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EVALUATION
OF ANALYTICAL METHODS
IN BIOLOGICAL SYSTEMS

general editor: R.A. de Zeeuw

PART B

HAZARDOUS METALS IN HUMAN TOXICOLOGY

edited by

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PREFACE

Metals have played an important, yet dual role in the history of man. On the one hand their increased industrial use has contributed substantially to technological development, and on the other they have long been recognized as potential hazards to human health. Since the early days of civilization, man has been aware of the toxic properties of minerals such as copper, lead, mercury, antimony and some non-metallic elements such as arsenic. As a result they were, and still are, frequently involved in poisonings, accidental, suicidal and homicidal. Increasing industrial development has increased the opportunities for hazardous exposures to metals, as evidenced by frequent occurrences of occupational diseases. More recently, we have come to realize the deplorable role that metals can play in cases of environmental pollution.

Notwithstanding their long history in human toxicology, our knowledge and understanding of the fate of metals in the human body and their potential toxicity is still fragmentary. To a large extent, this may be attributed to the fact that accurate, precise and selective methods for the detection and quantitation of metals in biological materials at trace levels have not been readily available.

Fortunately, the last two decades have seen major developments and innovations in analytical methodology that also seem to hold potential for the trace analysis of body fluids and tissues for metals. However, it has also been recognized that many of the proposed techniques have limitations and pitfalls, in particular with regard to interference by the biomatrix.

As reliable analytical data are a prerequisite for the correct interpretation of toxicological findings and in the understanding of the role of metals in human toxicology, it was felt that a critical review and evaluation of the currently available techniques for the bioanalysis of metals would be highly valuable. Such an evaluation should not only be limited to the techniques as such but should also consider factors such as sample pre-treatment, digestion techniques, recoveries and losses, sample throughput, cost and automation. However, as the reader should also be able to interpret analytical data, relate them to other biological or physiological findings and put them into perspective with regard to health and disease states, this book will also deal with the toxicokinetics and dynamics of metals, distribution and elimination, biological changes, symptomatology, etc.

Thus the purpose of the book is to allow the scientist faced with a particular analytical problem to make an objective assessment of the methodologies open to him, to let him chose the best method for this task, then to interpret his analytical findings correctly and, finally, to put these findings into the correct perspective with regard to human toxicology.

This book is the second volume in the series Evaluation of Analytical Methods in Biological Systems. I am indebted to Dr. Antoine Vercruysse for transforming an idea into a coherent, well balanced book and to the authors for their cooperation and dedication in the preparation of the various chapters. I also thank Dr. Bryan S. Finkle, Salt Lake City, UT, U.S.A., for many helpful suggestions and advice in defining the scope of the book.

It is hoped that this volume will be a valuable reference source for many scientists involved with the analysis of metals and the interpretation of these findings in human toxicology. Suggestions and criticisms will be welcomed.

Groningen, The Netherlands — Rokus A. de Zeeuw

CHAPTER 1

METALS IN HUMAN TOXICOLOGY

A. VERCRUYSSE

CONTENTS

1.1. INTRODUCTION

The name metal is a very broad term. Over two thirds of the elements can, chemically, be called metals. Within this term we find other groups, such as alkali, alkaline earth, heavy and light metals. Nor do we get a more clearly defined group on the basis of physical properties. This enables us, for instance on the basis of hardness, to distinguish the best known and most used metals from the non-metals. However, again we are soon confronted with elements that posses properties of both metals and non-metals. Hence we are obliged to create a class of transition elements. The term toxic elements has been used to describe the interaction of this kind of element and their compounds with living material. This also is not a well defined group; indeed, a toxic effect on living material can be expected from all elements. However, it is a fact that the term toxic metals often implies the heavy metals. Nevertheless, the specific gravity of an element is not the basis on which to define its toxicity. Other workers use the term trace elements, but this is not a clearer definition.

We get into even greater trouble when we discuss the essential character of the elements. Toxicity has a relative meaning which enables us to compare the elements, but it is not a unique property of an element or a group of elements. From the point of view of man, the animal or vegetable kingdom, we may attach a potentially higher toxicity to particular elements. We must evaluate toxicity

on the basis of the interaction of the element with biosystems. The elements start their interaction with the functional groups of macromolecules as ionized structures. Several workers[1-5] have called attention to the possibility of a more logical division into groups or the discovery of an increasing or decreasing toxicity within one particular group of the Periodic System. These proposals of divisions into groups parallel the development of research in toxicology and the biochemical background of the effect.

In this book we consider the analytical toxicology of a number of elements that are important for man and his environment, the choice being made of lead, mercury, cadmium, arsenic, thallium, tellurium, selenium, nickel and chromium. The main objective is to deal with analytical methods applicable to materials of human origin and applicable in the fields of forensic, industrial, eco-, clinical and fundamental toxicology.

1.2. HUMAN TOXICOLOGY

The toxicology of an element is dependent on the kinetics of the interaction of the element in ionized form or as an organic compound of the element with the human organism. The potential toxic character or the final toxicity depends on the resultant of this interaction, in which both the element concerned and the organism, with its anatomical and physiological characteristics, have a mutual influence.

The kinetics of the interaction between an element in ionic form or as a part of an organic molecule and the actual position of the effect on a biochemical mechanism in the cell have different stages. All the toxicokinetic factors are completely analogous with those known in pharmacokinetics. Different toxicokinetic models have been published, e.g., for Cd[6].

Toxicokinetics can be considered in three stages: (1) a stage of entry and resorption; (2) a stage in the organism where transport, distribution, accumulation, biotransformation and the effect take place; and (3) a stage in which the chemical leaves the organism. In each of these stages we find the element in a suitable chemical and physical form to interact with the anatomical characteristics and the physiological properties of the organs or systems.

1.2.1. Resorption

In this stage the physical and chemical properties of the molecules and media in which they are present introduce the first important data with relation to the discussion of toxicity. The possible contacts of man with the elements Pb, Hg, Cd, As, Tl, Cr, Ni, Se and Te and their compounds differ widely. These ele-

ments occur in very distinct concentrations in nature, in air, water, soil, food and beverages, so that the background concentrations differ throughout the world. Man's activities and living and food habits create fluctuations in the concentrations of the elements to which groups of the population or individuals are exposed. Many figures concerning potential exposure and concentrations in air, food and beverates and exposure in the working environment have been published for Pb[7-10], Hg[7-9], Cd[7,9], As[7-9], Tl[7,9], Cr[7,9], Ni[7-9], Se[7-9] and Te[7,9].

The nature of the compounds (organic or inorganic) in which an element occurs leads to different toxicities of the element. The different chemical and physical properties that exist among compounds have important consequences for resorption (solubility, lipid solubility), transport, distribution, accumulation and the final effect (the binding characteristics with macromolecules, the possibility of chelation). Numerous influences can change radically the chemical form of the element. Hg and As are the most illustrative examples upon which physical and biological factors in the environment act. These elements are transformed in the environment into organic molecules with generally much higher resorption rates in humans. In addition the chemical characteristics, the physical state of the compound is also a factor that affects the resorption. The particle size is important during resorption in the bronchial tubes and also in the gastrointestinal tract. The solubilized form of a chemical is ideal for resorption through the skin and the gas phase is ideal for resorption into the lungs. The importance of the particle size of Pb during resorption in the lungs has been demonstrated in man[11].

Resorption, in a qualitative and quantitative sense, differs in the gastrointestinal tract, the lungs and the skin. Physiological properties such as the respiratory minute volume can increase resorption significantly. The lung minute volume increases by a factor or three between rest and during heavy work. The characteristics of gastrointestinal resorption are such that that the resorption of Pb may be very low owing to the formation of insoluble compounds.

The exact mechanisms that govern resorption of the discussed elements in man are often only speculative. Thus it is postulated that lead is absorbed by means of the calcium-binding protein, where a competitive effect with calcium resorption can occur[12]. Also there is competition among the several elements themselves and also between these elements and other chemicals, drugs and components of nutrition. The quantitative aspect of resorption in man is documented best for Pb and Hg and less so for As, Cd and Tl. There are almost no data available for Cr, Ni, Se and Te.

The absorption of Pb into the gastrointestinal tract is of the order of 10%[13], and up to 50% for children[14]. Resorption into the lungs is dependent on the particle size and is of the order of 30 ± 10%[13]. Resorption through the skin is

possible for organic compounds[15]. The total intake of Pb in an average population in an industrial community is 14 ± 4 µg/day (lungs) and 20-40 µg/day (orally)[16].

Very important differences exist among mercury compounds. Data on the resorption of these compounds are not available, but we have figures for representatives of general groups (inorganic mono- and divalent, elemental, organic), although it would be dangerous to generalize these figures within the groups. Elemental mercury diffuses very rapidly into the lungs but resorption is very low in the gastrointestinal tract[17]. Contradictory data are available on resorption through the skin. In principle, resorption by this route must be possible. Of inorganic mercury compounds, the divalent mercurichloride has been most studied. This compound resorbs to the extent of about 1.4-15.6% orally[18]. The Task Group on Metal Accumulation[18] has also pointed out the possible resorption of inorganic mercury compounds through the lung, but exact data are not known. According to Fridberg and Nordberg[19] the resorption of short-chain organic compounds is very important; organic compounds with very rapid metabolization in man have not been studied so extensively. Methylmercury compounds are 80% resorbed into the lungs. The oral resorption of these compounds, which are mainly present in food and bound to proteins, is very effective[20,21]. Although no quantitative data are available, these compounds, owing to their lipid character, can penetrate through the skin. The exposure to mercury of certain populations is widely dependent on the composition of their food, the consumption of fish being an especially important factor. For consumption of uncontaminated fish, the Swedish Expert Group[22] found a daily intake of 1-20 µg/day, with contaminated fish giving figures 10-20 times higher. The amount of inorganic mercury in food does not rise above 10 µg/day and is less variable.

The resorption of Cd into the lungs is subject to great variation, levels of 10-50% being found. The particle size seems to have a major influence, followed by the character of the compound[23]. Oral resorption, according to a study by McLellan et al.[24], is of the order of 5% but can be influenced by interactions with other factors (lack of Ca, Fe and protein deficiency, hormones)[25-28]. No data are available on resorption through the skin. The daily intake of Cd in food, in an uncontaminated environment, is of the order of 25-60 µg/day for a person of 70 kg[23]. The concentration and the form in which Cd is present in the air are variable. They are lowest in rural areas (0.006-0.036 $\mu g/m^3$) and highest in cities (0.002-0.05 $\mu g/m^3$). A cigarette smoker is exposed to 0.1-0.2 µg per cigarette.

The resorption of arsenic compounds into the lungs in man is poorly documented. The only experiment is that of Holland et al.[29] with lung cancer patients. Of the total dose added, 5-8% was resorbed. From the gastrointestinal tract dis-

solved arsenic(III) is 80% absorbed, and arsenic(V) and organic forms are also resorbed to a large extent. There should be the possibility of resorption through the skin.

The total daily intake of arsenic compounds varies considerably with the amounts of fish and other seafood consumed. Intake through beverages depends on the amount present in drinking water, so that published figures on intake have only local value.

Lie et al.[30] studied the resorption of thallium(I) nitrate by different routes in animals. They noted very good resorption (100%) by the oral route and in the respiratory tract. The daily intake of thallium is very low in humans.

The resorption of Cr through the lungs is mainly a problem of industrial toxicology. We have few data and the problem is complex because of the several possible valence states of this element. In evaluating the oral resorption we have to take into account the existence of tri- and hexavalent chromium. The extent of oral resorption is about 10%.

Human toxicological data on the resorption of Ni are scarce but orally and from the lungs it is about 10%.

Resorption of Se and Te from the lungs is not documented. Stewart et al.[31] found an oral resorption of 80% for Se. There are no data for Te in humans.

1.2.2. Transport, distribution and accumulation

After resorption, the molecules are transported by the bloodstream and pass into the cellular fluids, where they exert their toxic effect. Most of the elements considered here show a high affinity for certain active groups on plasma proteins. In general, these elements are transported bound to the macromolecular fraction of the plasma. Between the different elements there exist qualitative and quantitative differences in binding properties. In addition to binding with plasma proteins, binding with low-molecular-weight components and cellular elements of the blood also occurs. The distribution may be limited by the binding, and on the other hand binding in the tissues causes accumulation.

Lead is 95% bound to the red cells and 5% to plasma components. In the red cells themselves it is bound to haemoglobin[32]. After resorption lead is present in an exchangable compartment and in a one in which it accumulates. Bone tissue is the accumulator organ. The transport of lead into the foetus has been demonstrated[18].

As in all other phases, the behaviour during transport, distribution or accumulation differs for inorganic, organic and elemental mercury. Berlin et al.[17] showed that elemental mercury has a high affinity for the brain. However, the biotransformation of elemental into divalent mercury occurs very rapidly so that

the effect in the brain is very short in the case of a single exposure. Elemental mercury passes through the placenta[33]. Inorganic mercury distributes between the plasma and the red cells and is bound to the thiol groups of the proteins. It is known that divalent mercury is not distributed into the foetus. The kidney is the main organ where Hg^{2+} accumulates. Methylmercury derivatives are also bound to the plasma components and the red cells but in a different ratio to other mercury compounds. The concentration is ten times higher in the red cells than in the plasma[20]. The organs of accumulation of methylmercury are the liver, the kidney and the brain, it distributes evenly between mother and foetus.

Intra- and extracellular cadmium is bound to a metallothionein (mol. wt. 10,500 daltons); in the circulation 70% is found in the red cells. The metallothionein is primarily synthesized in the liver and in many other tissues[34]. Half of the cadmium stored in man is found in the liver and the kidney, the kidney takes one third of the amount[23]. Distribution into the foetus has been demonstrated in animal experiments[35]. In man it is found that the placenta contains 10-fold higher concentrations than the blood of the mother[36].

Data on the transport and distribution of arsenic in humans are incomplete, mainly because the different possible valences were not taken into account. Arsenic is transported both in plasma and bound to the red cells, but the extent of exposure seems to influence the ratio between the two[37]. Arsenic(III) is accumulated in the liver and the kidney[38]. Distribution into the foetus has been demonstrated in animals although the placenta forms an exellent barrier[39]. Thallium(I) shows properties identical with those of potassium in biological media. The main reason is that the ionic diameters and charges are identical. The distribution of Tl^+ is analogous to that of K^+ and it distributes especially in the red cells. There appears to be a difference in distribution in chronic and in acute intoxication. Tl^+ accumulates preferentially in the muscle of the heart (very short), the kidney, bone tissue and hair[40-42]. Tl^+ passes rapidly into the foetus.

Chromium is transported in the circulation in both the cells and plasma[43]. In the plasma there exist two fractions, an albumin fraction (60-70%) and a fraction bound to a β-globulin (30-40%)[44,45]. The higher the concentration in the mother the higher it is in the foetus[46]. The distribution of chromium has only been studied in animals. In the rat Cr distributes into the reticuloendothelial system, liver, spleen and bone marrow[47].

Nickel is transported in the circulation bound with albumin, a α_2 macroglobulin (nickeloplasmin) and different ultrafiltrable fractions[48,49]. The distribution and accumulation differ for acute and chronic intoxication. Parker and

Sunderman[50] found in the rabbit, after brief exposure, a distribution into the kidney and further into other tissues. Nickel passes into the foetus[51].

In the plasma selenium is bound to three kinds of proteins. Especially α- and β-globulin[52]. In the red cells the concentration is much higher than in plasma[53] and is there associated with glutathione peroxidase[54]. The kidney is the preferential place of accumulation of selenium[55]. Animal studies have demonstrated its distribution into the foetus[56,57].

In rats 90% of the tellurium in the circulation is bound to the red blood cells, but in other animals this proportion is not always the same. Distribution into the foetus is possible[58]. In short-term exposure the kidney, the lungs and the liver are the accumulator organs; in chronic intoxications Te has been found in bone tissue[59].

1.2.3. Biotransformation

The biotransformation of the elements and their compounds differs widely according to their nature (inorganic or organic). Biotransformation reactions are mainly oxidations or reductions, cleavage of the carbon binding, methylation or binding with macromolecules or metallothionein. It is generally accepted that the biotransformation of the elements is carried out with the same enzyme systems as are all other metabolic reactions on toxica. Toxicity can be higher or lower after biotransformation.

Inorganic lead stays in the organism in the same valence state, Pb^{2+}; the metabolic changes involve binding to macromolecules. Tetraethyllead is biotransformed to Pb^{2+} ions and tri- and diethyllead.

Many mercury compounds are very extensively metabolized. Elemental mercury is biotransformed first to Hg^{+} and further to Hg^{2+} [60]. This transformation has direct consequences on the symptoms of the intoxication and gives differences, for example, in chronic and acute intoxications.

The biotransformation of methylmercury has been extensively studied in animals and was demonstrated in humans in intoxication cases in Iraq by Bahir et al.[61]. The intensity of the biotransformation varies in different tissues, being highest in the kidney and lowest in the brain[62]. Other alkylmercury compounds are metabolized much more rapidly than the methyl derivatives. The influence on the toxic effect of transformation of organic compounds to inorganic Hg^{2+} is still not clear. The possibility of the methylation of Hg^{2+} by intestinal bacteria was described by Rowland et al.[63]. This transformation has to be taken into account when considering the kinetics of every mercury compound.

Apart from fixation on macromolecules and metallothionein, no biotransformation is known for Cd^{2+}.

Arsenic is very extensively biotransformed in biological material. These biotransformations mainly involve changes in valence. Oxidation of As^{3+} to As^{5+} and reduction of As^{5+} are possible. However, the reported data are confusing and contradictory[64]. More recent studies[65,66] have demonstrated the possibility of the methylation of arsenic compounds. The end products of this methylation processes (dimethyl and monomethyl compounds) are formed from both As^{3+} and As^{5+}. The liver seems to be the place where this biotransformation occurs. Biotransformation seems to have no effect on Tl^{+}, so this element remains in the monovalent state.

Hexavalent chromium is metabolized to the trivalent state, but no data for humans are available.

The divalent character of nickel is not changed in biological materials. Nickel carbonyl is oxidized to Ni^{2+} and CO[67].

The biotransformation of selenium occurs in two stages. It is first reduced and then methylated into mono- and dimethyl compounds[68]. The latter compound is volatile and is exhaled. A final methylation forms trimethylselenium derivatives, which are excreted in ionized form in the urine[69]. Selenomethionine and cysteine are formed and incorporated in proteins.

From certain tellurium compounds elemental Te is formed through reduction and is precipitated as a blue-black deposit in the tissues[70]. Dimethyl compounds are also formed, which have a specific odour[71].

1.2.4. Excretion and biological half-life

The excretion of the above elements and their compounds takes place mainly through the kidney (urine) and the bile and gastrointestinal mucosa (faeces). Less important are the hair, nails and volatile metabolites. The factors that affect this excretion are the physical and chemical properties of the molecules and the anatomical and physiological properties of the excretion tissues.

Lead is excreted to the extent of 75% through the kidney after glomerular filtration[71]. The exact figure for excretion through the bile is unknown but the total gastrointestinal excretion is about 16%. The faeces also contain 90% of the non-resorbed orally ingested lead[10]. Lead is present in appreciable amounts in the hair.

The biological half-life of lead is dependent on the hypothesis used in the kinetic model. The model worked out by Rabinowitz et al.[72] gives a realistic interpretation of the kinetics. This model describes three compartments with different half-lives. In the first compartment (blood and rapidly exchangable soft tissue) lead has a half-life of 19 days, in the second compartment (soft tissue and readily exchangeable bone tissue) 21 days and in the third compartment (the skeleton) 20 years.

The excretion of elemental mercury after metabolization is identical with that of Hg^{2+}. The excretion model is a multi-compartment system[18] with a small compartment (the brain) with a half-life of greater than 1 year. The main excretion routes of Hg^{2+} are the urine and faeces. Excretion through the kidney is a tubular secretion after reabsorption of the filtered mercury[73]. Excretion through the bile is secondary and involves a mercury-protein complex. Which one is the most important is dependent on the dose: with a higher dose the main excretion route is in the urine.

Of the different mercury compounds, methylmercury is the most studied. It is excreted to the extent of 90% in the urine as methylcysteine and methylglutathione compounds[74]. For methylmercury an important enterohepatic recirculation occurs[75]. The biological half-life of methylmercury with a non-toxic exposure is about 70 days[22]. Some of the mercury is excreted through the hair and the milk[61].

