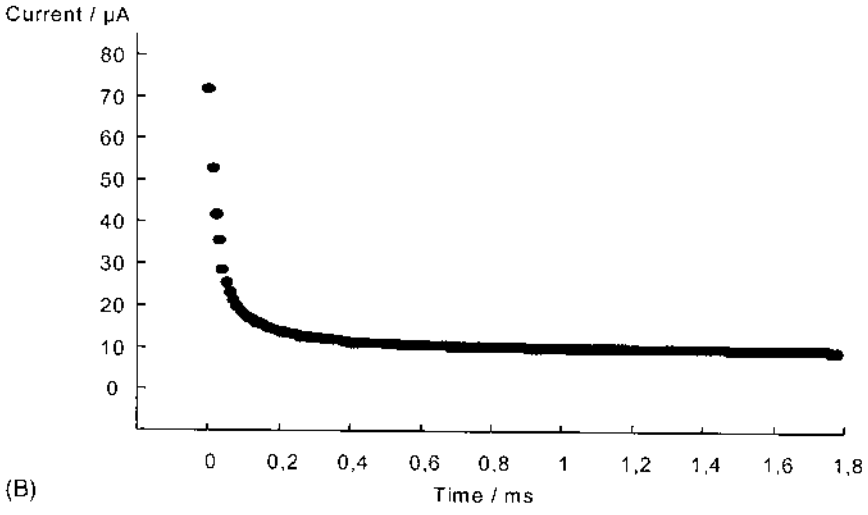


FIGURE 4 (A) Schematic diagram of the experimental setup of the capacitance biosensor. (B) Current transient obtained after the potentiostatic pulse is applied.

ments. The electrode surface can be isolated by, for example, covering it with long-chain alkenethiols that readily self-assemble to materials like gold. A capacitance still can be measured although it is not a double-layer capacitance in the usual sense. The capacitance change is measured by applying a fast potentiostatic pulse to the electrode and evaluating the resulting current transients, which are altered by any changes that affect the metal-binding proteins.

Thus, monitoring of heavy metals with this type of biosensor is based on the conformational change that occurs when the metal ions bind to the protein. The conformational change results in change of the capacitance. A schematic representation of the detection principle is shown in Figure 3.

4.3.1 Biosensor Preparation

Biosensors were prepared according to a previously published protocol (30). The biosensor was inserted as the working electrode in a specially constructed three-(four)-electrode flow cell with a dead volume of 10 µl, as shown in Figure 4A. The electrodes were connected to a fast potentiostat described elsewhere (62). A platinum foil and a platinum wire served as the auxiliary and the reference electrodes, respectively. An extra reference electrode (Ag/AgCl) was placed in the outlet stream. The buffer solution was pumped by a peristaltic pump and samples were injected into the carrier buffer flow. The working electrode had initially a rest potential of 0 mV versus the Ag/AgCl reference electrode. Measurements were made by applying a potential pulse of 50 mV and current transients were recorded following the applied potential step according to Equation 2 (see Fig. 4B).

$$i_{(t)} = u/R_s \exp(-t/R_s C_1) \tag{2}$$

where $i_{(t)}$ is the current at time t, u is the amplitude of the potential pulse applied, R_s is the resistance between the gold and the reference electrodes, C_1 is the total capacitance over the immobilized layer, and t is the time elapsed after the potential pulse was applied.

5. PRACTICAL EXAMPLES

5.1 Whole-Cell Biosensors

A bacterial sensor for lead was used to investigate levels of bioavailable metals in soil. Fifteen soil samples from the Haarlem, Arnhem, and Rotterdam municipalities were analyzed with the *R. metallidurans* AE2450 lead sensor. Results obtained with this Pb sensor and by chemical analysis are summarized in Table 3. Five grams of each soil sample was mixed in a reconstitution medium (0.2% gluconate, 20 mM MOPS, pH 7.0, 20 µg.ml^{-1} tetracycline). β-Glycerophosphate was used in the medium to avoid lead precipitation. An aliquot of 2 ml was