The excretion of cadmium occurs through the urine and the faeces, but very slowly and only as a fraction of the total body burden. The cadmium-methallothionein complex is filtered through the glomeruli and reabsored in the tubuli. In this way Cd is accumulated in the cortex of the kidney. The excretion in the urine increases when the kidney function is impaired[76]. The biological half-life of Cd in the liver and the kidney is about 10 years.

Inorganic arsenic is excreted mainly in the urine and partly in the faeces[77]. According to Mealey et al.[77], excretion occurs in three distinct phases, which correspond with three possible compartments (with half-lives of arsenic of 2.8 h, 28 h and 9.6 days, respectively). There are different excretion patterns for As^{5+} and As^{3+} [78]. The organic forms of arsenic, present in food and especially in seafood, are excreted very rapidly, in about 2 days, in the urine[79]. The biological half-life of organic arsenic is about 20 h[80]. Another route of excretion of arsenic is the hair and skin[81].

Thallium is excreted in urine and faeces. Barclay et al.[82], in a comparative study, found a human excretion rate of 3%/day and noted a pronounced fluctuation during the day.

The urine is the most important route of excretion for chromium. Mertz et al.[83] described three different compartments with biological half-lives of chromium of 0.5, 5.9 and 83.4 days, respectively. No quantitative and qualitative toxicological data are known.

The excretion of nickel has not been well studied and the biological half-life has been studied only in animals.

Quantitative figures for the excretion of selenium and tellurium in humans are unknown. These elements are excreted through the urine and faeces.

1.2.5. The dose-effect relationship

The biological effects of lead have been extensively described. The most important effects are the inhibition of enzyme systems, especially in the synthesis of haemoglobin (inhibition of Ala-D, ferrochelatase, coproporphyrinogen-decarboxylase). As a result of this inhibition, the effect on the haemopoietic system can be explained. The exact biochemical basis of the effects in the gastrointestinal tract, the kidney and the central nervous system have still not been established. There is no proof that Pb is a carcinogen and its mutagenic effect is debatable. Zielhuis[84] described a dose-response relationship between blood lead concentration and certain biochemical changes. Ala-D in the red blood cell is already inhibited in 50% of the population at lead concentrations of 31-40 µg per 100 ml, this inhibition is pronounced in 50% of the population for lead concentrations of 51-60 µg per 100 ml. An OMS Report[85] gives a limit value of 40 µg of lead per 100 ml of blood for workers in exposed conditions.

The effect of mercury can be explained by the binding of Hg^{2+} to macromolecules and thiol groups. Many enzyme systems are inhibited in this way. The most important effect of Hg^{0} is registered on the nervous system and the brain, the kidney is the target organ for inorganic divalent mercury and the central nervous system is attached by organic methylmercury. The biological monitoring of an exposure to mercury is very difficult because of its variable concentrations in food[86]. In a chronic exposure to elemental mercury, intoxication starts at a level of excretion of mercury in the urine of 50 µg/g creatinine. Exposure to Hg^{2+} is difficult to evaluate through biological monitoring.

Competition with other metals in enzyme systems is the reason for the toxic effect of cadmium. Cadmium replaces zinc as a cofactor in certain enzyme systems. In addition, cadmium is reactive towards the thiol groups of the proteins. Acute intoxications are characterized by local effects on the lungs and gastrointestinal tract. The clinical symptoms of chronic intoxication are deformation of the skeleton, itai-itai desease and pathological conditions of the lungs and kidney. The biological limit values are cadmium in blood 10 µg/l and urine excretion 10 µg/g creatinine. A qualitative and quantitative determination of the protein excretion gives the possibility of early diagnosis of the intoxication.

Fowler[64] reviewed extensively the different interactions of different arsenic compounds with enzyme systems. A large number of enzymes are inhibited by arsenic compounds (ATPases, phosphatases, monoaminooxidases, protein synthesis, etc.). As^{3+} fixes on thiol groups while As^{5+} has a lower activity for these groups. The toxicity of soluble arsenic compounds is highest and the toxicity decreases in the order AsH_3 > As^{3+} > As^{5+} > R-As-X. Many workers[87-89] have published epide-

miological studies and concluded that there is a high risk of lung and skin cancer. However, it is to early to say if arsenic is a carcinogen or a co-carcinogen.

One of the most plausible reasons for the toxicity of Tl^+ is substitution with K^+. The clinical symptoms are especially located in the nervous system and alopecia is a known effect. The exact biochemical defect of chromium is not known. Clinical symptoms are local effects and the allergic reactions are significant. Different workers[90-92] have described bronchopulmonary cancers.

Nickel is an essential element and has an interaction with the iron metabolism[93]. Effects on the respiratory tract are the clinical symptoms of an intoxication and in the same way way as Cr is it an allergen. The question on its carcinogenic action has not been resolved.

Selenium is also an essential element and different functions of this element have been described. Data in human toxicology are very scarce; attention has been given to its possible neurotoxicity. Selenium should exert some properties as an anti-carcinogen[94].

When we consider carefully human toxicological data for most elements, we have to conclude that they are very incomplete. Qualitatively we can make some good assumptions on the biochemical basis of the intoxications that occur with these elements. Quantitatively only Pb, Hg and Cd are relatively well documented. Very important questions remain unsolved and there is clearly a need for further research in this field.

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CHAPTER 2

EXPOSURE TO TOXIC METALS: BIOLOGICAL EFFECTS AND THEIR MONITORING

A. SINGERMAN

CONTENTS

2.1. INTRODUCTION

There are about 20 metals or metal-like substances considered to be toxic for humans, including antimony (Sb), arsenic (As), beryllium (Be), cadmium (Cd), cobalt (Co), chromium (Cr), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tellurium (Te), tin (Sn), tungsten (W) and vanadium (V).

Toxic metals may give rise to symptoms from the central nervous, the haematopoietic, the cardiovascular or the respiratory systems. Some of them have been recognized to be carcinogenic, teratogenic or mutagenic, towards either man or animals.

The effects of a metal on a cell arise as the consequence of the binding of the metal to the different ligands in the cell. When a certain number of binding sites are involved a functional change may occur, and when the critical concentration of the metal in the cell is reached the functional change becomes adverse.

The critical concentration for a cell as defined by the Task Group on Metal Accumulation (TGMA) is the concentration at which undesirable functional changes, reversible or irreversible, occur in the cell. The lethal concentration was defined by TGMA as the cellular concentration sufficient to cause death of the cell[1].

The various types of changes induced by metals can be assessed either by clinical observation or through functional tests as well as by morphological and biochemical techniques. The metals may give rise to either local or systemic effects. After repeated exposures the metal accumulates and a subclinical or a clinical effect may appear. It is then of the major importance to detect the biochemical alterations at subcellular levels before the clinical symptoms appear.

Biochemical changes may give important indications on the mechanism of action of metals on the cell. The metals may interact with the cell membrane and with intracellular organelles, binding to certain ligands, thus interfering with the integrity of the cell. The binding of a metal with different ligands on cell membranes, enzymes and other cellular constituents probably constitutes the first step of the biochemical change known as "biochemical lesion".

The biochemical lesion is considered to arise mainly as a consequence of the interference of metals with enzyme systems, which in turn leads to functional changes. The processes developed at the cellular membrane level are susceptible to the action of metals, and when the metal reaches the inside of the cell a number of enzymatic activities may be changed. Changes are due to the binding of the metal at certain regions on the enzyme molecule. That region of the enzyme surface where the reactants are bound, interact and are chemically altered is known as the active centre. The changes may originate in a direct interaction at the active centre, but may equally well originate in interactions between metals and ligands not directly involved in the active centre. Changes in the electrostatic charge may be produced together with shifts in the ionization constant of the active centre. A change in the enzymatic reaction may also be due to structural changes in a protein or to the combination of the metal with the coenzyme.

The active region is usually only a small fraction of the total enzyme molecule, but the regions surrounding the active centre can influence the activity of the centre through either steric or electrostatic effects.

An active centre may contain several individual sites on which dissociable acceptors, activators or coenzymes are bound. A metal may therefore interfere in the binding of any component or disturb the coordination between the sites. As the individual sites can be complex and comprise a number of groups, the metal may block the reaction by several different mechanisms.

The cellular membrane is the most important site of action for metals. Biological membranes are organized assemblies consisting mainly of proteins and lipids, acting as highly selective permeability barriers. As they contain a great amount of lipids, particularly phospholipids, small amounts of a metal may lead to changes in the surface tension and in the charge of the lipidic films. Such changes result, in turn, in alterations of the permeability and the metabolic activity of the surface enzymes.

The metal is usually absorbed by ligands on the external surface of the membrane. Most of these ligands are essential for the membrane to keep its properties as a diffusion barrier and are also necessary for the normal function of the membrane enzymes. These enzymes are important elements for the active transport and biosynthesis of the membrane constituents. Owing to its localization, they are particularly sensitive to the action of metals.

As the membrane is the major barrier to the entry of inhibitors into the cell, it is important to determine if the enzymes involved are in the cell, within the membrane, or outside. Some enzymes are at or near the cell surface and are susceptible to influences that do not extend within the cell. When the enzyme is both at the membrane and inside the cell, specific inhibition may be achieved even if the inhibitor does not penetrate into the cell, and a specific functional change in the membrane may be brought about.

Enzymes are not homogeneously distributed throughout the cell but are concentrated in compartments or subcellular structures. This is of great importance for the accessibility of the metal to the enzyme. Substrates and enzymes may coexist in the cell without reaction, probably owing to spatial separation. If enzymes are unavailable to their substrates they are probably protected from the action of metals. However, although the enzymes are localized in compartments, the segregation between cell fractions is not considered to be complete.

Toxic metals are well known as enzyme inhibitors, reacting chemically particularly with proteins or nucleic acids. They may cause structural changes in proteins resulting in denaturation, alteration of bioelectric properties, impairment of the transmission of nerve impulses and loss of transport or other vital functions.

The activity of very large molecules such as haemoglobin, intermediate molecules such as metallothionein and small molecules important for their redox potential such as ascorbic acid or glutathione may all be affected by toxic metals.

An important characteristic of toxic metals is the reversible formation of complexes with organic ligands. The different types of binding to different ligands constitute the effects of toxic metals at the biochemical level.

Interference with biochemical activities in the cell depends on the type and number of ligands and on their structural and functional organization, e.g., whether active or passive binding sites are affected. Effects on biochemical systems may be reversible or irreversible depending on the type of binding. Metals may compete for the same binding site with different affinity and different intrinsic activity. Interactions between binding sites may also influence the response at the biochemical level. The functional result may be additive, antagonistic or synergistic.

Toxic metals may interfere with essential trace metals by competing for binding sites. Some enzymes contain a metal that is bound in such a specific manner that it cannot be removed without loss of enzyme activity. Toxic metals may displace the essential metals from such metalloenzymes, thus bringing about a decrease in enzyme activity. In addition to the interaction of metals with enzymes per se, the rate of enzymic reactions may be reduced by binding of metals with coenzymes or substrates.

When the metal affects the cell permeability or cellular structures such as lysosomes, there may be an increase in the enzymes in intra- or extracellular fluids, but some enzymes may also be directly "activated" subsequently to metal interactions.

Some effects of toxic metals may also involve a mechanism in which the metal acts as a hapten and the protein-bound metal acts as an antigen, resulting in an allergic type of effect such as metal fume fever and skin sensitization due to nickel and chromium.

Metals such as lead, mercury and cadmium may disrupt different pathways of oxidative phosphorylation, a process associated with the integrity of the mitochondrial membrane. Oxidative phosphorylation - more accurately called respiratory-chain phosphorylation - is the mechanism by which the free-energy decrease accompanying the transfer of electrons along the respiratory chain is coupled to the formation of the high-energy phosphate groups of adenosin triphosphate (ATP). The enzymes of electron transport and oxidative phosphorylation are located in the mitochondria, ATP being the major product of the mitochondrial biochemical activity. The ATP donates its terminal phosphate group to specific acceptor molecules, providing the energy necessary for chemical work (the biosynthesis of cell macromolecules), osmotic work (the active transport of inorganic ions and cell nutrients across membranes against gradients of concentration) and mechanical work (the contraction of muscles).

As the energy of ATP is delivered to those energy-requiring processes, the ATP undergoes cleavage to ADP and inorganic phosphate. The ADP is then rephosphorylated at the expense of energy-yielding oxidation of fuels to yield ATP, thus completing the cellular energy cycle.

Both oxidation and phosphorylation are tightly coupled processes taking place within the mitochondria. Electron transport and the coupled generation of ATP may be dissociated and this dissociation is called uncoupling of oxidative phosphorylation. Trialkyllead, inorganic lead, cadmium and mercury are known to act as uncoupling agents at low concentrations.

Although toxic metal ions combine with organic molecules interacting with different ligands such as amino, imidazole, phosphoryl, carboxyl and hydroxyl residues, the interference of metals with cellular biochemical systems is often due to interaction at important sites such as the sulphydryl (SH) groups of enzyme systems. In fact, they interact with the total system including enzyme, substrate, cofactors and activators. The binding of metals to any of the components of the system may affect the overall function.

The presence of the thiol groups seems to be essential for the full activity of a number of enzymes, particularly for dehydrogenases. Inactivation of SH groups may occur in different ways, mercaptide formation being considered as the predominant reaction underlying metal inhibition. Mercaptide formation may be reversed by the addition of an excess of monothiols or dithiols, and full activity restored.

Mercury (Hg^{2+}), antimony (Sb^{3+}), lead (Pb^{2+}) and cadmium (Cd^{2+}) are among the metal ions that react with enzyme thiol groups to form with mercaptides[2-4]. Cadmium[5] and mercury[6] are known to bind to a small protein called metallothionein, the binding probably involving protein SH groups.

Arsenic (As^{3+}) reacts with thiols to form mercaptides (arylthioarsenites), the reaction being reversed by the addition of monothiols. The arylthioarsenites produced dissociate in the organism to form the toxic arsenoxide.

The enzymic oxidation of pyruvate, α-ketoglutarate and succinate is most susceptible to the action of organic arsenicals. Inorganic arsenic (As^{3+}) also affects thiol enzymes, being less effective on α-ketoacid oxidases.

It has been shown that enzymes blocked by arsenicals are those requiring a dithiol cofactor known as dihydrolipoic acid, essential for the α-ketoacid oxidases such as pyruvate oxidase. The enzyme system is inhibited through the formation of stable coordination compounds (cyclic thioarsenite complexes) with the cofactor, and can be reactivated by addition of a dithiol such as dimercaptopropanol, which competes with the arsenical by dissociating the complex and reversing oxidase inhibition.

Interaction with SH groups of the erythrocyte membrane leading to membrane damage is probably the main characteristic of the action of toxic metals. The mode of action of toxic metals is still a matter of great controversy. It has been suggested that the mechanism of toxicity may be mediated through peroxidation of membrane lipids[7,8], which is consistent with the hypothesis that organometallic compounds and metals such as mercury, cadmium and lead, undergoing univalent redox reactions, may promote tissue damage through (a) radical-induced mechanisms or (b) irreversible inhibition of enzymes such as glutathione-peroxidase (GSH-Px) and superoxide dismutase (SOD), involved in the defence against the toxic effects of oxygen[9].

Channa Reddy et al.[10] have reported that the transferase and peroxidase activities of glutathione transferases (GSH-Trs) were markedly inhibited by cadmium, methylmercury, mercury and lead ions, Cd^{2+} being the most potent inhibitor of all the metal ions investigated. However, the mechanism of inhibition does not seem to be clear. Although it appears reasonable to assume that these metal ions might compete with the enzyme protein for glutathione (GSH), the inhibition could not be reversed by the addition of GSH, suggesting that it is not due to competition for GSH. These findings led the authors to speculate that the inhibition may be due to interaction with one or more essential groups on the protein molecule.

According to Sandstead[11], some of the effects of toxic elements and the protective effects of essential elements might be attributed to competition for binding sites on ligands having important roles in homeostasis.

2.2. PRINCIPLE OF MONITORING BIOLOGICAL EFFECTS

The basis of modern occupational medicine consists in measuring the biological response of man to the characteristics of his work environment. For this purpose it is necessary to make routine measurements on health and environmental indices with a view to detecting changes in the health status of workers and their environment. A careful analysis and evaluation of results is required in order to be able to take prevence action, which is of fundamental importance in the field of occupational medicine.

Environmental monitoring is carried out with the aim of assessing the doses of hazardous agent(s) to which the worker is exposed at his place of work, while biological monitoring provides baseline data for measuring early adverse effects of exposure, understanding by biological monitoring the measurement of levels of toxic agent(s) and/or other associated parameters indicating the organism's response to them, in biological materials. This procedure provides quantitative evidence about the absorption and retention of suspect substances in the body, reflecting the magnitude of exposure.

The two procedures are regarded not as alternatives but as complementary, and should be carried on in parallel in order to evaluate effective integrated exposures. It should be remembered that the early detection of health impairment at a reversible stage is needed so that protective action can be taken before illness develops.

Changes in biochemical parameters as measured in various biological fluids may often be among the more sensitive indicators of early changes in health due to hazardous agents in the work environment. The quantitative evaluation of biochemical parameters is essential for the establishment of dose-effect/response relationships.

Advances in analytical technology have facilitated the identification and quantification of a great number of biochemical parameters, many of which are still a matter for research study. It is interesting that automatic analysis with microtechniques has become a valuable tool in health evaluation by measuring several parameters simultaneously.

As has already been stated, most toxic metals interfere with the action of enzymes, bringing about changes in enzymatic activity. Thus, increased enzyme activity in plasma or serum may indicate lesions of specific organs or tissues. The presence of enzymes known as C-enzymes (present in the cytoplasm of cells) allows the detection of early changes related to membrane permeability before M-enzymes (located in mitochondria) - indicating more extensive cell damage - are released. Changes in the activity of enzymes not specific for any organ or tissue, but involved in different metabolic pathways, may also be detected. In some instances the enzyme activity may be decreased (enzyme inhibition).

The field of enzyme diagnosis has been enlarged by the investigation of the molecular fractions of enzymes called isoenzymes. The changes detected in isoenzymograms have higher predictive validity than changes in total enzyme activity. Both increases and inhibition of urinary enzymatic activities and changes in their corresponding isoenzymes patterns are also of great diagnostic value.

Serum enzymology has become an important tool in the early diagnosis of potential harmful effects of toxic metals. Serum enzyme levels and isoenzymes patterns, serving to identify damage to different organs, are usually included in screening profiles for monitoring purposes, and urinary enzyme activities and their isoenzymograms - reflecting early renal damage - are also included. Subliminal injuries can thus be detected.

Enzyme profiles therefore afford a means of identifying the target organ involved in a toxic process.

An elevation of serum enzyme activity is usually interpreted as the efflux of enzymes from a damaged organ into the circulation. However, when the enzymes are located in subcellular fractions, e.g., mitochondria, no increase in serum

levels is shown until a latent period elapses, when the injury is more severe. Enzymes of cytoplasmic origin, which are easily released, such as ornithine carbamyl transferase (OCT) and sorbitol dehydrogenase (SDH), are early indicators of liver cell injury, while the passage of glutamate dehydrogenase (GDH) into the circulation is evidence of a mitochondrial lesion.

Serum enzyme tests may then provide information not only about the severity of the cell damage but also about the subcellular site of the damage.

Metal-induced enzyme deflections can manifest in the form of an inhibitory action on certain enzymes, leading to decreased activity, either by displacement of essential metals intrinsic to the enzyme through the ionic metal (Pb^{2+}, Hg^{2+}) or as a result of the affinity of the metal compound to reactive ligands (SH groups) on the enzyme surface.

Changes in serum levels of γ-glutamyl transferase, ornithine carbamyl transferase, sorbitol dehydrogenase, alcohol dehydrogenase, arginase and aminopeptidase may provide evidence of disturbances in liver function.

As the normal serum levels of sorbitol dehydrogenase are extremely low, its increased activity is of great diagnostic value. Elevations of serum ornithine carbamyl transferase precede the changes in transaminase levels, thus becoming an early indicator of hepatic disturbances. An increase in serum β-glucuronidase has often been used as an index of liver injury.

Sometimes, combined changes in serum levels of more than one enzyme may increase predictive power. This is the case for the De Ritis quotient (SGOT/SGPT), which indicates only membrane permeability disturbance when it is less than unity. As SGOT is partly an M-enzyme, a De Ritis quotient greater than unity may indicate mitochondrial injury.

The determination of urinary enzymes may provide a useful index of kidney damage. Changes in urinary enzymes excretion precede changes in kidney physiological function. The measurement of enzyme excretion in the urine is a more sensitive and an earlier means of detection of kidney damage than the usual functional tests.

Urinary glutamic oxaloacetic transaminase (UGOT) measurement seems to be a reliable method for the detection of tubular injury. It is not influenced by an increase in serum glutamic oxaloacetic transaminase (SGOT) of hepatic or cardiac origin. In rats poisoned with chromium, uranium and mercury, the UGOT level has provided the best indicator for the quantitation of kidney damage[12]. It is considered to be a renal test providing reliable information on proximal convoluted tubule injury. As the highest specific activity of acid phosphatase is found in the glomeruli, increased urinary activity of this enzyme can indicate an active glomerular lesion.

High activity of aminopeptidase has been shown in the proximal tubules and increased activity of this enzyme in the urine can be found in cases of tubular lesions induced by mercury.

Increases in urinary lysozyme and ribonuclease activity, indicating tubular damage, have been found in cadmium-exposed workers.

Serum transaminases levels are not subject to significant changes on exposure to lead except at hazardous levels[13,14]. Serum aldolase has shown to be consistently elevated in individuals exposed to lead without signs of toxicity and the magnitude of the enzyme increase has been found to be correlated with the urinary output of lead[15]. Subnormal levels of serum alkaline phosphatase and cholinesterase have been found in lead-exposed workers[16], the activity of alkaline phosphatase being inversely related to the degree of haematological deviation[17].

Abnormal activities for serum transaminases[18], lactate dehydrogenase[18], alkaline phosphatase[19], and cholinesterase[20] have been observed in mercury exposed subjects. A decrease in serum lactate dehydrogenase is often found in subjects exposed to elemental mercury[18], while alkaline phosphatase activity is increased[21]. In early stages of overexposure to mercury serum cholinesterase has been found to be depressed[20].

A rise in serum transaminases and a fall in serum lactate dehydrogenase have been reported as early signs of exposure to manganese[22].

Many other biochemical changes, some of them associated with genetic abnormalities, are considered below in more detail for individual metals.

2.3. THE ENZYME INDUCTION PHENOMENON

Some toxic agents can induce the endoplasmic reticulum (mainly of the liver) to produce more enzymes. Although the resulting metabolites may prove to be more toxic than the original chemical, enzyme induction is regarded in general terms as a defence mechanism, often being not specific. This may explain, for instance, the interference of drugs with the response to occupational chemicals and vice versa.

The stimulation of drug-metabolizing systems of the liver is brought about by enhanced neosynthesis of microsomal enzymes. Microsomal inducers seem to accomplish their stimulatory effects on microsomal proteosynthesis by accelerating the formation of DNA-directed RNA.

The susceptibility to toxic agents may be deeply influenced by microsomal enzyme inducers. The specificity and intensity of microsomal enzyme induction varies for the different inducing agents[23].

Ethanol and cigarette smoke are known to be microsomal stimulators. Adaptive microsomal changes, which include enhanced ethanol and drug metabolism, have

been found following chronic ethanol consumption[24]. Although the major part of ethanol oxidation is catalysed in liver cells by alcohol dehydrogenase (ADH), there is another metabolic pathway not mediated by cytosolic ADH but through a microsomal ethanol oxidizing system (MEOS), leading to an increase in the activity of a number of drug-detoxifying microsomal enzymes with a concomitant increase in the content of cytochrome P-450 and NADPH cytochrome P-450 reductase[24-26]. As some toxic metals such as beryllium[27] and tetraethyllead[28] have also been shown to stimulate microsomal hepatic activity, while cadmium[29], inorganic lead[30] and mercury[31] are known as microsomal activity inhibitors, unexpected interactions may derive from the presence of ethanol.

That cigarette smoke contains significant amounts of microsomal activators has been shown by the accelerated biotransformation of phenacetin into *p*-acetamidophenol observed among heavy smokers with respect to non-smokers[32,33], and by the finding of enhanced metabolism of nicotine in smokers[34].

The microsomal metabolizing function may not only be stimulated but also inhibited by a number of factors, some being environmental and occupational agents responsible for modifying the activity of the enzyme systems involved.

Depression or total inhibition of hepatic microsomal enzyme systems brings about a stimulation of toxicity when biotransformations result in the formation of innocuous metabolites, while in cases of metabolic activation of the xenobiotic, the inhibition of microsomal enzymes means a delayed formation of toxic metabolites.

The chemically induced microsomal enzyme inhibition may be mediated by different mechanisms: depression of de novo enzyme synthesis, increased turnover rate of enzyme, conformational change of the enzyme molecule, probably due to binding with the inhibitor, or damage to endoplasmic membrane structures.

It is known that alkyllead compounds (TEL and TML) exert their toxic action on the central nervous system through their dealkylated metabolites (triethyl- and trimethyllead). It has been shown that iproniazid protects experimental animals against tetraalkyllead compounds by inhibiting the dealkylating enzyme systems of hepatic microsomes and consequently blocking their biotransformation into trialkyllead metabolites[35]. Lead, zinc and mercury have shown in vitro inhibitory action on hepatic enzyme systems[26].

The cytochrome P-450 from hepatic microsomes in its reduced form has shown a high affinity for carbon monoxide (CO)[36,37]. When attached to CO, the cytochrome P-450 is unable to bind substrates (xenobiotics) and therefore the whole microsomal oxidation-reduction cycle becomes inoperative and hepatic metabolism of xenobiotics is then inhibited. Carbon monoxide is present in concentrations between 1 and 5% in the gaseous phase of cigarette smoke[38,39]. It competes with oxygen (O_2) not only for haemoglobin but also for cytochromes, particularly

cytochrome P-450, binding to Fe^{2+}, thus leading to the inhibition of microsomal enzymes[40].

Inhibition of microsomal drug-metabolizing enzymes may also result from acute ethanol intoxication[41], by a mechanism of enzymatic competition.

A number of investigators have studied smokers and non-smokers with regard to social characteristics and physiological variables, and found that smokers consume more alcoholic beverages than non-smokers[42,43]. Others have found definite correlations between cigarette smoking and the consumption of coffee and alcohol[44]. In this respect it should be pointed out that alcohol, cafeine and nicotine are considered to be xenobiotic agents liable to modify the activity of hepatic microsomal enzyme systems[45], and consequently the toxicity of other xenobiotics.

As the above and many other compounds appear to affect the microsomal enzyme system, it is of major importance to be aware of the potential that exists for joint action among compounds that inhibit or stimulate microsomal enzyme activity.

Exposed workers may be more susceptible or more resistant to subsequent exposures or to simultaneous exposures to different compounds, and the overall effect will depend on whether the enzyme system is stimulated or inhibited.

Trace metals have been found to be unique regulators of haeme and heame proteins. They appear to control both the synthesis and the degradation of the metalloporphyrin, through initial repression of δ-aminolaevulinic acid synthetase (ALA-S) (the rate-limiting enzyme in haeme synthesis), and induction of haeme oxygenase (haeme-Ox) (the rate-limiting enzyme in haeme degradation). There is experimental evidence that the rate of biosynthesis and degradation of haeme is a function of metal ion concentrations at appropriate regulatory sites in the cells[46].

The role of haeme in cellular respiration is well known, but this metalloporphyrin is also essential for the oxidative detoxification of a great number of endogenous and exogenous chemicals. The ability of metals to deplete cellular haeme content emphasizes the biomedical significance of these elements.

The induction of haeme-Ox is mediated by different metals such as cobalt, copper, chromium, manganese, iron, nickel, zinc, cadmium, mercury, lead and selenium. This induction phenomenon reflects a direct action of metals on the enzyme regulatory site. The induction of the "de novo" formation of haeme-Ox resulting in a depletion of cellular "free haeme" and of haeme proteins (mitochondrial respiratory cytochromes such as cytochromes P-450, P-448 and b_5) underlines the interest derived from the study of this particular enzyme inducing action from the biochemical, pharmacological and toxicological viewpoints[46-49].

Frydman et al.[50] have shown the induction of microsomal haeme-Ox (the membrane-bound enzyme catalysing the oxidation of the α-methene bridge of haeme IX with the formation of biliverdin IX-α) by Co^{2+} in rat liver, together with the induc-

tion of biliverdin reductase (the enzyme that catalyses the reduction of biliverdin IX-α to bilirubin IX-α)[51].

Copper (Cu^{2+}) has also been shown to be a potent inducer of haeme-Ox[52], and so have other metals mentioned above[46].

After experimental exposure to metal ions, impaired P-450-dependent oxidation of xenobiotics has been observed, owing to the depletion of cytochrome P-450, as already mentioned. This may also occur naturally: in the newborn human the liver content of cytochrome P-450 and drug-metabolizing ability are markedly decreased, as is ALA-S activity, while haeme-Ox activity is much higher than that in adults. The high activity of haeme-Ox and the low levels of ALA-S are considered to be responsible for the low content of P-450 in newborns, contributing to the overproduction of bilirubin. A reduction in bilirubin glucuronidation with a subsequent decrease in bilirubin excretion would explain the commonly occurring jaundice in newborns.

A number of workers have reported that certain metal ions such as Co^{2+}, Ni^{2+} and Hg^{2+} are able to modify the cellular content of glutathione (GSH) in the rat liver[53-56], and selenium has also been reported to increase cellular GSH levels[56,57].

A vast body of literature has been published on the interrelationships between selenium and the selenoenzyme glutathione peroxidase. Chung and Maines[58] have reported the effects of selenium on two other enzymes in the rat liver, namely γ-glutamyl cysteine synthetase, which is believed to constitute the rate-limiting enzyme in the pathway of GSH biosynthesis, and glutathione disulphide reductase (GSSG-Red), which is known to catalyse the reduction of GSSG to GSH. They observed increases in the activities of both enzymes after repeated exposure to low doses of selenium (the precise molecular mechanism has not been established), accompanied by elevated cellular levels of GSH and GSSG. The in vitro studies carried out indicate that the increased activities of the synthetase and the reductase do not reflect activation of preformed enzymes, but an increased production of the reductase and the synthetase. However, the possibility of selenium-mediated decreased catabolism of the enzymes cannot be excluded.

Cobalt and nickel are known to produce an initial decrease in cellular glutathione content followed by an increase of several-fold above the normal values[46].

Cadmium and zinc have been shown to induce the synthesis of metallothionein (a low-molecular-weight protein containing one third of its amino acids as free cysteine residues) in the liver, while mercury induces metallothionein synthesis in the kidney of rats[59-61]. Eaton et al.[56] have reported that the effects of metal ions on renal metallothionein content differed in some instances from those observed in the liver, but were similar in general terms, and zinc seemed to be the most effective inducer of metallothionein in both tissues.

2.4. WHERE AND WHAT TO MONITOR

Biological tests have demonstrated reliability in evaluating the degree of exposure to environmental contaminants, either in the field of occupational medicine or in studies on air pollution. Samples of blood, urine, faeces, breath, hair, nails, saliva, sweat and other specimens of biological origin are submitted for analysis, and the results obtained are conveniently evaluated in order to draw some conclusions about the effects of suspected toxic substances at the cellular or subcellular level.

The analysis of biospecimens has largely replaced methods of environmental monitoring by air sampling. Although the analysis of air samples from a working atmosphere may assess the degree of exposure to a toxic agent, it does not show either how much of the toxic substance has been absorbed or what the effects on the cells are.

Exposure to a toxic substance brings about an increase in the amount of the substance and/or its metabolites in body fluids. However, unless the biological threshold levels (BTLVs) for the appearance of adverse effects can be assessed, any increase in a toxic agent in biological media is meaningless. In fact, it would be desirable to fix not only the lowest levels that cause symptoms but also other parameters that will permit the detection of the biochemical lesion, i.e., the biochemical changes resulting from the interaction of the toxic agent with different ligands located on cell membranes or intracellular organelles, before the appearance of clinical symptoms.

In the interpretation of the results of biospecimen analysis, consideration should be given to the considerable individual variations that occur in biological levels from identical exposures. Such differences among individuals can be largely compensated for by means of collective tests, yielding average figures derived from a number of subjects under equivalent exposure circumstances.

Certain analyses are subject to limitations owing to endogenous levels in the biospecimen. A knowledge of the normal limits of variation of the substance is thus a prerequisite to obtaining information on the magnitude of overexposure.

The analysis of biospecimens is supplementing to an increasing extent the traditional environmental surveys, and biological samples are replacing air samples. A specimen of blood or urine may yield more information about total exposure than air samples. Also, the measurement is made directly on the individual responses to the toxic agent.

Of course, biological samples present more analytical difficulties than air samples, as it is necessary to isolate and determine extremely small amounts of a substance in the presence of large amounts of organic matter and other interfering substances. Serious errors can be committed by-any laboratory, but the

probability of their occurrence can be minimized by good practice. A numerical value can only be given some meaning when it results from a carefully performed analysis in which the best available method was used.

Various internationl committees have been set up with the aim of performing intra- and inter-laboratory studies on different analytical methods, establishing the optimum working conditions, limits of detection, precision, accuracy, specificity and the correct way to collect and store biological specimens.

No laboratory can give correct information on a bad sample. When biological specimens are to be collected for the monitoring of workers exposed to toxic metals (usually blood and urine samples), they may be grossly contaminated or otherwise incorrectly taken. For many years there has been controversy on how and when urine samples should be collected and how large the sample should be, and a great deal has been written on the reliability of urine analysis. For the monitoring of exposure to toxic metals 24-h specimens are desirable. When they are collected correctly, the urinary levels of the toxic metals tend to show smaller fluctuations than smaller samples[62,63]. The results from a single voiding of urine are meaningless[64]. The analysis of a spot urine sample may give a completely different result than a 24-h sample. The variability of spot samples is well known to impair the quality of the analysis, decreasing the significance of the values obtained from such specimens[65]. The basis on which the results from urinary excretion tests are expressed may alter their interpretation significantly. The results may be expressed as rate of excretion or adjusted to either a constant specific gravity or creatinine concentration. The latter may serve a reliable index of the adequacy of 24-h urine collection[66].

Blood analysis is generally believed to provide a better index than urine analysis for expressing the degree of exposure to a toxic metal. In order to avoid sample contamination or deterioration, specific recommendations should be followed in each case for both blood collection and blood storage.

Blood and urinary lead levels are valuable diagnostic tools in assessing excessive absorption of lead. An increase in either value represents a reliable guide to early lead exposure[67]. A high correlation exists between lead concentrations in air and lead values in body fluids.

Increased urinary mercury is an early signal of exposure, but no correlation or a poor correlation has been shown between mercury in air and urinary mercury excretion[68-70]. High individual variations and large fluctuations from day to day have been observed in urinary mercury excretion for workers under similar exposure conditions[70,71]. However, on a group basis, urinary excretion is roughly proportional to elemental mercury vapour concentration in air[72]. The same applies to mercury in blood levels.

As regards cadmium there appears to be no evidence of a quantitative relationship between urinary cadmium levels and the degree or duration of exposure. No correlation has been shown between urinary cadmium excretion and the clinical pattern of long-term exposure[73,74]. Little or no information is given by blood cadmium levels. Both blood and urine values can be used only as rough measures of the rate at which cadmium is being stored as indicated by exposed experimental animals[75].

Analysis of urine for arsenic in groups of exposed subjects may provide a good index of absorption. However, no biological limits have been established to differentiate safe from potentially dangerous absorption. A number of workers[76-78] have reported urinary excretions of arsenic 10-100 times greater in asymptomatic exposed people than in control groups. Further studies are required in order to assess the relationships between arsenic in air and urine concentrations and symptoms. Arsenic in blood levels do not seem to be specially meaningful.

The value of urinary manganese excretion in exposed subjects is doubtful, as only a minute fraction of the amount absorbed is excreted via the kidney[79]. Blood manganese levels are a better criterion for the assessment of absorption. Exposed workers with only slight symptoms may attain blood manganese levels as high as four times normal[80]. A reliable test based on the determination of manganese in faeces has been proposed for the monitoring of exposed workers[81], taking into account that the bile is the main route for removal of manganese.

Chromium is normally present in urine in only trace amounts; a substantial rise has been found in exposed workers. Blood and urine chromium levels appear to remain elevated for years after exposure[82]. However, according to Smith[63], blood and urine determinations of the metal in workers exposed to chromium do not give useful information on the degree of exposure. Others have found that the excreted fraction of filtered chromium and consequently its renal clearance are considerably higher in exposed workers than in subjects not occupationally exposed[63], the clearance increase being strictly correlated with the duration of working life. The clearance therefore appears to be good index of absorption.

Although in the biological monitoring of industrial personnel for absorption of toxic metals the two most frequently used body fluids are blood and urine, and most emphasis has been placed on blood and urine determinations, other types of biospecimens may also give useful information, e.g., saliva and hair.

Salivary fluid, obtained from the parotid glands in a standardized way, may be a useful biological indicator of the absorption of toxic metals. The levels of mercury in concurrently obtained specimens of blood, urine and saliva from exposed workers have shown a highly significant correlation between the blood and salivary concentrations of mercury[65]. As saliva is in direct equilibrium with the capillary network supply to the glands, the concentration of mercury in saliva can be directly related to its concentration in the circulating blood.

Analysis of hair has been used for many years to detect arsenic intake. Increased levels of arsenic in the hair of certain segments of the population have been shown to correlate with excessive concentrations of arsenic in drinking water[84]. Analysis of hair for metals has become a routine procedure for the monitoring of either industrial or environmental exposure[85], as human hair is a readily available specimen which may reflect the presence of excess of toxic metals in the body[86]. The amounts of trace metals internally deposited in hair may serve as a good index of the duration of exposure and the extent of storage. Hair analysis tests for the screening of excessive metal absorption have been described for lead[87,88], cadmium[89], arsenic[85], mercury[90], nickel[91] and other trace elements[86].

Lead concentrations in hair (and teeth) have been used as indicators of long-term exposure, but the information does not seem adequate to assess reliability and usefulness[92].

Large amounts of cadmium have been found in hair segments of workers regularly exposed[93]. However, hair analysis seems to be of little value in cadmium-exposed subjects owing to external contamination that is difficult to remove, as shown by Nishiyama and Nordberg[94].

A relationship between arsenic levels in hair and in ambient air has been reported by Hammer et al.[85] and Bencko et al.[95], who found that the mean concentration of arsenic in hair reflects the degree of arsenic air pollution in communities. Arsenic in hair seems to increase with the magnitude of exposure and decreases, returning to normal levels within a short period, after arsenic exposure has ceased. Arsenic levels in hair may be within the normal range among retired workers formerly heavily exposed and still showing symptoms or sequelae[96].

Increased storage of mercury in the body, as reflected by elevated levels of mercury in the hair, is regularly found in human consumers of fish contaminated with organic mercurials, although a definite correlation between blood and hair levels of mercury has not been shown[97,98].

A study carried out in Iraq during a methylmercury poisoning epidemic[99] did not allow any calculation of risk to be made from the data obtained by analysis of hair segments. Measurements of mercury levels in head and body hair, finger nails and toe nails from dentists and dental surgery assistants showed that hair and nail mercury levels were significantly higher in staff handling mercury than in a control group[100].

Nechay and Sunderman[91] concluded that measurements of nickel in hair may supplement nickel analyses of serum and urine as indices of the body burden of nickel.

The highest concentrations of chromium in humans are found in hair, as reported by Mertz[101].

2.5. INTERPRETATION OF RESULTS

When problems of interpretation are overcome, useful information can be obtained from biological analyses, including the best possible assessment of total exposure to a toxic agent in some instances and an indication of early damage to tissue in others. The most obvious question raised by an analytical result is whether it is normal or above-normal; the term "unexposed" is preferable to "normal". Reliable data on "normals" may be difficult to find for less common parameters, and consequently the amounts to be expected in a biological specimen submitted for analysis.

The highest concentration of a toxic substance that can be found in a biological medium without damage to health has been defined by Vigliani as the maximum allowable biological concentration. The subject of biological threshold limits for toxic metals and related parameters has been a matter for discussion by a number of international scientific committees and groups of experts. As long as the sensitivity of analytical techniques continues to increase and new enzymatic and other biochemical changes are being found, it is more and more difficult to fix a limit between normal and abnormal. On the other hand, the finding of some anomalies is not always easy to interpret and should not necessarily be considered as an adverse effect with respect to health.