TABLE 3 Characterization of the Soil Samples by the BIOMET Lead Sensor and by Chemical Analysis

Sample TNO ID	Sample municipal ID	Origin	Pb equivalent (mg/kg dw)	Total content (mg/kg dw)[a]	Pb in exchangeable (mg/kg dw)[a]	Pb in carbonate fraction (mg/kg dw)[a]
822523-001	1.3	Haarlem	2.29 ± 1.27	640	0	127
822523-002	2.4	Haarlem	2.21 ± 0.51	300	0.3	99
822523-003	3.4	Haarlem	1.89 ± 0.51	1500	0	321
822523-004	4.1	Haarlem	1.64 ± 0.15	370	0	78
822523-005	5.2	Haarlem	2.88 ± 0.59	470	0	88
822526-001	003A	Arnhem	1.53 ± 0.29	240	0	50
822526-002	006A	Arnhem	1.69 ± 0.51	400	0	99
822526-003	010A	Arnhem	3.50 ± 1.76	460	0	126
822526-004	011A	Arnhem	2.98 ± 0.00	980	0	342
822526-005	014A	Arnhem	2.05 ± 0.73	700	0.7	160
822526-006	017A	Arnhem	3.29 ± 0.73	860	0	236
822526-007	020A	Arnhem	1.69 ± 1.03	3200	0	25
822526-008	021A	Arnhem	6.69 ± 0.62	4400	0	576
422278-001	Almstraat	Rotterdam	n.d.	1100	0	667
422278-003	Schiekade	Rotterdam	13.4 ± 4.57	2700	0	1420
422278-004	Strekkade	Rotterdam	6.34 ± 1.13	1020	0	137

n.d., not determined because of lack of soil material.
[a] The chemical analyses were performed by Tauw Milieu b.v.

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further diluted 1:2 and 1:4, and 20 µl of the soil solution was mixed with 180 µl of the reconstituted bacterial sensor (at a concentration of 10^7–10^8 CFU/ml). Duplicate soil/solution/bacteria samples were incubated in a Lucy 1 luminometer at 23°C and bioluminescent measurements were automatically performed every 30 min for 8 h. A calibration curve was generated by adding 20 µl of $Pb(NO_3)_2$ solutions to the 180-µl bacterial solution. The following concentrations were tested: 0, 0.6, 1, 2.5, 5, 10, 15, and 25 µM lead (equivalent to 0, 0.124, 0.207, 0.518, 1.04, 2.07, 3.11, and 5.18 ppm lead). The equation derived from the calibration curve fitting was used to determine the concentration of lead equivalent in the soil sample and is presented in Figure 5. The soil dry weight (dw) was determined separately by drying the soil samples at 105°C for 18 h.

The detection limit of the lead sensor was around 0.85 mg lead/kg dw. Most of the values obtained with the soil samples were low but not negligible, except sample 021A from Arnhem and the two soil samples from Rotterdam. There was no direct relationship between the total content of lead of the soils, or the $CaCl_2$-extractable fraction, and the quantity of lead measured with the bacterial sensor (Fig. 6). Almost no lead could be detected in the $CaCl_2$ fraction by conventional analytical methods. This $CaCl_2$ fraction is sometimes considered the ''bioavailable'' fraction for living organism and, from our data, it is greatly underestimated. However, the bioavailable concentration measured with our bacterial sensor was directly related to the quantity of lead found in the sodium-acetate-extractable fraction (Fig. 7). This fraction corresponds to the fraction of

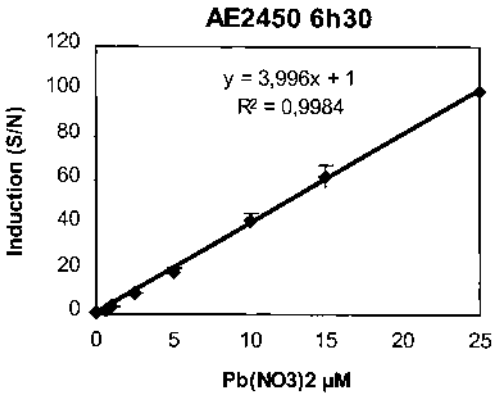

FIGURE 5 Calibration curve of the lead AE2450 Pb-BIOMET sensor in the presence of increasing concentration of lead nitrate after 6.5 h contact. The average bioluminescence of two measurements was plotted as the ratio of the bioluminescence observed in the presence of increasing lead concentrations to the bioluminescent signal obtained in the presence of MilliQ-treated water.