An International Study Group of Experts[102] has proposed the term "recommended health-based biological limits", that is, the no-adverse-effect level of toxic substances or their metabolites in human biological materials. It seems that the health of the workers can be better protected when individual health-based biological limits are applied instead of group average levels. A few countries have also included the prevention of health impairment in offspring as a criterion in determining exposure limits.

Practical difficulties are also found when dealing with biological specimens in relation to the method of expressing the results. Further problems arise because of the multiplicity of parameters analysed and the lack of agreement on the method of expressing results.

A problem of paramount importance concerns the experience of laboratories performing this kind of analysis. Such laboratories should have experience in microanalysis and trace element analysis on the one hand, and experience in enzymology on the other, in order to identify and eliminate errors in the analytical procedures used. Constant quality control is needed in each individual laboratory and interlaboratory control is also essential. Whenever possible, methods and recommendations of international standardization committees should be followed.

New techniques are still being developed for the determination of metals in biological materials. The latest would be the methods for the in vivo measurement of metals. The method of partial body in vivo neutron activation analysis, among others, has been shown to be particularly useful for the detection of cadmium in the living person[103]. In vivo determinations of liver and kidney cadmium by neutron capture X-ray analysis are being performed with transportable measurement systems developed at the University of Birmingham, U.K.[104,105].

The need for the development of metal-speciation techniques has been emphasized by many investigators, as different compounds of the same metal may be responsible for different kinds of risks. It is therefore recommended[106] that whenever possible the exposure should not be expressed as element levels, but as the levels of specific compounds. This point still needs further research.

2.6. INDICES FOR LEAD EXPOSURE

Lead induces certain biochemical injuries at cellular or subcellular levels. This is a good example of a metal acting on the permeability of the erythrocyte membrane. Pb^{2+} causes inhibition of the active transport of K^+ and a specific increase in membrane permeability. Studies carried out on erythrocyte membranes of workers with lead exposure have shown a decrease in the activity of Na^+-K^+/ATPase (this enzyme hydrolyses ATP, thus providing the energy necessary for the active transport of Na^+ and K^+), as well as a rapid loss of intracellular K^+ and a decrease in the intracellular content of ATP[1-3].

A great deal of research work has been carried out with the aim of establishing possible biochemical sites for the action of lead on the biosynthesis of haemoglobin. Rimington[4,5] gave the first evidence that anaemia in the presence of increased lead absorption is a consequence of the inhibition of haeme synthesis. According to the numerous studies carried out since then, it may be concluded that lead can affect all steps in the pathway of haeme synthesis, although the degree of inhibition of each enzymatic system varies considerably. In vitro experiments indicate that δ-aminolaevulinic acid dehydratase (ALA-D) and haeme synthetase (Hem-S) are the two most sensitive enzymes to the action of lead. Some controversy has arisen, however, in connection with the inhibition of the latter[6,7].

The sensitivity of ALA-D to Pb^{2+} has given rise to the development of a test based on the determination of the activity of this enzyme on circulating erythrocytes as a means of detecting lead absorption at a very early stage[8-15]. ALA-D activity inversely correlates very closely with blood lead levels and gradually becomes depressed and at a slow rate under the influence of a small uptake of lead[12].

It has been found that lead affects not only the haeme moiety synthesis but also the globin synthesis and, as shown by Pernis and Zanardi[16], the percentage of haemoglobin A_2 increases in human adults and laboratory animals with increasing lead absorption. Bonsignore et al.[17] found a significant increase in foetal haemoglobin F in lead-exposed workers, attributed to the possibility of derepression of the gamma gene.

Lead also affects the activities of enzymes other than those directly related to haeme biosynthesis. A number of investigators have described changes in the activity of various enzymes, e.g., increases in transaminases[18,19] and aldolase[20] and decreases in alkaline phosphatase[21,22] and cholinesterase[23].

A group of workers with slight to moderate exposure to lead and practically symptomless[24] presented no evidence of significant changes in the serum activity of aldolase, cholinesterase and transaminases, but a significant decrease was shown in the total activity of lactate dehydrogenase as well as the partial inhibition of its cathodic molecular fractions.

According to Urbanowicz et al.[25], in industrial lead exposure an increased urinary excretion of 5-hydroxyindole acetic acid becomes evident earlier than the respective δ-aminolaevulinic acid and coproporphyrin maximum excretions. The test may be applied for the detection of abnormal lead absorption during the first weeks of exposure. However, there is no agreement among different investigators on the relationship between lead in blood (PbB) levels and hydroxyindoleacetic acid (HIIA) excretion. The mechanism of action is not well understood, and the analysis itself does not seem suitable for epidemiological studies.

Chisolm and Silbergeld[26] have found increased excretion of homovanillic acid (HVA) in the urine of young children with increased lead absorption. No significant correlations between HVA, free erythroporphyrin (FEP) and chelatable lead (PbU-EDTA) were found, suggesting that the so-called "HVA effect" is not related to lead effects on haeme synthesis. In other words, the inhibitory effect of lead on haeme synthesis is unrelated to its apparent effect on HVA metabolism. These studies in children seem to provide the first evidence of a dose-related effect of lead in man on a metabolite, which may originate in neuronal tissue, namely HVA. Maas and co-workers[27,28] found in monkeys and human adults that HVA is the major catabolite of dopamine, and that 33% of urinary HVA is derived from the central nervous system. The data obtained in Chisolm and Silbergeld's study suggest that the effect of lead on urinary HVA is reversible and dose-related, and may serve as potential marker for lead.

Experimental studies carried out on rats fed a diet containing low doses of lead for 7 months[29] showed a number of enzymatic changes which might be induced either by the direct effect of lead or by the metabolic adaptation to the lead damage. The changes included decreased glutamate dehydrogenase and malate de-

hydrogenase and increased glucose-6-phosphate dehydrogenase. The rats showed increased urinary excretions of lead and δ-aminolaevulinic acid.

A previously unrecognized enzyme specifically cleaving the phosphate bond of pyrimidine 5'-nucleotides in human red cells has been described by Valentine et al.[30] and called pyrimidine 5'-nucleotidase (Py 5N). The same group[31] discovered that patients with lead poisoning exhibited a decreased activity of red cell pyrimidine 5'-nucleotidase, and this finding applied both to severely intoxicated patients[32] and to individuals with mild exposure[31].

In order to determine the value of this enzyme as a biological index of lead exposure, Buc and Kaplan[33] investigated the Py 5N activity in red cells together with the classical parameters in subjects with varying degrees of lead absorption. The red cell Py 5N activity was found to be decreased in all cases, even when most of the other biological tests remained negative. It was concluded that red cell Py 5N represents a reliable and sensitive index of lead exposure.

2.6.1. Interpretation of blood and urine lead levels

The reader is referred to Chapter 4 for the methodology of the determination of lead in blood and urine.

2.6.1.1. Lead in blood

PbB in human subjects without known exposure seems to be fairly constant throughout the world, ranging from 10 to 35 µg per 100 g on average[34-36]. Consistent differences have been shown between children and adults[37,38], men and women[39] and urban and rural populations[40,41].

The group of experts who met at the 2nd International Workshop on Permissible Levels for Occupational Exposure to Inorganic Lead[42] could not agree on "what level should be regarded as a health-based permissible level for occupational exposure", but it was agreed that for male workers individual PbB should not exceed 60 µg per 100 g in the light of knowledge available to the group. However, it was considered desirable to reduce individual exposure below this level, taking into account the effects on the haematopoietic system at concentrations above 45-50 µg per 100 g and on nerve conduction velocity at concentrations between 50 and 60 µg per 100 g. Because of potential effects on the foetus, a safe practice would be to avoid the employment of women of child-bearing age on lead work where blood levels might regularly exceed 40 µg per 100 g. Experiments carried out by Hayashi[43] on pregnant and non-pregnant rats led him to conclude that avoidance of lead exposure should be the rule during pregnancy.

It should be remembered that some individuals may show increased vulnerability (hypersusceptibility) due to genetic traits (sickle cell anaemia, thalassaemia,

G6PD deficiency, etc.), malnutrition, combined exposure to other occupational and environmental factors, ingestion of different drugs and alcohol, smoking of cigarettes, etc. For such individuals the given blood lead concentrations related to other biochemical changes may be altered. In such cases an additional safety margin may be desirable, as the unusually sensitive must also be protected.

According to Zielhuis[44], "the lead content of whole blood (PbB) is the most valid indicator of total uptake and of health risk and, therefore, the PbB level is the primary indicator of both total exposure and health risk in screening individuals and population groups". A Biological Quality Guide (BQG) was proposed and accepted by the Commission of European Communities (CEC) as a guideline for a general survey of population exposure, carried out in 1979 and in 1981 in the nine member countries[45]; total exposure is not unacceptable if 98% of PbB levels are <35 μg per 100 ml, 90% <30 μg per 100 ml and 50% <20 μg per 100 ml. Taking into account the susceptibility of young children, a lower guideline was proposed for preschool children: 98% <30 μg per 100 ml, 90% <25 μg per 100 ml and 50% <20 μg per 100 ml. The maximum limit of 30 μg per 100 ml has been adopted by the WHO[46] for occupational exposure of females of fertile age.

2.6.1.2. Lead in urine

Blood samples are to be preferred for the biological monitoring of lead exposure as they are more stable and more indicative than urine samples. PbB is entirely specific and reliable in indicating the extent of the absorption of lead[47]. When urine is chosen to be analysed rather than blood it is necessary to sample more frequently. Usually the frequency is doubled with respect to blood sampling.

On the other hand, PbU provides the only analytical criterion for the determination of absorption in exposure to alkyllead compounds (TEL and TML), and although a BTLV for PbU in workers exposed to alkyl derivatives has not been yet assessed, PbU represents the most suitable test for detecting dangerous absorption of TEL[48].

The PbU of unexposed individuals varies from non-detectable levels up to 80 μg per 1000 ml, with an average of about 30 μg per 1000 ml[49]. More recent studies have revealed much lower levels. Tsuchiya et al.[36] obtained a mean value of 12 μg per 1000 ml for a group of 2300 policemen in Japan.

Haeger-Aronsen[50] obtained mean values of 14.0±9.0 μg per 1000 ml for PbU in unexposed people, and when concentrations were adjusted to creatinine excretion the mean value was 8.6±5.6 μg per gram of creatinine.

Moderate lead absorption gives rise to an increase in PbU with values ranging between 100 and 150 μg per 1000 ml within a few weeks[49]. According to Tsuchiya and Harashima[51], for a 48-60-h working week an average air lead concentration of 100 $\mu g/m^3$ would lead to an average PbU level of 150 μg per 1000 ml.

It should be noted, however, that "normal" rates of PbU excretion may be found even when the exposure is high[52]. For subjects who have been removed from exposure some months prior to diagnosis in whom PbB has almost returned to normal levels, a Ca-EDTA provocation test may provide useful information. If PbU excretion within 24 h after intravenous infusion of 1 g of chelating agent exceeds 1000 µg, the subjects are considered to have had abnormally high lead exposure in the past[53]. The upper limit in healthy adult subjects would be less than 600 µg of lead excreted over 4 days after a 1-g injection of Ca-EDTA[54].

2.6.1.3. Lead in other biological samples

It has been stated that sweat contains lead in concentrations comparable to those in urine[47].

Lead in hair has also been proposed for surveying exposed workers. Lead levels in hair may reflect long-term exposure. A procedure based on anodic-stripping voltammetry (ASV) has been described[55] and recommended for its simplicity, speed and the small amounts of sample required (1-15 mg). The lead concentrations in the hair of persons working in a battery factory were found to range between 81 and 740 ppm (mean = 321 ppm), while those in the hair of controls not industrially exposed ranged from 5 to 46 ppm (mean = 39 ppm). The results were similar to those obtained by atomic-absorption spectrometry (AAS) and by the dithizone method. Jenkins[56] reported values for some toxic metals in human hair with tentative normal and toxic levels. The values for lead ranged from 0 to 70 ppm for normal and from 0 to 1880 ppm for exposed individuals.

Neutron activation analysis (NAA) has been preferentially used for the identification of metals in hair in the field of criminology.

The use of saliva has been suggested as an alternative method to blood or urine sampling for the biological monitoring of lead. Salivary lead determination by AAS offers the advantage that interference from sodium is negligible because of its lower concentration in saliva[57]. Lead concentrations found in human parotid saliva ranged from 0.3 to 1.0 µg/ml. However, the use of saliva as an appropriate medium for detecting lead exposure is still a matter of controversy[58].

Teeth (either deciduous or extracted) can also be used for the biological monitoring of lead exposure. Lead has been analysed by AAS with direct atomization from the solid state[59], after separating enamel from dentine[60]. Increased concentration of lead in teeth persists even after blood lead levels have decreased[61]. It has been suggested that the lead concentration in the dentine of shed deciduous teeth is a good indicator of past exposure to lead during infancy and early childhood.

Finger nails and toe nails have been used for the monitoring of exposure to lead, although they have not been employed as extensively as hair[62]. Hair and

nail samples are relatively easy to collect, are small and require no special containers or refrigeration for storage and transport. The methodology to be applied is almost the same, usually AAS.

2.6.2. ALA-D activity in erythrocytes

δ-Aminolaevulinic acid dehydratase (5-aminolaevulinate hydrolyase, E.C. 4.2.1.24), catalysing the conversion of δ-aminolaevulinic acid (ALA) into porphobilinogen (PBG), is inhibited by lead rather specifically, with one known exception in the case af alcoholism[63]. Because of the direct effect of tobacco smoke on ALA-D activity, it is recommended to take smoking habits into account when epidemiological studies are carried out[64].

The inhibitory action of lead on ALA-D has been demonstrated in vivo and in vitro by a number of investigators[65-70].

Measurement of ALA-D activity in erythrocytes is a relatively simple procedure, determining the amount of PBG formed per unit time by a standard amount of enzyme source. The simplest technique, described by Bonsignore et al.[71], requires the incubation of blood (source of enzyme) with ALA (substrate) at 38°C. The amount of PBG formed after incubation for 1 h is measured by means of a colour reaction in the presence of Ehrlich reagent.

Many investigators have modified the procedure and results from different laboratories are not comparable, as shown by an interlaboratory study carried out through an European standardized method has been developed, tested in a collaborative study and agreed upon by nineteen laboratories[73]. The results of these tests compare very favourably with PbB determinations. The interlaboratory coefficient of variation was 10%.

The inhibition of ALA-D has shown a negative correlation with PbB. Hernberg et al.[74] showed a negative linear regression over a range of 5 to 90 μg per 100 g of PbB, when ALA-D was plotted on the logarithmic scale. Wada et al.[75] reported a limiting PbB level of about 15 μg per 100 ml, below which ALA-D showed no correlation with PbB levels.

Depression of ALA-D activity in erythrocytes is not only a sensitive but also a specific index of exposure to lead. As stated above, ALA-D activity in human blood also decreases with the elevation of blood ethanol but returns to normal at the same rate as the ethanol level[63].

The European standardized method[73,76] gave a range of ALA-D activities from 30 to 60 Units per litre for normal blood samples, with an average coefficient of variation of only 3% and a standard deviation of 2%. One European Unit is the amount of ALA (μmoles) converted into PBG per minute per litre of red blood cells at 37°C:

μmoles ALA min^{-1} $LRBC^{-1}$ = U/L*

The main features of this method are reproducibility, precision, low cost per analysis and easy implementation (no elaborate equipment is required).

The interlaboratory comparison shows that laboratories using the standardized European method for ALA-D activity can obtain comparable results. Reference quality control blood is not available for ALA-D owing to the instability of the enzyme, and a fresh blood sample has to be obtained from individuals whose blood ALA-D activity has been previously determined (ALA-D activity is stable with time).

2.6.3. Erythrocyte porphyrins

Protoporphyrin (PP or PP IX) is formed in the mitochondria during the differentiation of the erythrocyte in the bone marrow. The conversion of PP to haeme requires the insertion of iron into the PP ring. It appears that Pb^{2+} does not prevent the mitochondria from incorporating iron but rather from using it. In over words, it may be postulated that Pb^{2+} either inhibits haeme-S or inhibits some other system in such a way that iron is not provided in a proper form to the enzyme[78,79].

PP is elevated not only in lead exposure but also in iron deficiency anaemia, while ALA-D activity is not affected by iron deficiency. Lamola and Yamane[80] were able to demonstrate that the PP accumulating in lead exposure and in iron deficiency anaemia is in fact ZnPP and not free PP.

A number of methods have been proposed for determining the concentration of erythrocyte porphyrins in blood. Because of the difficulties arised from the lack or readily available standards the complexity of the methodology and the lack of agreement among laboratories, a Symposium on Porphyrin Measurements - Laboratory and Clinical Aspects, was sponsored by the Capital Section of the AACC and the Clinical Chemistry Service of the NIH (U.S.A.) in 1976. A group of invited experts in the field of porphyrin chemistry held discussions on the methodology for protoporphyrin measurements, among other points of interest.

All the known methods consist in fluorimetric assays of PP extracted from blood in various ways with different solvents, most of them being micromethods requiring a few microlitres of blood[81-84]. However, all of these methods seem to have been displaced by the use of a new instrument specially designed for the

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*It has been recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) that "the Unit in which enzymic activity is expressed be the amount of activity that converts one mole of substrate per second"[77]. This unit is called the katal (kat): 1 kat = 6 x 10^7 U; 1 U = 16.67 nkat; 1 mU = 16.67 pkat.

rapid assay of ZnPP in unprocessed blood. This instrument, calles a haemofluorimeter[95], operates with only one drop of blood obtained from a finger puncture, which is placed on a cover slip and inserted in the sample holder of the instrument, which also holds a permanent blank and a fluorescence standard (usually Rhodamine B). The ZnPP concentration is automatically and instantaneously computed and the value is displayed on a digital readout as micrograms of ZnPP per 100 ml of blood of a standard haematocrit. The whole procedure takes about 5 sec. The values obtained with the haematofluorimeter correlate well and linearly with those given by currently accepted extraction methods[86]. The median ZnPP values found by Olsen et al.[87] in a group of 1050 adults (40-year-old suburban population) were 28 µg per 100 ml of blood in women and 25 µg per 100 ml of blood in men. In fact, the average PbB level of the unexposed population will determine the PP level as there is an interrelationship between the two.

High-performance liquid chromatography (HPLC) has been applied[58] to the analysis of porphyrins. A rapid HPLC method has been developed for accurate determination of ZnPP and PP in blood, and the results compared with those obtained by conventional methods. The total analysis time required is about 15 min per sample.

The results obtained suggest that the determination of ZnPP is a sensitive test for the detection of mild increases in PbB concentrations in lead-exposed workers. However, it should be remembered that the results are elevated in iron deficiency states and may also be influenced by heavy alcohol intake[89], as is the PbB level[89]. It is recommended that positive tests be supplemented by another test that is not influenced by either alcohol consumption or cigarette smoking. According to Zielhuis[42], although ZnPP is considered to be the best secondary parameter, it cannot replace PbB except for screening purposes.

The ALA-D/PP ratio has been suggested by Beritić et al.[90] as a valuable measure of the intensity and duration of lead exposure, while Alessio et al.[91] consider the determination of erythrocyte PP to be a useful test not only for the monitoring of exposed workers but also for subjects with past exposure. In a more recent publication, Alessio et al.[92] considered erythrocyte protoporphyrin IX (EP) and ALA-D activity to be useful tools for establishing "the persistence and extent of an active deposit of lead in the organism, while PbB is of very limited use". EP and ALA-D are therefore suggested as two good indicators for the identification of subjects who have stopped working with lead, but having been severely exposed in the past.

Haematofluorimetric readings for ZnPP have shown to be fully equivalent to the erythrocyte PP levels obtained by extraction techniques (correlation coefficient $r = 0.98$)[93].

Zielhuis[94] estimated that not more than 5% of the population will show a perceptible increase in erythrocyte PP when PbB levels are 30 µg per 100 ml in adult males, 25 µg per 100 ml in adult females and 20 µg per 100 ml in children.

Mean normal values for erythrocyte PP as given by Alessio et al.[91] are 29±8 µg per 100 ml RBC with ranges between 15 and 60 µg per 100 ml RBC. For workers with past exposure the mean values have found to be 145±85 µg per 100 ml RBC with ranges between 25 and 350 µg per 100 ml RBC.

The haematofluorimetric readings have been shown to be linearly related to ZnPP concentration over the entire range from 30 µg per 100 ml of blood in normals to 1100 µg per 100 ml of blood in severely exposed individuals[85].

Sassa et al.[79] reported that over 95% of the population with a PbB level of 60 µg per 100 ml showed a PP concentration of 140 µg per 100 ml RBC or above.

2.6.4. Urinary coproporphyrins and δ-aminolaevulinic acid

The measurement of coproporphyrins in urine is usually carried out by extraction of the porphyrins into either ethyl acetate-acetic acid[95] or diethyl ether[96], followed by transfer into hydrochloric acid. The absorbance is measured at 401 nm with the corrections recommended by Rimington and Sveinsson[97]. Under these conditions uroporphyrins are not extracted and therefore do not interfere. An alternative method has been reported in which the fluorescence of the acid aqueous phase is measured after adsorption on to magnesium hydroxide[98]. The method of Schwartz et al.[99] in which the hydrochloric acid extract is measured either by spectrophotometry or by fluorimetry has also been used extensively for the determination of urinary coproporphyrin.

Electrophoresis on Cellogel[100] revealed the urinary uroporphyrin with eight carboxylic groups (UPU) and the coproporphyrin with only four carboxylic groups (CPU) by the intense pink fluorescence of the bands when examined under UV light. The technique is highly sensitive.

An increase in CPU is not specific to lead exposure, but may be considered as a sensitive indicator of lead absorption[101].

The normal levels of CPU are usually below 100 µg per gram of creatinine. Mean values have been estimated at 38.0±24.0 µg per gram of creatinine[50]. CPU starts to increase at a PbB level of approximately 40 µg per 100 ml[101] and above this value it is proportional to PbB provided no interfering factors are present, e.g., haemolytic anaemia, hepatitis or cirrhosis. For PbB levels of 60 µg per 100 ml, the CPU concentration may reach about 200 µg per gram of creatinine.

The basic method for the measurement of urinary δ-aminolaevulinic acid (ALAU) was developed by Mauzerall and Granick[102]. After separation of ALA and PBG by column chromatography, ALA is eluted, complexed with acetylacetone and determined spectrophotometrically by means of a colour reaction with Ehrlich reagent. A number of modifications and simplifications have been reported[103-107]. Cavalleri et al.[108] described a technique adapted to an AutoAnalyzer. The specificity of

the method was evaluated by comparison with the chromatographic technique, and sensitivity, accuracy and precision were studied. The procedure seems to be particularly useful for the screening of lead-exposed workers.

There is no doubt that the specificity of ALAU is better than that of CPU[109-111]. Increased ALAU excretion has been observed at blood lead levels of 40-50 µg per 100 g[40,74,112,113], and an increase in ALAU becomes very marked when PbB levels exceed 40-60 µg per 100 g, as shown by Sakurai et al.[114].

In unexposed individuals ALAU usually does not exceed 4.5 mg per gram of creatinine. Mean values have been estimated to be 1.52±0.59 mg per gram of creatinine[50]. On a group basis, a concentration of around 10 mg of ALAU per gram of creatinine corresponds to a PbB level of 60 µg per 100 g[115].

A satisfactory correlation has been found between ALAU and PbB[115,116] or PbU[11,111,117].

2.6.5. Red-cell pyrimidine 5'-nucleotidase

As already stated, lead absorption is accompanied by an acquired deficiency of erythrocyte pyrimidine-specific 5'-nucleotidase (Py 5N). When sufficiently severe, the lead-induced deficiency gives rise to findings similar to the hereditary disorder.

Pyrimidine 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5) in normal haemolysates dephosphorylates hydrolytically uridine and cytidine 5'-monophosphates (UMP and CMP), releasing inorganic phosphate.

The original method of Valentine et al.[39] for determining the enzyme activity consists in measuring the amount of inorganic phosphate released from CMP or UMP during a 2-h incubation with a previously dialysed haemolysate. This procedure is laborious and unsuitable for large numbers of assays.

A simple and rapid radiometric assay for Py 5N was developed by Torrance et al.[118], in which [^{14}C]CMP serves as substrate. The CMP that is not dephosphorylated to cytidine is bound to a barium sulphate precipitate which forms in the deproteinization process. The cytidine remains in solution and is counted. The method is simple and reproducible and can be carried out on large numbers of samples.

Buc and Kaplan[119] developed a radioassay for Py 5N activity. The radioactive uridine released after incubation with [5-^{3}H]uridine 5-monophosphate (UMP) is separated on DEAE-cellulose paper and counted. This method is also very simple, does not require preliminary dialysis of the haemolysate and is 50-fold more sensitive than that based on the measurement of the inorganic phosphate. With this procedure it is possible to detect 1 nmol of released uridine with good accuracy. It requires only 25 µl of non-dialysed haemolysate and incubation for

1 h. After separating UMP from uridine by paper chromatography, uridine is counted in a liquid scintillation spectrometer.

Mean normal values for Py 5N activity as determined by the Valentine et al.[30] method have been calculated as 7.5±0.8 μmol Pi/h/g Hb. The range of values for exposed workers with mild exposure was shown to be 1.7 - 5.3 μmol Pi/h/g Hg. Maximum inhibition of Py 5N is reached at a level of about 200 μg of Pb per 100 ml of packed cells.

Py 5N activity is recommended to be systematically investigated along with ALA-D activity in subjects potentially exposed to lead.

2.6.6. Other secondary parameters

It has already been stated that significant biochemical changes are associated with genetic abnormalities, which have been evidenced in workers showing hypersusceptibility to certain industrial contaminants. A number of investigators have studied the problem of hypersensitivity responses to xenobiotic agents, originating from hereditary disorders known as inborn errors of metabolism. The inherited sensitivity to toxic environmental hazards has been extensively described in the literature[120-127]. Most of these genetic deviations become apparent only upon exposure to specific chemicals.

Erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is one of the most widely studied genetic anomalies. Red cells with defective G-6-PD activity show impaired reduction of glutathione, and consequently cellular integrity is not ensured. A vast clinical documentation has shown severe haemolytic phenomena in lead-exposured subjects even when the PbB level was relatively low[120-131]. The hyperhaemolysis could possibly be explained by the additional insult of the red cell arising from the inhibitory action of Pb^{2+} on the membrane Na^{+}-K^{+}/ATPase and the lead-induced reduction of the glutathione (GSH) level.

A simple test[132] has been devised for diagnosis of the genetic G-6-PD deficiency and for predicting hypersusceptibility of lead-exposed workers. The test is based on the reduction of oxidized pyridine nucleotide (NADP). Minute amounts of pyridine nucleotide fluoresce intensely when reduced (NADPH) and activated with longwave UV light.

To the blood sample is added a reaction mixture containing the substrate (glucose-6-phosphate), the coenzyme (NADP), a lytic agent (digitonin) and a buffer solution (potassium phosphate, pH 7.4). A control baseline spot is made on a filter-paper, and additional spots are made after specified periods of incubation. After the spots have dried, inspection under longwave UV light will show a bright fluorescence for normal G-6-PD activity. No appreciable fluorescence appears when G-6-PD-deficient blood is tested.

The International Committee for Standardization in Hematology (ICSH) recommended methods for red-cell enzyme analysis[133], including G-6-PD. Indications for storage of samples, preparation of haemolysates, performance of enzyme assay and normal values were given. Mean normal values for G-6-PD activity at 37°C are as follows: without correction, 12.1±2.09 U/g Hb; and corrected for 6-PGD activity, 8.3±1.59 U/g Hb.

Another genetic anomaly to be taken into account in the biological monitoring of workers with the risk of lead exposure is the presence of abnormal haemoglobins, such as HbA_2, which can also be associated with lead-induced anaemia.

HbA_2 is a slowly migrating variant of the normal HbA and is present in normal blood at levels up to 2.5% of the total. A tendency for an increase in HbA_2 has been shown in the early stage of lead exposure[16]. Foetal haemoglobin (HbF) has also been shown in adults exposed to lead[17].

HbF and HbA_2 may be determined by the spectrophotometric measurement of their peroxidase activity after separating HbA_2 by chromatography on DEAE-cellulose and obtaining HbF through a microtechnique of alkaline denaturation[134]. In normal adults, the mean percentages for HbA_2 and HbF have been calculated to be HbA_2 = 1.97% (S.D. 0.53%) and HbF = 0.59% (S.D. 0.175%). Both values are increased in thalassaemia to 4.7% for HbA_2 and 2.31% for HbF.

A simple method for the determination of HbA_2 has been reported by Vettore et al.[135]. HbA is separated from HbA_2 by electrophoresis on Cellogel in a discontinuous buffer at alkaline pH. The strips of cellulose acetate containing the Hb fractions are dissolved in 80% acetic acid. The percentage of HbA_2 is calculated from the spectrophotometric absorbance at 396 nm.

The average concentration of HbA_2 determined by this method in normal subjects was 2.31±0.37%. In subjects with heterozygous β-thalassaemia the mean value was increased to 4.64±0.53%.

None of the methods givies figures for lead-exposed workers. The authors claim that the electrophoretic method is simple, rapid, precise, relatively inexpensive, reliable and reproducible.

With increasing levels of PbB a decreased activity of adenosine triphosphatase (Na^+-K^+/ATPase) has been shown[1-3,136]. Although a very low negative correlation (r = -0.26) has been found between PbB level and the enzyme activity in red cell membranes, on a group basis an increase of PbB from 32 to 38 µg per 100 ml decreases the enzyme activity by about 25%. Although this test is still being used for research purpose it has no application as a routine test for biological monitoring of lead-exposed workers because the methodology is complex and time consuming.

Many other tests have been proposed as contributions to the early detection of lead exposure, but they remain in the field of experimental and research work

because of either technical difficulties or poor correlation with PbB levels, or simply owing to the absence of a clear dose-effect/response relationship.

2.7. INDICES FOR MERCURY EXPOSURE

Some toxic metals undergo oxidation-reduction reactions, the most studied of which are those for mercury. After inhalation, elemental mercury vapour crosses the alveolar membranes and enters the bloodstream, dissolving in the plasma. Owing to its high diffusibility and high lipid solubility, the dissolved mercury crosses the red cell membrane where it is oxidized to Hg^{2+}, which may react with different organic ligands.

The brain uptake is about ten times greater in animals exposed to mercury vapour than in those given an equivalent dose of ionic mercury[1-3]. This led to the idea that, although oxidation within the red cell is rapid, a certain amount of dissolved vapour persisted in the plasma long enough to reach the blood-brain barrier. On entering the brain tissue, it was subject to tissue oxidation processes and ionic mercury was trapped, becoming bound to tissue proteins.

There is experimental evidence that catalase is directly involved in the oxidation of mercury vapour[4]. As some subpopulations are genetically acatalasemic[5], they may react differently than normal individuals to mercury vapour.

The affinity of mercury for SH groups is probably the basic biochemical property of mercury and mercury compounds. The binding to SH groups of proteins in membranes and enzymes is responsible for the interference with membrane structure and function and with enzyme activity. It is assumed that mercury reacts selectively with thiol groups located on the surface of enzymes, although other groups may take part in this reaction. The complex mercury-enzyme is enzymically inactive but it may become active again if mercury is removed. The cell membrane is the first point of attack by metals. The mercurials cross the membrane at different rates. As the mercurial slowly penetrates to compartments within the membrane, the permeability to $Na^+ + K^+$ increases and the Na^+-K^+ activated membrane ATPase is inhibited. As the mercurial passes through to the interior of the cell, the membrane recovers its original permeability properties. The more rapidly the mercury compounds penetrate the cell, the more transient are the membrane effects[6].

In a survey carried out on a group of mercury miners a marked inhibition of red cell membrane Na^+-K^+/ATPase has been demonstrated[7].

Mercury also interacts with phosphoryl groups of cell membranes[8] and with amino and carboxyl groups of enzymes[9]. Metallothionein from patients treated with mercurial diuretics has been found to contain mercury bound more firmly than either cadmium or zinc[10,11]. Mercury also interacts with nucleic acids by adding not only to the phosphate groups but also to the bases[12].

Mercury accumulated in the kidney is partly in the form of a metallothionein-like complex[13,14]. In the rat, binding by this protein seems effective in repeated exposure to Hg^{2+} owing to the induction of higher levels of the metallothionein-like protein by mercury[15]. The same probably applies to exposure to elemental mercury vapour, as it results in enhancement of the metallothionein level in the kidney.

The reversible formation of complexes with organic ligands has been the subject of intensive investigation in recent years, and the potential for the formation of a variety of complexes in vivo is indicated by the high stability constants for ionic inorganic mercury and for methylmercury cations, forming complexes with virtually any organic molecule of biological importance[16].

Mercurials are non-specific enzyme inhibitors and many of the mercurial binding sites are inactive, i.e., no detectable effects ensue[8].

Studies by Kosmider[17] suggested that lactate dehydrogenase (LDH) would be one of the most sensitive enzymes to the action of mercury, its inactivation being related to the presence of SH groups.

Studies carried out on a group of mercury miners[7] have shown an increase in the total activity of serum and urinary LDH and an increase in the cathodic molecular fractions 4 and 5. No significant changes were found in the activity of other enzymes studied, such as transaminases and cholinesterase.

Methylmercury salts accumulate to a high degree in erythrocytes. Studies by Takeda et al.[18] showed that the methylmercury radical is bound to the cysteine residues of haemoglobin. The binding would be readily reversible either for methylmercury or for inorganic mercury ions[19,20]. The erythrocyte to plasma ratio is approximately 1 for inorganic mercury and about 10 for methylmercury.

Experiments carried out by Hirota et al.[21] have shown that the activity of glutathione-peroxidase (GSH-Px) is inhibited by methylmercury ions and suggest that the measurement of this enzyme activity could be a good parameter for indicating an early adverse effect of methylmercury.

It has been postulated[22] that the neurotoxicity of methylmercury may involve free radicals formed by the breakdown of methylmercury and does not result solely from the intact organometallic compound. There is also evidence that high doses of methylmercury inhibit its conversion into inorganic mercury.

More research work would seem desirable in order to establish the biochemical lesions in humans and develop sensitive biochemical tests for the early detection of mercury exposure, particularly for elemental mercury vapour and short-chain alkyl mercurials (the most important forms of mercury from the standpoint of risk to human health).

2.7.1. Interpretation of blood, urine and hair mercury levels

The reader is referred to Chapter 5 for the methodology of the determination of mercury in blood, urine and hair.

2.7.1.1. Mercury in blood

Magos et al.[23] found that the average blood levels of mercury in individuals having no history of exposure were 0.54 µg per 100 g as total mercury. Data from Suzuki et al.[24] indicate average of 1.7 µg per 100 g of blood in unexposed individuals. Yamamura et al.[25] reported for blood samples in normal subjects an average content of mercury of 0.9±0.6 µg per 100 g. Yoshida et al.[26] reported average total mercury in blood of 2.5 and 2.0 µg per 100 g in control male and female subjects, respectively.

A WHO study group[27] has established that people who do not usually eat fish and without occupational exposure show mercury levels in whole blood of $\leq$ 0.5 µg per 100 ml. For moderate consumption of fish the blood levels of mercury rise to 1.0-2.0 µg per 100 ml, and for heavy fish eaters blood mercury levels of 10-20 µg per 100 ml may be observed.

In people with long-term exposure to methylmercury, Tsubaki[28] reported blood levels ranging from 0.2 to 80.0 µg per 100 g, while Yamamura et al.[25] found that 5 out of 47 workers exposed to elemental mercury in small plants manufacturing thermometers showed blood levels over 10 µg per 100 g. In 30 workers exposed to metallic mercury Yoshida et al.[26] found average levels of total mercury in blood of 10.4 µg per 100 g.

Mercury exhalation found in animals after exposure to elemental mercury vapour[29] has also been confirmed in man[30]. The relationship of mercury levels in blood and in exhaled air has been studied in individuals exposed to metallic mercury vapour[31] and a correlation coefficient of 0.867 ($p < 0.01$) was found. The possibility has been considered of using the mercury levels in exhaled air as an efficient index of elemental mercury levels in blood.

According to Clarkson[32], not only do blood samples give the best estimate of mercury body burden, but mercury blood levels may bear a constant ratio to the concentration of mercury in the target organ.

Yoshida et al.[26] found increased inorganic mercury levels in plasma when exposure was due to metallic mercury while for ethylmercury exposure an increase in red cells organic mercury was found.

2.7.1.2. Mercury in urine

Urinary mercury values may serve as useful guides in early periods of exposure. It appears that in prolonged exposure a decrease in urinary mercury excretion may occur because of renal injury and/or other factors.

A level of 100 µg per 1000 ml has been suggested as the urinary threshold limit, but clinical evidence of exposure has been seen with both lower and considerable higher urinary levels than 100 µg per 1000 ml. The threshold limit for organic mercury has been suggested to be fixed at less than one tenth of the limit used for inorganic mercury vapour, keeping urinary mercury levels below 10-15 µg per 1000 ml[33].

In unexposed subjects Yamamura et al.[25] reported average urinary mercury content of 3.5±2.3 µg per 1000 ml, while in exposed workers the mercury urinary excretion exceeded 300 µg per 1000 ml in 13 subjects out of 47.

Fish intake habits are not taken into account when reporting mercury levels in urine, as mercury in fish is in the form of methylmercury, which contributes very little to mercury in urine.

The highest concentrations of mercury in air have been found in studies of mining operations, where the mercury urinary excretion may attain levels as high as 2.175 mg per 1000 ml. Donovan[34] reported levels of urinary mercury ranging from 30 to 700 µg per 1000 ml in a non-mercury-related mining operation.

Observations by Goldwater et al.[35] on workers exposed to mercury vapour suggest that some individuals may tolerate much higher levels of mercury than the average person, excreting over 2.0 mg of mercury per day without showing adverse symptoms. Piotrowski et al.[36] found that the concentration of metallothionein in rats is equivalent in mercury binding capacity to a level of mercury associated with the onset of damage to the kidney. Therefore, toxic effects will appear once the binding capacity of metallothionein has been exceeded. This would be a possible biochemical explanation for the development of tolerance to inorganic mercury, and also for the fact that urinary excretion of mercury may continue for years after cessation of exposure without any accompanying symptoms of toxicity[37].

Analyses for total, ionic and elemental mercury have been proposed for the monitoring of workers with urinary mercury excretion levels exceeding 250 µg per 1000 ml[38]. The risk of mercury poisoning increases as the rate of absorption of mercury exceeds the rate of oxidation and binding capacity of the body's detoxification mechanisms so that elemental mercury begins to appear in the urine.

On a group basis, blood mercury levels may correlate with urinary excretion, and the latter is roughly proportional to elemental vapour concentration in air[39].

According to the WHO study group[27] the concentrations of mercury in blood and urine can only be used to evaluate exposure to metallic mercury vapour and not to other forms of mercury.

2.7.1.3. Mercury in hair

Yoshida et al.[26] have found that the average total mercury concentration in hair for 24 unexposed people was 5.5 ppm (µg/g) in males and 3.3 ppm in females,

while the average total mercury content of hair in 24 male workers involved in the manufacture of thermometers was 10.3 ppm. These investigators did not find any significant correlation between total mercury in hair and inorganic mercury in blood, but did find a significant correlation between methylmercury levels in hair and blood.

Concentrations of mercury in hair seem to be linearly related to levels of mercury in red blood cells in individuals exposed to methylmercury compounds, the concentration in hair being roughly 300 times the blood concentration. In individuals with a low consumption of fish, values of less than a few milligrams per kilogram of hair are found. Heavy consumption of contaminated fish may result in values of 20-50 mg/kg[27].

The concentration of mercury along the length of the hair will not be uniform if the blood level is not steady. The most recent hair growth will reflect current blood levels of mercury. Analysis of different segments of hair may give a rough history of exposure to mercury, taking into account that hair grows at the rate of 1.0-1.5 cm per month.

The concentration of total mercury in indicator media and the equivalent long-term daily intake of mercury as methylmercury associated with the earliest effects in the most sensitive group of adult population have been reported as follows[40]: for an equivalent long-term daily intake of 3-7 μg/kg body weight, the blood mercury levels ranged from 20 to 50 μg per 100 ml and the hair levels between 50 and 125 μg/g.