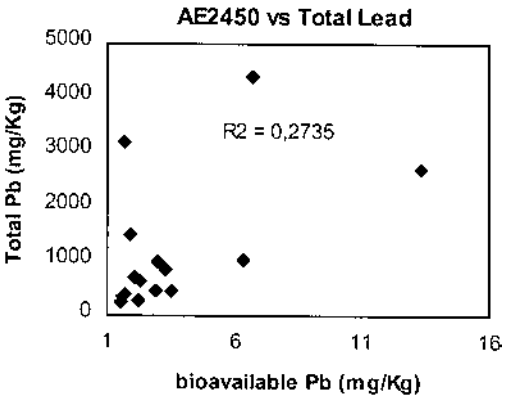

FIGURE 6 Analysis of the soil with the AE2450 Pb-BIOMET sensor plotted against the total amount of Pb after $CaCl_2$ extraction procedure. R^2, coefficient of linear regression.

metal bound to $CaCO_3$. In other words, this means that the lead present in those soils was available for the soil bacteria and able to induce a bioluminescence signal. The soil samples from Haarlem and Arnhem (except number 021A) have very little available lead. Sample 021A from Arnhem and both samples from Rotterdam had a higher available lead concentration, the highest being the soil

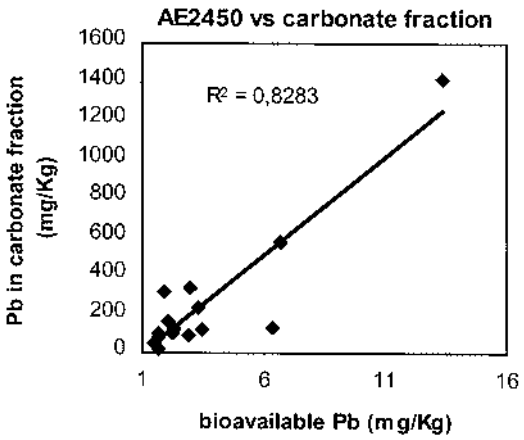

FIGURE 7 Relationship between the bioavailable Pb fraction measured with the AE2450 Pb-BIOMET sensor and the amount of Pb after Na acetate, pH 5 extraction procedure. R^2, coefficient of linear regression.

sample from Schiekade. The vial supposed to contain a soil sample from Almstraat was empty and could not be tested. The bioavailable fraction of lead was also much lower than the amount of lead chemically detected, which means that most of the lead was not available to the bacterial community. As a consequence, none of the samples were toxic for our soil bacteria.

The relationship between the lead concentration found in the Na-acetate-extractable fraction and the bioavailable concentration determined with the BIOMET lead sensor remains to be confirmed using other lead-polluted soil to generate more points in the region of high available lead content.

5.2 The Capacitance Biosensor

To date members of two classes of metal-binding proteins have been tested for capacitance biosensor construction, namely, the synechococcal metallothionein, SmtA (14), and the mercury-resistance protein, MerR (25).

5.2.1 A GST-SmtA-Based Biosensor

Metallothioneins are small proteins that sequester metal ions in a ''cage'' structure. In animal metallothioneins there are two domains, each of which can sequester three or four metal ions (65,66). Metal binding is associated with a large conformational change in the protein, as the sulfhydryl groups of cysteins coordinate the metal ions. There have been two reported bacterial metallothioneins, one in *Pseudomonas*, a gram-negative soil organism (13), and another in the cyanobacterium *Synechococcus* (14). The former has not been studied after first being reported, and the latter, the *smtA* gene product, is thought to be required for zinc homeostasis. The SmtA protein has been overexpressed as a glutathione-S-transferase–metallothionein fusion protein (67), and this was used as the biological component of the capacitance biosensor.

The electrodes were prepared as previously described (30) and were optimized and characterized with regard to selectivity, sensitivity, stability, and potential for regeneration (63).

Three different protein immobilization methods were tested: (a) covalent coupling with 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC), (b) entrapment in a polymer (polyethylene-glycol-diglycidyl-ether), and (c) cross-linking with glutaraldehyde (30). The best results (highest sensitivity) were obtained by covalent coupling with EDC. The sensitivity of the biosensors was found to be dependent on both the nature of the buffer and the pH. The highest signals were obtained in borate buffer at pH 8.75, while signals were lowest in HEPES buffer (pH 7.5) and the signals decreased with decreasing pH of the buffer (63).

A typical calibration curve recorded for metal ion concentrations in the 10^{-15}–10^{-3}-M range is presented in Figure 8. The first part of the calibration

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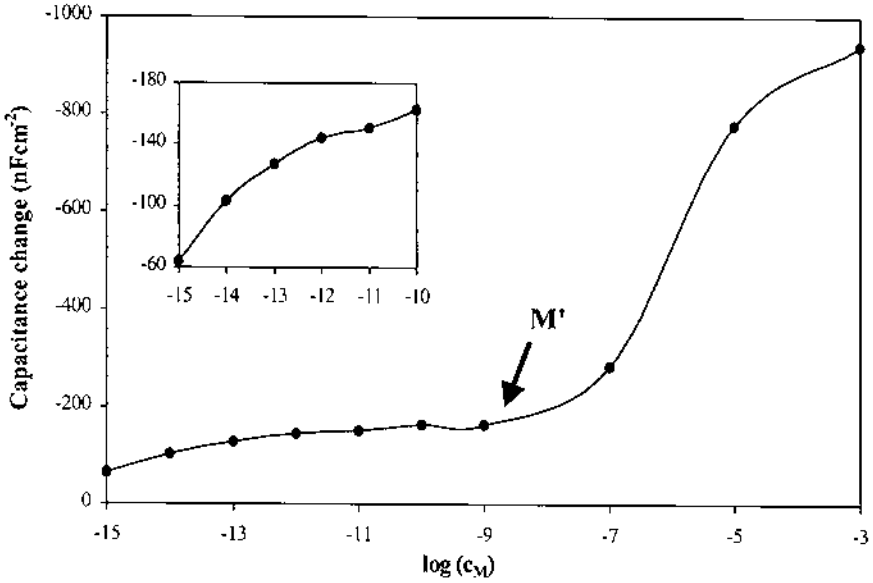

FIGURE 8 Typical dependence of capacitance change (in nF/cm^2) with the logarithm of metal ion concentration (c_M). Experimental conditions: 10 mM borate buffer, pH 8.75, flow rate 0.5 ml/min, room temperature. M′ marks the transition in the shape of the capacitance curve.

curve, showing gradual capacitance changes (up to a concentration of metal ion designated M′), was attributed to the titration of the Cys and His residues of the protein, while the second part, above M′, displayed a radical capacitance change and was thought to be due to significant conformational changes in the protein structure. The selectivity of the GST-SmtA electrodes was tested toward copper, cadmium, mercury, and zinc. The electrodes responded to all four heavy metals from femtomolar concentration levels. The selectivity pattern was: $S_{Cu} > S_{Cd} > S_{Hg} > S_{Zn}$. The calibration curves recorded for copper, cadmium, mercury, and zinc were similar, but they behaved differently in terms of the value of M′, beyond which the radical conformational changes occured. The dependence of M′ with the metal ion for the GST-SmtA and MerR electrodes, respectively, is presented in Table 4.