The time-weighted average air concentrations associated with the earliest effects in the most sensitive adults following long-term exposure to elemental mercury vapour as well as the equivalent blood and urine concentrations have also been reported[39]. For 0.05 mg/m^3 in air, blood mean values were 3.5 μg per 100 ml and urinary excretion 150 μg per 1000 ml, and non-specific symptoms were shown. When the mercury in air reached values of 0.1-0.2 mg/m^3, blood levels were 7-14 μg per 100 ml and urine levels 300-600 μg per 1000 ml, and tremor appeared. These values reflect exposures for 1 year or more.

2.7.1.4. Mercury in other biological samples

Sweating constitutes a significant route of elimination for mercury[41], a fact that has not been fully appreciated and not well documented, as the concentration of mercury in sweat may be sufficiently high to be taken into account in the overall mercury balance in workers exposed to elemental mercury vapour.

As stated in the general part of this chapter, salivary fluid obtained from the parotid glands is a biological indicator for absorption of mercury, the concentration of mercury in saliva being correlated with the mercury blood levels. However, this test has not yet entered routine monitoring work[42]. In any case, the method of saliva collection, although simple, has to be well standardized[43,44]

2.7.2. Other secondary parameters

Kidney damage may be evidenced in mercury exposure by increased proteinuria. Joselow and Goldwater[45] reported that in a group of workers exposed to elemental mercury vapour the mean urinary protein excretion (90 mg per 1000 ml) was significantly higher than the mean protein excretion in an unexposed group (53 mg per 1000 ml), and the urinary protein correlated with urinary mercury levels. Kazantzis et al.[46] also reported increased urinary excretion of protein in workmen exposed to elemental mercury vapour. The kidney does not seem involved after exposure to methylmercury compounds[47]. From electrophoretic findings it has been inferred that the mercury renal lesion is of the mixed (tubular-glomerular) type[48], albumin accounting for up to 55% of the total protein excreted.

A serum disproteinaemia with a reduced A/G ratio is the usual picture in mercury exposure. A fall in γ-globulin content with associated rises in α- and β-globulin levels has been shown in most of the studies. Abdel-Kadar et al.[49] found that albumin and consequently the A/G ratio were significantly lower in exposed workers than in controls, while γ-globulin was significantly higher.

In a study carried out on a group of mercury miners[7], the most interesting findings concerned (1) the serum LDH isoenzymes patterns, which showed a shift towards a predominance of the cathodically migrating bands, with the appearance of sub-bands of unknown origin; (2) the urinary LDH isoenzymes patterns, showing a partial or total inhibition of bands; and (3) the membrane ouabaine-sensitive ATPase (Na^+-K^+/ATPase), showing strong inhibition in most of the samples.

Other enzymatic changes have been reported[49], such as decreases in the activity of SGOT and SGPT, and increases in the activity of serum and urinary α-esterases, serum cholinesterase and urinary ribonuclease, in mercury-exposed workers. A significant correlation was found between the enzyme activity and blood mercury levels.

A tendency towards an increase in transaminases has been shown by Cigula et al.[50], not only in mild exposure but also in the general population due to environmental contamination. The increase was attributed to the hepatotoxic action of mercury. A decrease in serum LDH activity is frequently found in exposure to elemental mercury[51]. Increasing enzyme levels of alkaline phosphatase pointing to liver damage are regularly found in protracted exposure to mercury[50].

Although many enzymatic changes have been shown by different investigators in mercury exposure, either in experimental animals or in humans, none of them has yet been found to be capable of being applied as a biological test of exposure.

2.8. INDICES FOR CADMIUM EXPOSURE

One of the main problems arising from cadmium absorption is the chronic tubular damage evidenced by a tubular proteinuria. The first observations indicated the excretion of low-molecular-weight (LMW) proteins. According to Smith et al.[1], the excretion of a LMW albumin characterized the effects of cadmium on workers exposed to the metal dusts and fumes.

The absorption of cadmium in man and experimental animals induces kidney damage with proteinuria characterized preponderantly by α_2-, β- and γ-globulin fractions and low albumin. A peak located in the β region was found to be due to the presence of a LMW protein[2]. The proteinuria was explained by postulating that cadmium is transported by metallothionein (a LMW Cd- and Zn-binding protein) to the proximal tubules, acting there upon enzymes which are essential for reabsorption functions, and thus bringing about an impairment in the reabsorption of serum proteins. According to studies by Friberg[3] and by Bonnell et al.[4], the proteinuria can appear long after cessation of exposure.

It has been shown[5,6] that cadmium urinary excretion in rabbits is low at the beginning and, after a certain period of time, a sudden increase in cadmium excretion occurs concomitantly with the appearance of tubular proteinuria. A similar phenomenon has been observed in mice[7], i.e., a sharp rise in cadmium urinary excretion at the time at which the detection of urinary proteins indicated a renal tubular impairment. These results are in agreement with observations made in humans, as reviewed by Friberg et al.[8]: exposed workers without proteinuria excrete small amounts of cadmium, while high excretions of up to 1.0 mg per 24 h are found in those individuals with proteinuria.

Until recently, it was very difficult to establish whether a correlation exists between urinary cadmium excretion and either liver or kidney accumulation of cadmium. It was suggested that a continuous accumulation occurs, the biological half-time of cadmium being extremely long. At the time at which tubular damage is evidenced by the proteinuria, urinary excretion of cadmium increases very markedly.

The modern technique of in vivo measurement of metals has allowed some light to be thrown on the liver and kidney accumulation of cadmium.

Roels et al.[9] reported a study carried out on a group of workers from two Belgian zinc-cadmium smelters, in which a number of parameters were measured, namely in vivo cadmium concentrations in liver and kidney, blood and urinary levels of cadmium, total proteinuria, albuminuria, β_2-microglobulinuria, etc. The results obtained showed that kidney disfunction is likely to develop in cadmium workers when the concentration of cadmium in the liver ranges between 30 and 60 ppm and is almost always observed in those with levels of cadmium in

the liver above 60 ppm. In the renal cortex, the critical concentration of cadmium ranges from 160 to 285 ppm; above 285 ppm there is a high probability (>95%) that all the subjects will show signs of renal dysfunction. Once cadmium-induced renal dysfunction has developed the results suggest a progressive decrease in renal cortex cadmium concentration, particularly when increased β_2-microglobulinuria is diagnosed.

From the study of the correlations between the parameters examined it can be concluded that the risk of developing cadmium-induced renal dysfunction appears to be very low when the critical cadmium urinary excretion of 10 µg/g of creatinine is not regularly exceeded, which corresponds to an average cadmium body burden of 160-170 mg.

The technique of in vivo measurement by neutron activation was first described by the Birmingham University Group[10] employing neutrons from a cyclotron, and then adapted to portable devices based on radioisotopic neutron sources[11].

Cadmium levels in blood probably reflect recent exposure, as the elimination of cadmium from blood seems to be relatively fast, the metal being bound mainly to proteins within the erythrocytes.

Some LMW proteins such as β_2-microglobulin (β_2-m), free light chains and retinol-binding protein (RBP) have been isolated from the urine of patients chronically exposed to cadmium and with evidence of tubular damage[12-14]. β_2-m has also been isolated from the urine of patients with Itai-itai disease[15].

In cadmium-exposed rabbits Piscator et al.[16] showed low β_2-m levels in serum with an increased urinary excretion, which would indicate a decrease in tubular reabsorption of filtered β_2-m.

In humans exposed to cadmium, Kjellström and Piscator[17] found a significantly increased urinary excretion of β_2-m whereas total protein was still within normal levels, the increased excretion of β_2-m not being related to serum levels.

Japanese investigators[18-20] have claimed that increased urinary excretion of β_2-m in cadmium-exposed people may be due to overproduction of β_2-m.

Interactions of cadmium with phospholipids such as phosphatidylethanolamine and phosphatidylserine have been reported[21], suggesting that they might provide a biochemical basis for the toxic effects of cadmium on mitochondria, kidney tubules and nerve membranes.

Cadmium may either activate or inhibit a number of enzymes both in vitro and in vivo. The activities of δ-aminolaevulinic acid dehydratase, pyruvate dehydrogenase and pyruvate decarboxylase are among others enhanced by cadmium, while δ-aminolaevulinic acid synthetase, alcohol dehydrogenase, arylsulphatase and lipoamide dehydrogenase are inhibited by cadmium.

The detection of the cadmium proteinuria is of diagnostic significance especially after cessation of exposure, in which case the metal is not excreted into

the urine, although a large amount may be accumulated in the kidneys. Even when there is no evidence that renal function continues to deteriorate, proteinuria persists.

On the other hand, the interpretation of the results is not easy. No good correlations seem to exist between the degree of exposure and biological levels of cadmium especially concerning urinary excretion. The usefulness of this parameter is the subject of controversy. It is generally accepted that a high cadmiuria is indicative of renal damage induced by the metal, and moderate cadmiuria in the absence of proteinuria is considered to be a sign of exposure.

The proteinuria, characterized by changes in the electrophoretic patterns, is considered to be the main biochemical finding indicating tubular damage.

In clinically healthy workers moderately exposed to cadmium for less than 20 years, Roels et al.[22] have shown glomerular-type proteinuria, while in workers exposed for more than 20 years, mixed-type proteinuria (glomerular + tubular) was shown. It appears that in the early stage of cadmium absorption the glomerular dysfunction precedes the tubulopathy, which is found only in workers highly exposed to cadmium and excreting more than 1 g of protein daily[23].

An experimental study carried out on rats[24] confirmed previous observations on man, and strengthened the hypothesis that the cadmium-induced proteinuria classically considered as a tubular-type proteinuria is in fact a mixed-type proteinuria, involving not only the tubule but also the glomerulus. Combining electrophoresis and gel filtration it has been possible to reveal an increased excretion of low- and high-molecular-weight proteins.

The following model for cadmium transport and accumulation in the kidney has been proposed[25]: exposure to cadmium induces synthesis of metallothionein in the liver and this LMW protein appears to be involved in the transport of cadmium in blood. Cadmium circulates in the blood bound to metallothionein in both erythrocytes and plasma. Metallothionein is cleared from the plasma and is taken up in the kidney. In the kidney there will be a continuous release of cadmium from catabolized metallothionein. Released cadmium will probably stimulate the synthesis of metallothionein. There is also the possibility that cadmium may bind to reabsorbed metallothionein, which in turn will be catabolized, this procedure being repeated time and again and preventing the secretion and excretion of cadmium. Cadmium in the kidney will thus have available binding sites all the time. The result is a continuous accumulation of cadmium in the kidney. When exposure to cadmium is excessive the renal cadmium will eventually reach a level, the critical concentration, where the binding capacity of renal metallothionein for cadmium is exceeded and tubular dysfunction will occur.

2.8.1. Interpretation of blood and urine cadmium levels

The reader is referred to Chapter 6 for the methodology of the determination of cadmium in blood and urine.

2.8.1.1. Cadmium in blood

According to Friberg et al.[8] the true average level of cadmium in whole blood among non-exposed persons is not known, but can be assumed to be around 0.1 µg per 100 g or less. Most of the studies carried out on normal subjects have used different variations of AAS for the determination of cadmium in blood levels. Vens and Lauwerys[26] reported average values of 0.95 µg per 100 g with a standard deviation of 1.0 µg per 100 g and Szadkowski[27] found mean values of 0.35 µg per 100 g of blood. Ediger and Coleman[28] reported the mean normal level to be 0.06 µg per 100 g. Friberg et al.[8] suggested that the average normal level of cadmium in blood should be considered to be well below 1.0 µg per 100 g, emphasizing the difficulties in the accurate determination of low concentrations of cadmium in biological materials. It is obvious that a high blood cadmium indicates high exposure. Blood cadmium is generally regarded as reflecting recent exposure rather than body burden, but a good correlation has sometimes been seen between cadmium in urine and cadmium in blood after long-term low-level exposure or after cessation of exposure[29]. A blood value above 1.0 µg per 100 g is suggested to be an index of significant cadmium exposure, although it is not always possible to evaluate the risk of renal damage on the basis of blood levels of cadmium.

2.8.1.2. Cadmium in urine

Large differences in normal urinary excretion of cadmium have been reported, but most investigators have given average values of 1.0-2.0 µg per 24 h. Suzuki and Taguchi[30] did not find any relationship between age and excretion of cadmium (others did find increases with age), obtaining a mean excretion of 2.4 µg per 1000 ml for men and 2.0 µg per 1000 ml for women. An average cadmium excretion of 0.39 µg per 24 h was found by Piscator[31] in Stockholm, while in a polluted area of Sweden the mean urinary excretion was found to be 2.1 µg per 1000 ml. Brouwers and Lauwerys[32] have reported a urinary cadmium excretion of less than 2.0 µg per 1000 ml in unexposed people. The levels were much higher in exposed workers, but no correlation with concentrations of cadmium in the air was found. Imbus et al.[33] reported a mean value of 1.59 µg per 1000 ml with ranges between less than 0.5 and 10.8 µg per 1000 ml in normal persons (spectrographic method). Szadkowski et al.[34] reported a mean value of 1.25 µg per gram of creatinine, with a range from 0 to 5.0 µg/g in normal subjects.

Cadmium excretion in exposed workers has been studied by a number of investigators[35-42], who reported cadmium excretions from near zero to around 1.0 mg per day. When proteinuria is present during exposure, there is always an increase in the excretion of cadmium. The same occurs in experimental animals. After exposure has ceased, cadmium excretion will decrease whereas proteinuria will persist. When evaluating urinary cadmium excretion in exposed workers it is not always clear whether tubular dysfunction is present. Piscator[31] reported that in cases with long exposure times (more than 5 years) but without signs of renal dysfunction, the urinary cadmium level was generally below 10.0 µg per gram of creatinine, whereas in workers with signs of renal dysfunction (changes in electrophoretic patterns and increases in β_2-m) the urinary excretion of cadmium was higher than 10.0 µg per gram of creatinine. In workers slightly exposed to cadmium for short periods of time at a plant manufacturing electrolytic zinc, Singerman[43] found a mean value for urinary excretion of cadmium of 4.0 µg per 1000 ml (7.4 µg per gram of creatinine) with a corresponding proteinuria of 68.4 mg per 1000 ml (106.6 mg per gram of creatinine) and no renal damage. The highest values for cadmium and proteins were found to be 7.8 µg per 1000 ml (17.3 µg per gram of creatinine) and 121.5 mg per 1000 ml (196.4 mg per gram of creatinine), respectively.

Cadmium accumulates in the kidneys, and during the period of accumulation only small amounts of cadmium are excreted in the urine. On a group basis, this excretion appears to be related to the body burden of cadmium. When a certain concentration is reached in the kidneys, cadmium excretion increases considerably and the accumulation rate of cadmium in the kidney decreases. The critical concentration for renal tubular damage has been estimated to be about 200 µg per g wet weight in the cortex[29].

2.3.1.3. Cadmium in other biological samples

Cadmium in hair. In spite of the difficulties in the analysis of metals in hair, the possibility of using hair as an indicator of exposure to cadmium has been investigated and almost all studies used AAS. Mean normal concentrations of cadmium in untreated hair as reported by Schroeder and Nason[44] were 2.77±4.37 µg/g in males and 1.77+1.64 µg/g in females. Studies by Nishiyama[45] on hair pretreated with detergent showed the following mean values for cadmium: in Yugoslavia (rural area), 0.54+0.27 µg/g; and in Sweden, 0.44±0.14 µg/g for males and 0.87+0.26 µg/g for females.

For exposed workers hair analysis is still considered to be of little value, as external contamination is great and, as shown by Nishiyama and Nordberg[46], it is virtually impossible to remove by washing procedures.

Cadmium in saliva. Cadmium may also be excreted in saliva. Concentrations of up to 0.1 µg/g have been reported by Dreizen et al.[47], but this test has not yet found application in biological monitoring of workers.

In general terms it can be stated that, for long-term low-level exposure, a urinary cadmium excretion above 10 µg per 1000 ml or 10 µg per gram of creatinine may indicate either impending or actual renal tubular impairment, and a blood level above 1.0 µg per 100 g indicates significant cadmium exposure.

It is important to point out that smoking habits may considerably influence cadmium accumulation, and are responsible for significant differences in blood cadmium levels and urinary cadmium excretions. Cadmium in blood levels of 0.84±0.26 µg per 100 ml in smokers and 0.62±0.28 µg per 100 ml in non-smokers have been reported[48]. Kjellström[49] found cadmium excretions of 0.36-0.56 µg per gram of creatinine for smokers and 0.13-0.43 µg per gram of creatinine for non-smokers. As cigarette smoke is considered to be the major source of cadmium exposure to man[50], it has to be taken into account when evaluating laboratory results.

2.8.2. Proteinuria

Cadmium proteinuria has been characterized by means of gel filtration, ion-exchange chromatography and immunological methods[29]. A large number of LMW proteins were present in the urine of cadmium-exposed workers[23,51], including β_2-m, ribonuclease, muramidase and immunoglobulin chains. Large amounts of retinol-binding protein (RPB) were also found by Peterson and Berggård[14].

This increase in the urinary excretion of LMW proteins is regarded as the first sign of cadmium-induced renal dysfunction. The quantitative determination of the LMW proteins, and particularly β_2-m, increases the diagnostic sensitivity. It has been shown that increased amounts of β_2-m may be excreted without any increase in total protein excretion or any major changes in the electrophoretic pattern of urinary proteins[17]. However, it is important to point out that in a health survey carried out in areas environmentally contaminated by cadmium in Japan[52], it was found that the increase in urinary excretion of LMW proteins is not specifically induced by cadmium or other toxic metals but may be related to other factors such as ageing.

Piscator[53] developed a method for the determination of total urinary protein, based on the biuret reaction after precipitation of protein with Tsuchiya's reagent (phosphotungstic acid in hydrochloric acid-ethanol solution). The urine sample (1-2 ml) is mixed with Tsuchiya's reagent, the precipitate is centrifuged, washed and dissolved in sodium hydroxide solution and, after addition of Benedict's reagent (copper sulphate in sodium citrate and sodium carbonate solution), the absorbance is read at 330 nm. Human albumin is used as a standard.

Studies carried out on cadmium-exposed workers using this method[54] showed that the urinary protein excretion was related to exposure time (mean values of 100 mg of urinary protein per 24 h for a 5-year exposure time to 955 mg per 24 h for more than a 30-year exposure time). The daily excretion of protein for unexposed people ranged from 25 to 80 mg, with an average of 50 mg. The cadmium-exposed individuals excreted from 70 to 2600 mg per 24 h.

For the electrophoretic examination of urinary proteins, Piscator[54] has used paper electrophoresis since 1959. In most instances this method allowed the differentiation of a characteristic tubular pattern from glomerular and normal patterns. In later studies, cellulose acetate electrophoresis[55], electrophoretic multifractionation on a special support of cellulose acetate known as RS Cellogel[43], disc electrophoresis[56] and isoelectric focusing[57] have been used to diagnose cadmium-induced proteinuria. In each of the above techniques, the increase in excretion of LMW protein is shown by peaks in certain ranges of electrophoretic mobility. Isoelectric focusing in thin-layer polyacrylamide gel[57] allows the quantitative determination of β_2-m. Other electrophoretic techniques such as electrophoresis in agarose and starch gel have been studied by Piscator and Pettersson[29], but they preferred paper electrophoresis.

In cadmium-exposed workers a typical tubular pattern is shown, characterized by an albumin content of less than 25%, large α_2 and γ fractions and a distinct peak in the β_1-β_2 region. The β-fraction appears as the largest of the urinary protein fractions. These results have been reported for workers with daily excretions of protein above 150 mg. Normal urinary proteins regularly show a distinct albumin fraction and an otherwise diffuse pattern.

The high increase in β_2-m excretion indicates that the quantitative analysis of β_2-m may be the most sensitive method for detecting tubular damage induced by cadmium at an early stage. In healthy subjects with normal renal function β_2-m is constantly produced, being eliminated almost exclusively by the kidneys, predominantly by glomerular filtration. After glomerular filtration more than 99.9% of excreted protein is reabsorbed in the kidney tubules, where it is catabolized. An increased excretion of β_2-m in the urine is therefore a sensitive indicator of proximal tubular disfunction.

The development of a radioimmunoassay[58] has made possible the measurement of β_2-m concentrations in normal urine samples. The sensitivity of the original method goes down to below 2 µg per 1000 ml, the coefficient of variation is 8.2% and the recoveries are about 100%. The normal average urinary excretion is about 100 µg per 24 h[59].