The stability of the GST-SmtA electrode was studied over 15 days. Calibration curves were recorded for copper in the 10^{-15}–10^{-10}-M range. Between measurements the sensor was stored in 100 mM borate buffer pH 8.75, at 4°C, and could be regenerated with 1 mM EDTA just before new measurements were made. However, a 40% loss of activity was observed after 15 days of storage.

TABLE 4 Dependence of M′ with Metal Ion for GST-SmtA- and MerR-Based Biosensors, Respectively

	M′ (M)	
Metal ion	GST-SmtA	MerR
Cu^{2+}	10^{-6}	10^{-6}
Hg^{2+}	10^{-9}	10^{-8}
Cd^{2+}	10^{-9}	10^{-7}
Zn^{2+}	10^{-10}	10^{-7}

M′ is the metal ion concentration at which a discontinuity in the change of capacitance with metal ion concentration was observed.

When a higher concentration (100 μM) of copper was used, the electrode could not be regenerated with EDTA. Therefore, the regeneration of the GST-SmtA electrodes was studied in different buffers (borate and HEPES) and at different pH values (8.75, 8.0, and 7.5). The pH of the buffers used was found to influence strongly the possibility of regeneration of the electrodes. At pH 8.0 electrodes could be regenerated even after injection of 100 μM copper in both borate and HEPES buffers (63).

5.2.2 A MerR-Based Biosensor

MerR is the regulatory protein responsible for inducible expression of mercury resistance proteins (25). Mercury binds to the dimeric MerR protein, and there is genetic evidence that this results in a conformational change in the protein (68,69), although no direct physical studies of the protein have been done owing to its relatively low solubility. We have been able to prepare native MerR protein in high yield, although care must be taken to keep it in a reducing environment by using buffers from which oxygen has been flushed and that are high in reduced sulphydryl compounds such as dithiothreitol. Electrodes containing MerR as the biological component were prepared in a similar manner to those described in the previous section.

Signals were again recorded for copper, cadmium, mercury, and zinc. The MerR electrodes were more selective for mercury than GST-SmtA–based ones, their sensitivity decreasing in the order: $S_{Hg} > S_{Zn} > S_{Cu} > S_{Cd}$. Recorded calibration curves were similar to those recorded for GST-SmtA electrodes, but the mercury signal was higher than the ones noticed for the other three metal ions throughout the studied concentration range. The dependence of M′ with different metal ions is shown in Table 4.

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6. CONCLUSIONS

As indicated at the beginning of this chapter, the development of biosensors for detecting heavy metals is still at an early stage compared with the development of biosensors for more orthodox biochemical compounds, and is certainly much less well developed than the detection of heavy metals by chemical methods. However, the progress recently made in the development of whole-cell and protein-based biosensors is encouraging and bodes well for the future. One of the great difficulties is the simple fact that metals are rarely found in environmental or industrial situations in pure form. Often other metals are present, or the metal salts are in a milieu of unusual pH or with high concentrations of interfering counterions. Until more work is done to address these problems, the use of biosensors may be more relevant to the study of metal-protein and metal-cell interactions in the laboratory rather than as a realistic solution to quantitation of heavy metal salts in the environment.

ACKNOWLEDGMENTS

Collaboration between the authors' laboratories was supported by a contract with the European Commission (ENV4-CT95-0141) and funding from the Swedish Institute. NLB thanks the Biotechnology and Biological Sciences Research Council, United Kingdom (grants G07943 and B10333), for support of the underpinning research in his laboratory and the Medical Research Council, United Kingdom (G.4600017) for bioinformatics support. PC thanks A. Bossus, B. Borremans, and A. Provoost for technical help. The European Commission (CT-1999-00008), the Swedish Natural Research Council, the Margit Stiernswärd, Bengt Lundquist, and Nils Hörjel foundations, and the Royal Physiographic Society in Lund are thanked for support to EC and IB. We gratefully acknowledge the contributions of colleagues, particularly Jon Hobman, Stephen Kidd, Jon Wilson, Kerry Glendinning, Niels van der Lelie, and Bo Mattiasson, to discussion of the work described in this chapter.

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