The method is performed using a standard kit containing labelled β_2-m standard, tracer and antiserum. The range of the standard curve normally used is 2-96 µg per 1000 ml. Only 0.1 ml of urine is needed for one analysis. During storage of

urine specimens β_2-m may degrade if the pH is lower than 5.6[59]. Either specific gravity or creatinine concentration in urine may be used for adjusting the β_2-m concentrations, unless 24-h samples are collected.

Only recently has β_2-m analysis been used on a large scale. The average β_2-m excretion among healthy adults reported by Evrin and Wibell[59] ranged from 100 to 125 µg per 24 h. These values are very close to those obtained by other investigators with the same procedure[17]. In subjects with cadmium in blood levels above 2.0 µg per 100 ml and a cadmium urinary excretion of more than 8.0 µg per gram of creatinine, Piscator[60] reported β_2-m urinary excretions of 142±126 µg per gram of creatinine. Kjellström[49] found increases in β_2-m urinary excretion up to 700 µg per gram of creatinine for exposed people in Japan.

The best way to obtain an accurate estimate of renal status would be to combine the determination of total protein excretion, the electrophoretic separation of different fractions and the determination of β_2-m excretion.

According to Kjellström and Piscator[17], urinary β_2-m should not be used for biological monitoring of cadmium-exposed workers, as available data indicate that tubular damage is irreversible, but it is a useful tool for the screening of tubular proteinuria.

Bernard et al.[61] developed a method for the determination of β_2-m in human urine (suitable also for the measurement of RBP in urine) that does not require the use of radioisotopes. If offers the same precision, specificity and sensitivity as the radioimmunoassay methods (RIA), being simpler and faster.

It is based on direct agglutination by β_2-m of latex particles on which an antibody against β_2-m is adsorbed (latex immunoassay). The agglutination is quantified by counting the remaining unagglutinated particles or by turbidimetry. The detection limit is 0.5 µg per 1000 ml and the analytical recovery of β_2-m in urine is around 97%.

However, as significant degradation of β_2-m occurs when the urinary pH is lower than 5.5 (acid urine samples), Bernard and Lauwerys[62] studied the possibility of obviating this difficulty by measuring the urinary excretion of RBP, which is a more stable LMW protein in acid urine samples, as an index of tubular dysfunction in cadmium exposed workers. They found that the RBP test in urine is as sensitive as the β_2-m test for screening purposes. The values found in a group of 150 healthy subjects were 376 and 139 µg per gram of creatinine for β_2-m and RBP, respectively (expressed as the geometric mean plus 2 geometric standard deviations).

Buchet et al.[63] adopted the following upper normal values for some urinary parameters for considering the renal function of a worker as normal: total proteinuria, <250; β_2-microglobulinuria, <0.2; and albuminuria, <12 mg per gram of creatinine.

2.9. INDICES FOR CHROMIUM EXPOSURE

Chromium occurs in most biological materials in the trivalent from (Cr^{3+}), strongly associated with proteins, nucleic acids (very high concentrations of chromium are found in nucleoprotein fractions) and a variety of low-molecular-weight ligands. The hexavalent form (Cr^{6+}) is more toxic than the trivalent form because of its oxidizing potential and its easy permeation of biological membranes (Cr^{6+} is able to penetrate the biological membranes, while Cr^{3+} is not).

Chromium is considered to be an essential trace element in man and in animals and plays an important role in insulin metabolism as the glucose tolerance factor (GTF). Its deficiency results in impaired glucose metabolism owing to the poor effectiveness of insulin. Impairment of glucose tolerance is the first symptom of chromium deficiency in experimental animals and it is possible that chromium deficiency is one of the causes of glucose intolerance in man[1]. The experimental results suggest that the impaired glucose tolerance in chromium deficiency may be due to a decreased response of the deficient animal to its endogenous insulin. A diabetes-like syndrome has been observed in chromium-deficient rats raised in a controlled environment.

In the rat, the chromium absorbed by the intestines is almost entirely bound to transferrin (the iron-carrying protein), and chromium in excessive doses is also bound to other protein fractions. In man, the bulk of an administered dose of radioactive chromium chloride is carried in the albumin fraction, and only 30-40% in globulins, of which transferrin is a part. Chromium disappears rapidly from the blood and is taken up by other tissues, where it is concentrated much more (by a factor of 10-100) than in the blood.

The metabolism of chromium depends heavily on its chemical form. Animal experiments suggest that one or more specific organic chromium complexes designated "glucose tolerance factor" are handled by an organism in such a way as to meet the criteria for an essential element. GTF is the specific chromium complex that seems to be physiologically active. The liver seems to be the site where chromium binding to GTF is regulated, as well as the site where vanadium is incorporated into transferrin and manganese is transformed into transmanganine-bound metal[2]. Although GTF has not yet been completely identified it is probably a dinicotinate-chromium complex.

Excessive chromium (either Cr^{6+} or Cr^{3+}) has been found to inhibit the activity of several enzymes, such as urease[3] and β-glucuronidase[4]. Glutathione reductase is inhibited within the red cell by Cr^{6+} [5].

Chromium is contained in nucleic acids in high concentrations; highly purified RNA fractions contain 50-140 μg/g[6,7]. The exact function of chromium in RNA is unknown. It is assumed that chromium is linked to the components of nucleic acid by coordination, but it is not known whether the bond involves Cr(0) or Cr(III)[6].

2.9.1. Interpretation of blood and urine chromium levels

The reader is referred to Chapter 9 for the methodology of the determination of chromium in blood and urine.

2.9.1.1. Chromium in blood

The concentration of chromium in blood has been reported to be 2.0-3.0 µg per 100 ml with an even distribution between red cells and plasma[8]. According to Baetjer et al.[9], the increase in blood values upon occupational exposure relates mainly to the erythrocytes.

The values obtained by Imbus et al.[10] for chromium in the blood of unexposed subjects (spectrophotometric method) were as follows: median = 2.65 µg per 100 g; mean = 2.76 µg per 100 g; range = 1.28-5.54 µg per 100 g. Versieck et al.[11] determined chromium in the serum of healthy individuals by neutron activation analysis. The mean value obtained was 0.016±0.0083 µg per 100 ml. Other values reported for chromium in serum ranged from 0.073 µg per 100 ml[12] up to 15.0 µg per 100 ml[13].

For chromate workers, the values found in the literature are as follows: 4.0-6.0 µg per 100 ml of whole blood[14]; 0-2.0 µg per 100 ml of plasma[9]; 3.0-14.0 µg per 100 ml of red cells[9].

2.9.1.2. Chromium in urine

Chromium is excreted in both urine and faeces, urine being the more important pathway. Nearly all chromium in urine is present in the form of low-molecular-weight complexes. Blood chromium coordinated to small-molecule ligands is filtered at the glomerulus, and up to 63% is reabsorbed from the filtrate in the tubules[15].

There is little agreement on the amount of daily urinary chromium excretion. Values as high as 860 µg per 1000 ml have been reported[16]. In a carefully controlled study carried out by Imbus et al.[10], a median value of 3.77 µg per 1000 ml and a mean value of 4.0 µg per 1000 ml with ranges from 1.8 to 11.0 µg per 1000 ml have been found. Pierce and Cholak[17] reported average excretion of 5.0 µg per 1000 ml for unexposed subjects. The lowest values are probably the most reliable and can be accepted with confidence. It is estimated that the normal urinary excretion of chromium goes up to about 10.0 µg per 24 h[18,19].

The excretion of chromium in chromium plating workers has been measured by Borghetti et al.[20]. At the end of every working day an increase in the urinary excretion of chromium was observed compared with the values at the beginning of work. The rapid urinary excretion of chromium allows the chromium to creatinine ratio in urine to be used as a daily exposure test. The highest values for chro-

mium urinary excretion were found in polishers: 37.2±27.4 µg per gram of creatinine (AAS with carbon rod).

According to Tola et al.[21], both water-soluble (Cr^{6+}) and water-insoluble (Cr^{3+}) chromium concentrations instead of the total chromium air concentrations should be measured, as water-soluble chromium is the more important from the biological standpoint.

The urinary excretion of chromium has shown to be a good indicator of short-term exposure to water-soluble chromium (Cr^{6+}) when the exposure level is above 50 µg/m^3, which represents the TWA (time-weighted average value) recommended by NIOSH (US National Institute for Occupational Safety and Health), expressed as chromium trioxide (CrO_3).

Excretions at levels above 30 µg per gram of creatinine represent levels of Cr^{6+} in air higher than the TWA. This applies only to short-term exposure.

When considering long-term exposure the carcinogenic risk has to be taken into account.

2.9.1.3. Chromium in other biological samples

Chromium in hair. Chromium is found in high concentrations in hair; values from 0.2 to 2.0 µg/g have been reported[22]. Hambidge[23] found 0.974 µg/g in the hair of newborns and 0.382 µg/g in maternal hair. High concentrations of chromium in the hair of newborns are the usual finding, with a gradual decline during childhood.

Creason et al.[24] reported values from 0.076 to 4.80 µg/g for chromium in the hair of children (aged 0-15 years), with a geometric mean of 0.56 µg/g, and from 0.06 to 5.30 µg/g for adults (over 16 years old), with a geometric mean of 0.62 µg/g.

Despite the paucity of human data on chromium levels in the hair of exposed workers, it is evident that significant increases may be of value in biological monitoring. On the other hand, blood and urine contents have indicated substantial increases in chromium exposure, and their values remain appreciably high for years after exposure. Although some enzymatic changes have been observed in chromium exposure, no defined biochemical lesion has yet been disclosed.

Cigarettes have been reported to contain 390 µg/kg of chromium[16] but no estimates of the inhaled amount from smoking are known. However, smoking habits have to be taken into account in the appraisal of exposure.

2.10 INDICES FOR ARSENIC EXPOSURE

Although arsenic has not been confirmed as an essential element, there is evidence that it is required for the growth of some species of animals.

It has been suggested that both the uptake and the toxic action of some metals may be determined by their chemical form[1]. It is also possible that particular forms of the metals may be produced within the alimentary tract under certain circumstances, thus modifying the toxic action. This applies well to arsenic, which is present in the environment mainly in inorganic forms while seafoods contain relatively higher levels in organic forms[2] and methylated species of arsenic have also been measured in fresh and salt water[3]. Organic forms of arsenic have shorter retention times, about half those of inorganic forms[2].

Since the original demonstration by Braman and Foreback[3] in 1973 of the occurrence of monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA) in human urine, several investigations have revealed that all mammalian species studied are capable of converting inorganic arsenic into these methylated forms[4-6]. It is assumed that at least 50% of ingested arsenic undergoes biomethylation, although the mechanism is not known, and inorganic arsenic in the environment may be methylated by microorganisms.

As the methylated forms of arsenic are much less toxic than their inorganic parent compounds, biomethylation would constitute an efficient mode of detoxification. This is the reason for the increased interest in studying methods, for the determination of methylated forms of arsenic in biological materials[7] and in drinking water samples[8], in which arsenic is present in both inorganic and organic forms.

Arsenic seems to concentrate in the skin, hair and nails because of its high affinity for sulphydryl (SH) groups. Vallee et al.[9] noted that even with low doses of arsenic, small amounts may be detected in tissues of ectodermal origin such as the hair and nails many months after it has disappeared from the urine and faeces.

It has been shown that inorganic arsenic crosses the blood-brain barrier in some anthropoids[10] but not in man. However, arsenic is transferred across the placenta in humans[11] and in rats[12].

It has been shown that enzymes inhibited by arsenicals are those requiring the dithiol cofactor dihydrolipoic acid, which is essential for the α-keto acid oxidases such as pyruvate oxidase. Arsenicals are known to block the entry of pyruvate into the tricarboxylic acid cycle. The oxidative decarboxylation of pyruvate is affected by trivalent arsenicals, which are inhibitors of the pyruvate dehydrogenase (probably combining with the dithiol lipoate moiety of the enzyme), thus leading to the accumulation of pyruvate. Trivalent arsenicals are also inhibitors of succinoxidase, probably due to their affinity for the thiol groups of the enzyme.

It is also known that arsenite may uncouple oxidative phosphorylation. In this case unstable arsenylated oxidation product undergoes irreversible hydrolysis and oxidation proceeds at an increased rate, but without the formation of the high-energy phosphate bond[13,14].

In vitro and in vivo studies have shown that As^{5+} exerts inhibition effects similar to those of As^{3+} on mitochondrial respiration and uncoupling of oxidative phosphorylation[15].

According to Vallee et al.[9] the interpretation of the in vivo action of arsenic compounds is difficult owing to the metabolic interconversions of various arsenicals. The pentavalent compounds (physiologically inactive in this form) are able to penetrate all parts of the body, including the central nervous system. They are excreted largely unchanged, but small amounts of them can be reduced in most tissues to the trivalent arsenoxides, in this way reaching otherwise inaccessible cells.

In radioactive tracer studies on arsenic in rats, 95-99% of the arsenic in the whole blood was detected in red cells, whereas in humans, monkeys and rabbits, arsenic was more uniformely distributed between cells and plasma proteins. This distribution was partly dependent on the form of arsenic (As^{3+} or As^{5+})[10,16]. The major carriers of arsenic in the plasma of mice have shown to be the α_1-globulins.

As^{3+} toxicity can be reversed by thiol compounds such as glutathione and cysteine in their reduced form[9].

Two modes of inhibition have been proposed for the inhibition by arsenic: (a) competition with phosphate during oxidative phosphorylation and (b) inhibition of energy-linked reduction of NAD[17].

2.10.1. Interpretation of blood, urine, hair and nail arsenic levels

The reader is referred to Chapter 7 for the methodology of the determination of arsenic in blood, urine, hair and nails.

2.10.1.1. Arsenic in blood

Very different figures have been reported by various workers. Liebscher and Smith[18] found an average concentration of arsenic in whole blood of 14.7 µg per 100 ml, with a range of 0.1-92.0 µg per 100 ml. Vallee et al.[9] found a range of 0.0-37.0 µg per 100 ml, while Heydorn[19] obtained mean arsenic concentrations in whole blood of 2.2 µg per 100 ml in Taiwanese subjects by neutron activation analysis.

Iwataki and Horiuchi[20], using a modified polarographic method, found that levels of arsenic in whole blood for 95% of a healthy Japanese urban population were less than 22.0 µg per 100 g and for 99% of the population the values were approximately 37.0 µg per 100 g.

Neuman and Singerman[21] obtained mean levels of 1.1±1.0 µg per 100 ml with ranges between 0 and 4.0 µg per 100 ml using the silver diethyldithiocarbamate method.

Damsgaard et al.[22] found a mean concentration of 1.1 µg per 100 ml in eleven normal human sera using neutron activation analysis. In the endemic area of Taiwan, with high levels of arsenic in drinking water, mean levels of 6.0 µg per 100 ml of whole blood were reported by Heydorn[19]. Whanger et al.[23] reported mean values of 12.2 and 25.1 µg per 100 ml of whole blood for residents in two different cities in Oregon, U.S.A., with arsenic-rich water.

Yamamura and Yamauchi[24], using AAS, found an average whole blood arsenic level of 3.3 µg per 100 g with a range of 1.2-5.5 µg per 100 g among 33 workers exposed to 90 $\mu g/m^3$ of arsenic, while a control group showed an average value of 0.7 µg per 100 g of whole blood with a range between 0.1 and 1.6 µg per 100 g.

2.10.1.2. Arsenic in urine

Most of the arsenic absorbed is excreted via the urine. The data found in the literature are variable for unexposed individuals, probably owing to the influence of the diet. Crecelius[4] reported that organic arsenic present in marine organisms is probably not metabolized in the human body but excreted via the kidneys in the same form as ingested, and high levels of urinary arsenic have been shown after shellfish ingestion.

Smith et al.[25] reported the concentrations of urinary arsenic (inorganic arsenic, MMAA, DMAA and total arsenic) found in a group of 41 male workers not exposed to arsenic as well as in a group of 83 smelter workers occupationally exposed to arsenic, mainly as arsenic trioxide as follows (µg per 1000 ml):

Total arsenic	As^{3+}	As^{5+}	MMAA	DMAA
(a) Mean urinary excretion of arsenic in the control group:				
21.2±2.04	1.3±1.58	1.3±1.59	3.4±1.63	11.5±1.47
(b) Low-exposed group:				
24.7±2.01	2.2±2.19	1.6±2.32	4.9±2.13	17.0±1.96
(c) Median-exposed group:				
51.8±1.61	4.8±2.08	2.4±2.86	9.7±1.90	32.7±1.71
(d) High-exposed group:				
66.1±2.14	8.6±2.62	3.1±3.64	20.8±2.55	64.1±2.42

As can be seen, dimethylarsinic acid (DMAA) was the major form of arsenic present in the urine of the smelter workers.

Yamamura and Yamauchi[26] reported the normal background levels of urinary arsenic for the different arsenic species as follows, for an unexposed group (n = 16) with 2 days restriction of ingestion of fish and shellfish prior to the

sampling: total arsenic, 2.15±0.70; As^{3+}, 0.24±0.07; As^{5+}, 0.08±0.07; MMAA, 0.62±0.18; and DMAA, 1.21±0.41 μg per 1000 ml.

According to Buchet et al.[27], "the determination of inorganic arsenic (As_i), monomethylarsenic (MMA) and dimethylarsinic (DMA) acids in urine appears to be the method of choice for the biological monitoring of workers exposed to inorganic arsenic, since these measurements are not influenced by the presence of organoarsenicals of marine origin". From the linear relationship found in their study between arsenic administered and arsenic excreted in urine, it was estimated that a time-weighted average (TWA) exposure of 50 $\mu g/m^3$ of arsenic would lead to an average urinary excretion of 220 μg of arsenic (As_i + MMAA + DMAA) per gram of creatinine.

Neuman and Singerman[21] found mean values of 4.5±3.9 μg per 1000 ml (5.5±1.1 μg per 24 h) with a range between 0 and 15.0 μg per 1000 ml (0-27.0 μg per 24 h) in an unexposed population, while in cases of hydroarsenicism the urinary excretion of arsenic attained values up to 1982 μg per 24 h.

It is interesting that in the older literature the values for urinary excretion of arsenic are significantly higher, which may perhaps be attributed to the analytical methodology and partly to the influence of dietary sources.

Pinto and McGill[28] found an average urinary arsenic level of 130 μg per 1000 ml and a median of 100 μg per 1000 ml in a group of unexposed workers, while in workers exposed from the same plant the average and median values were 820 and 580 μg per 1000 ml, respectively. Schrenk and Schreibeis[29] reported an average of 80 μg per 1000 ml with a range from 20 to 2000 μg per 1000 ml for unexposed people. Vallee et al.[9] found levels of 3.0-150.0 μg per 1000 ml (12.0-260 μg per 24 h) in unexposed people, while Iwataki and Horiuchi[20] reported 138 μg per 1000 ml (12-927 μg per 24 h) in a Japanese population sample.

2.10.1.3. Arsenic in hair

Arsenic is usually found in higher concentrations in hair and nails than in other parts of the body, which can be attributed to the high content of keratin in these tissues (the SH groups would be responsible for the binding of As^{3+}).

According to Smith[30], the average concentration of arsenic in hair of unexposed people was 81.0 μg per 100 g with a median of 51.0 μg per 100 g. For the 80% of the population examined the values were below 100 μg per 100 g, while concentrations above 300 μg per 100 g were considered as suspect of exposure.

Liebscher and Smith[18] reported concentrations of 46.0 μg per 100 g in Scotland and Kadowaki[31] reported 17.4 μg per 100 g in Japan, for unexposed subjects.

Values of up to 1500 μg per 100 g have been found in isolated cases of hydroarsenicism by Neuman and Singerman[21]. Terada et al.[32] determined hair arsenic concentrations in 39 Japanese subjects with hydroarsenicism (the well drinking

water contained 1-3 mg per 1000 ml of arsenic); 54% of the values were above 500 µg per 100 g and the maximum concentration was 8500 µg per 100 g of hair.

It has been stated that the content of arsenic in hair of exposed people (occupationally or not) can reach several milligrams per 100 g[33], and in a survey carried out on mine mill workers in Canada[34] 33% of the workers had over 1.0 mg per 100 g of arsenic in hair.

It is evident that the arsenic levels in hair are a relevant indication of exposure, provided that external contamination can be excluded.

2.10.1.4. Arsenic in nails

Values of 28.3 µg per 100 g have been reported in Scotland[18] and 89.2 µg per 100 g in Japan[31] for unexposed people, while values of up to 368.0 µg per 100 g were found in cases of hydroarsenicism[21].

2.11. INDICES FOR SELENIUM EXPOSURE

Selenium has been viewed for many years as a toxic substance. However, it is now considered to be a critical nutrient, deficiency of which may underlie a number of diseases. Selenium is said to be an essential trace element, with a 100-fold difference between the essential and toxic levels. According to Scott[1], the nutritional requirement for selenium lies between 0.1 and 0.3 mg/kg, whereas levels from 2 to 10 mg/kg may give rise to toxic symptoms.

Although it has long been known that animals need selenium for growth and reproduction, the U.S. National Research Council (NRC) did not establish a recommended dietary allowance for humans until 1978[2]. Several of the early studies reported that selenium may be a carcinogen[3] but, as reviewed by Shapiro[4], a number of workers have concluded that the assumption that selenium is carcinogenic is based on incomplete knowledge. According to the U.S. National Institute for Occupational Safety and Health (NIOSH)[5], "selenium has been mentioned for its carcinogenic, anticarcinogenic and teratogenic effects, but to date, these effects have not been seen in man".

In recent years selenium has been studied for its anticancer value. Schrauzer et al.[6] have reported that selenium possess antimutagenic properties and protects chromosomes against carcinogen-induced damage. However, this still remains a controversial research field.

The capacity of animals to reduce inorganic selenicals from selenium valences of +4 or +6 to the active selenide (Se^{2-}) places this element in a unique category. The affinity of Se^{2-}-dependent enzymes and proteins for toxic metal ions such as cadmium, mercury and arsenic appears to account for its antidotal value against excessive amounts of those toxic metals[7]. This property, previously assigned to SH compounds, may partly explain the anticancer value of high nutrient levels of selenium.

Selenium appears to play an invaluable role as a metabolic antidote to toxic metals such as arsenic, cadmium, mercury, methylmercury, copper and lead[8-13], and it should also be pointed out that compounds of some of these metals have been reported to detoxify or counteract toxic levels of selenium. The mechanisms involved in these reciprocal detoxifications are not fully understood.

The fact that man possess at least one important selenium-dependent enzyme, glutathione peroxidase (GSH-Px), seems to be in favour of the need for selenium as an essential trace element[14].

Selenium is probably the most toxic, weight for weight, of the essential nutrients. However, very few instances of human selenium intoxication have been recorded. The toxicity of selenium is thought to derive from selenite accumulation and its strong oxidative effects[15]. Selenium toxicity may be prevented by various SH compounds, tocopherol and some arsenic, mercury and copper compounds. On the other hand, the greater affinity of selenols compared with sulphydryl compounds for mercury and methylmercury may account for the known protective effect of selenium against mercury toxicity[16].

According to Ganther[17], inorganic salts of selenium are rapidly reduced in animal tissues to selenotrisulphides, selenopersulphides, hydrogen selenide, di- and trimethyl selenide and probably other low-molecular-weight metabolites and protein derivatives. The pathway of reduction of selenium seems to be mediated by glutathione (GSH) in the presence of glutathione-reductase (GSH-Red):

$$H_2SeO_3 \xrightarrow{GSH} GSSeSG$$
(selenotrisulphide derivative of GSH)

$$GSSeSG \xrightarrow[NADPH]{GSH\text{-}Red} GSSeH$$
(GSH-selenopersulphide)

$$GSSeH \xrightarrow[NADPH]{GSH\text{-}Red} H_2Se$$
(hydrogen selenide)

It is well known that erythrocyte plasma membranes are labile to lipid peroxidation (LPO) owing to their high polyunsaturated fatty acid content and their exposure to molecular oxygen. Peroxidation of erythrocyte plasma membrane lipids leads to haemolysis of the cells.

Reduced glutathione (GSH) is considered essential for the protection of haemoglobin and the erythrocyte membrane, and such protection requires not only GSH, but also the activity of enzymes such as GSH-Red and GSH-Px, as well as the production of NADPH.

The GSH-Px catalytic function of glutathione transferases (GSH-Trs) has been implicated by a number of authors in the protection of cellular membranes from damage due to LPO[18,19]. Until a few years ago, GSH-Px activity was thought to be due to a single enzyme (E.C. 1.11.1.9) containing selenium in a prosthetic

group[20]. This enzyme utilizes hydrogen peroxide as well as a variety of organic and lipid hydroperoxides as substrates, but is highly specific for GSH as the hydrogen donor[21]. A selenium-independent peroxidase, which has been named glutathione peroxidase II (GSH-Px II), has more recently been described[22], the activity of which does not decrease in selenium deficiency, and is directed only toward organic hydroperoxides.

It is generally thought that in the erythrocyte, GSH is continually oxidized by the action of GSH-Px on peroxide normally formed by the action of superoxide dismutase (SOD) on superoxide produced from oxyhaemoglobin.

As is known, maintenance of the normally high level of GSH in erythrocytes depends on the activity of GSH-Red, which utilizes NADPH for the reduction of GSSG to GSH, NADP being reduced to NADPH by the action of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6-PGD).

The activity of GSH-PX is dependent of the level of selenium in the diet. As GSH-Px can destroy lipid hydroperoxides, a lack of it could result in accumulation of those hydroperoxides which in turn have been shown to degrade membrane-bound cytochrome P-450[23], the terminal acceptor in the NADPH-dependent mixed-function oxidase system (MFOS) involved in the activation and detoxification of numerous xenobiotics.

Inhalation of selenium compounds at levels normally found in urban atmospheres does not seem to contribute significantly to body burdens of the metal. The major contribution to organ concentrations is probably provided by ingestion of selenium[24]. The work environment may be an additional source of exposure to selenium compounds, which can be either inhaled or deposited both on the skin and the clothes.

By means of the time-weighted average value (TWA) established by the American Conference of Governmental Industrial Hygienists (ACGIH) of 0.2 mg/m^3 of selenium in a simulation model, Medinsky et al.[24] calculated that the equilibrium concentrations in the tissues of workers could be three to four times higher than those resulting from ingestion, and concluded that this concentration of selenium in the working atmosphere could represent a hazard for some workers who may develop symptoms of long-term poisoning.

It has been shown in rats that the major excretory metabolite in urine is the trimethylselenonium ion[25], although inorganic selenium compounds have also been detected in urine after injection of radiolabeled selenite[26].

In man, the major excretory products of selenium metabolism are the same urinary metabolite, the trimethylselenonium ion, two other urinary organic metabolites not yet identified[27] and a volatile metabolite, dimethyl selenide. The presence of the volatile compound in expired air is significant only when the dose of selenium is close to levels that produce acute toxicity. Therefore,

exhalation of selenium (garlic odour of the breath considered by some workers to be the earliest and most characteristic sign of selenium absorption) is not taken into account in long-term exposure to low doses.

The measurement of GSH-Px activity in blood is often used as a means of detecting selenium deficiency because of the correlation between GSH-Px levels and the selenium nutritional status of both animals and humans. Similarly, the level of GSH-Px activity in blood could be an interesting parameter to be used for the evaluation of exposure to selenium, although it is not yet used in routine practice.

2.11.1. Interpretation of blood, urine and hair selenium levels

The reader is referred to Chapter 11 for the methodology of the determination of selenium in blood and urine.

2.11.1.1. Selenium in blood, urine and hair

Lane et al.[28] estimated the plasma and erythrocyte mean levels of selenium in a group of University-associated males and females to be plasma, 0.10 µg/g; erythrocytes, 0.73 µg/g of haemoglobin, 0.65 µg/g of protein.

Mean concentrations of selenium in the whole blood of individuals in 19 different locations within the U.S.A. as determined by Shamberger et al.[29], ranged between 15 and 25.6 µg per 100 ml. According to Allaway et al.[30], the mean selenium level in 19 U.S. cities was 20.6 µg per 100 g of whole blood, while in New Zealand Robinson and Stewart[31] found a mean level of 7.0 µg per 100 g of blood. No values for levels of selenium in the blood of exposed people have been found in the literature.

The urinary excretion of selenium, as estimated by Geahchan and Chambon[27] in France, in a group of 92 subjects non-occupationally exposed ranged from 2.6 to 47.0 µg per 1000 ml, with an average concentration of 12.3±8.21 µg per 1000 ml. Urinary selenium excretion has been used to indicate exposure in the environment and also occupational exposure. The values vary with the selenium content of the diet and geographical location. De Bruin[32] stated that the "reliability of urinary selenium excretion as index of total exposure is widely agreed upon". Values exceeding 100 µg per 1000 ml would indicate an abnormal degree of absorption, while urinary excretions above 500 µg per 1000 ml may indicate the development of toxic symptoms.

In fact, the information available on selenium exposed people is scarce. Hojo[33] studied the urinary selenium excretion in various groups of people. Factory workers exposed to toxic metals such as manganese, chromium, cadmium and mercury were found to excrete significantly larger amounts of selenium than the control

group. This was probably due to the fact that selenium is present in sulphide minerals, and may also be because the intake of other metals increases the excretion of selenium as metal selenides. According to the U.S. National Research Council, the selenium content of sulphide ores of mercury and zinc may be over 10%[34]. The values recorded by Hojo were as follows (means±S.D.):

Group	No. of subjects	µg Se per 1000 ml urine	µg Se per g creatinine
Control	21	57.9±26.3	59.9±24.5
Mn exposed	22	68.9±48.9	91.5±67.6
Cr exposed	14	106.9±45.4	146.3±85.7
Cd exposed	5	166.4±56.6	197.8±97.9
Hg exposed	1	288	172

The only data found in the literature about the selenium content of hair are the values obtained by Capel et al.[35] for a group of control children by AAS: mean level = 0.6 µg/g, with a range of 0.4-0.9 µg/g. No data were given for adults, either exposed or unexposed.

2.11.2. Glutathione peroxidase in blood

Glutathione peroxidase or glutathione-hydrogen peroxide oxidoreductase (E.C. 1.11.1.9) catalyses the conversion of the hydroperoxides of a variety of organic compounds such as fatty acids and thymine to the corresponding alcohols. This action against lipid hydroperoxides is particularly important in protecting cellular and subcellular membranes from oxidative damage.

The usual method[36] consists in measuring the enzyme activity by coupling of hydrogen peroxide with NADPH via glutathione reductase (GSH-Red). After preincubation of the reaction mixture (GSH-Red, EDTA, phosphate buffer, NADPH, GSH, sodium azide, and buffered haemolysed blood), for 5 min at room temperature, H_2O_2 is added. Every 30 sec for 5 min the concentration of NADPH is measured spectrophotometrically at 340 nm. Enzyme activity is expressed both as micromoles of NADPH oxidized per minute per gram of haemoglobin and micromoles of NADPH oxidized per minute per gram of protein; 1 Unit would then correspond to 16.67 nkat.

A modified method has been described by Lawrence et al.[37] and applied with good results by Chung and Maines[38].

2.11.2.1. Glutathione peroxidase activity

In a group of unexposed individuals, Lane et al.[28] found average values of 31 U per gram of haemoglobin (equivalent to 516.8 nkat) or 41 U per gram of protein (equivalent to 683.5 nkat) for the activity of GSH-Px, and this activity was related to the level of selenium in blood.

To our knowledge, GSH-Px blood levels in selenium exposed workers have not yet been recorded.

2.12. INDICES FOR TELLURIUM EXPOSURE

According to Weibust et al.[1], it is still not known whether or not tellurium is an essential element to humans and animals, although Vouk[2] states that "there is no evidence that tellurium is essential". The main source of tellurium for the general population is food, the daily intake having been estimated to be about 100 μg[3].

The toxicity of tellurium is considered to be low. The use of tellurium in industry does not appear to be hazardous in general terms owing to the characteristic garlic-like odour of the breath, sweat and urine, which indicates undue exposure[4]. However, it should be pointed out that its use is increasing in some modern industries and the potential hazard represented by radioactive tellurium isotopes which are formed in the fission of uranium and plutonium must not be forgotten[5].

The main route of exposure in industry is inhalation of dusts and fumes, although percutaneous absorption from dust is also possible. As the production and use of tellurium is related to other metallurgical processes, there is probably a multiple occupational exposure involving other toxic metals such as lead, arsenic, cadmium, thallium, selenium and zinc.

The chemical forms of tellurium present in the working atmosphere seem to be mainly elemental tellurium (Te^0), tellurium dioxide (TeO_2) and hydrogen telluride (TeH_2).

As shown in experiments carried out on rats[6], the metal is absorbed and then accumulates at a very slow rate, especially in the bone. The organ concentrating tellurium to the greatest extent in the short term is the kidney (radiotellurium seems to concentrate in the cortex), being the second highest concentration found in blood (90% of the blood tellurium isotope is bound to the erythrocyte proteins)[5].

Tellurium is removed from the body by methylation, It is excreted mainly with urine as dimethyl telluride $|(CH_3)_2Te|$, a compound with a garlic-like odour that also appears in the expired air (only small amounts, around 0.1%, are excreted via the lungs) of subjects exposed to elemental tellurium and Te^{4+} compounds.

The garlic smell of the breath and sweat persists for a considerable period after exposure.

The literature on the biochemical effects of tellurium in humans is rather scarce. Most of the research work has been carried out on rats and other laboratory animals.

Tellurium was shown to be teratogenic on the foetuses of rats fed a diet containing high levels of elemental tellurium and it seems that the metal acts directly on the embryo[7]. An increase in chromosome breakage incidence has been observed in human leukocytes treated in vitro with sodium or ammonium tellurite[8], but no animal studies on mutagenicity are known.

Experimental studies carried out on rats exposed to tellurium oxide and elemental tellurium aerosols[9-11] have shown the haemolytic action of tellurium (reduction in haemoglobin and erythrocytes, haematuria and bilirubinuria). Young et al.[12] demonstrated that both tellurite and selenite cause lysis of normal sheep erythrocytes in vitro, and that GSH-deficient sheep erythrocytes are considerably more resistant to haemolysis than normal cells. At the same time, lysis is preceded by a decrease in intracellular GSH level. On the other hand, diamide (a thiol oxidizing agent) inhibits tellurite-induced lysis, and addition of extracellular GSH to GSH-deficient erythrocytes results in a rapid lysis when tellurite (or selenite) is also present.

Tellurite-induced lysis of erythrocytes appears to occur only at high GSH/tellurite ratios. The mechanism of the lysis has not been explained, but it could result from the formation of one or more intermediate complexes between GSH and TeO_3^{2-} which would be further reduced leading eventually to elemental tellurium.

It should be pointed out that inherited erythrocyte GSH-deficiency also occurs in man, and either tellurite or selenite can be used as a means of detecting this deficiency.

Sodium tellurite has been found to reduce the activity of catalase and free thiol groups in blood[13]. Inhibition of catalase in erythrocytes was also observed by Sandrackaja[9,10] in rats exposed to Te^0 and TeO_2 aerosols.

Siliprandi et al.[14] reported some effects of tellurite on mammalian mitochondria oxidative processes. According to their results, tellurite would specifically inhibit the oxidation of NAD-dependent substrates (in intact mitochondria of rat liver and kidney) such as pyruvate, α-ketoglutarate and glutamate, without affecting the oxidation of succinate, α-glycerophosphate and ascorbate. The inhibition is considered to be a consequence of the interaction between tellurite and some mitochondrial SH groups, as the effect is reversed on the addition of dithioerythritol.

2.12.1. Interpretation of blood and urine tellurium levels

The reader is referred to Chapter 11 for the methodology of the determination of tellurium in blood and urine.

2.12.1.1. Tellurium in blood

The early data obtained for the concentration of tellurium in biological materials are considered to be very high and confusing, probably owing to analytical discordances. The values given by Schroeder et al.[6] for human serum (107 µg per 100 ml) and those reported by Soman et al.[15] for different organs and tissues are today considered to be too high. The values obtained in more recent studies, two to three orders of magnitude lower seem to be more reliable.

Van Montfort et al.[16] obtained values of 25 ng per 100 ml of whole blood in unexposed people using microwave-induced emission spectrometry. Some other investigators[1,17,18], trying different variations of AAS and NAA, were not able to detect any tellurium signal (the detection limits were 3 ng/ml for electrothermal AAS and 2 ng/ml for NAA) in whole blood samples from unexposed subjects.

2.12.1.2. Tellurium in urine

Tellurium was found at levels of 10-60 µg per 1000 ml in the urine of all workers exposed to tellurium-containing fumes in an iron foundry where the concentration in air was 10-100 µg/m^3, but not in any of the control group[19]. Tellurium has also been found in the urine of workers accidentally exposed to tellurium-containing fumes for not more than 30 min[20] at concentrations of 8-16 µg per 1000 ml.

However, urinary tellurium excretion is considered by some investigators to be of uncertain value in relation to exposure.

2.13. INDICES FOR THALLIUM EXPOSURE

Thallium and potassium ions seem to be biologically interrelated. Experiments with frog muscle[1] have shown that K^+ and Tl^+ cross cell membranes in a similar way and the active transport mechanism for K^+ into rabbit erythrocytes appears to transport Tl^+ also[2]. Thallium ions move between extracellular and intracellular compartments like potassium ions[3] and are excreted by the same routes via the kidney and into the gastrointestinal tract via the saliva and the bile. However, although the movements of both ions seem to be closely related, once inside the cell Tl^+ appears to be less readily released than K^+ [4]. Tl^+ can substitute for K^+ in causing activation of ATPase, which would indicate that the mechanism involved in the active transport of K^+ cannot differentiate between the two ions[5].

Skulskii et al.[6] have shown that Tl^+, in contrast to K^+, may readily penetrate the membranes of erythrocytes, and that the cell/medium distribution of Tl^+ in erythrocytes can be used to estimate the membrane potential. However, the interaction of Tl^+ with motochondria still remained controversial. In a subsequent study[7], evidence was presented that Tl^+ is able to permeate the mitochondrial inner membrane, and the movement of Tl^+ was shown to be electrophoretic. This fact allows the use of Tl^+ as a probe of the membrane potential, which is very important in theories about the mechanism of oxidative phosphorylation.

Tl^+ can therefore act as a lipid-permeable cation in mitochondria[7,8], distributing across the membrane at the same ratio as other lipid-permeable cations such as tetraphenylphosphonium. On the other hand, it distributes in red cells in a ratio opposite to that of Cl^- [9], which is in electrochemical equilibrium with the measured membrane potential.

Tl^+ interacts with Na^+-K^+/ATPase of erythrocytes[9] and has been shown to be transported by this enzyme in squid axon[10]. In a study on thallium interaction with gastric ATPase, which usually exchanges H^+ for K^+, Rabon and Sachs[11] have shown that this ATPase is able to use Tl^+ as a substitute for K^+, and that Tl^+ activates the ATPase with a higher affinity than K^+.

2.13.1. Interpretation of blood, urine and hair thallium levels

The reader is referred to Chapter 8 for the methodology of the determination of thallium in blood, urine and hair.

2.13.1.1. Thallium in blood

Few data are available on levels of thallium in biological media of unexposed subjects. Blood usually contains very little thallium, if any. In animal experiments thallium could not be detected in blood 20 min after injection. In rats chronically fed with thallium the highest concentration of metal was found in the kidney[12]. In man, the highest concentration of thallium is also found in the kidney[13].

2.13.1.2. Thallium in urine

Thallium is excreted via the kidney and intestine, and also partly by the salivary gland, hair and milk.

Although the levels of thallium in urine are not related to the degree of exposure, they may be of some help in diagnosis. Weinig and Zink[14] found excretions of 0.13-1.69 µg per 1000 ml in early morning urine samples of unexposed subjects.

2.13.1.3. Thallium in hair

The highest values of thallium in tissues of unexposed people were found in hair, where Weinig and Zink[14] reported concentrations of 4.8-15.8 μg/kg. They also found levels of 0.72-4.93 μg/kg for nails. The analyses were performed by mass spectrometry.

In the long term, hair and nails may provide an important route for the slow excretion of thallium from the body. The levels of thallium in urine and hair may be used as parameters of internal exposure.

In a series of surveys carried out by Brockhaus et al.[15] to establish the degree of thallium exposure in a population living around a cement plant emitting thallium-containing dust, 24-h urine samples were analysed for thallium by AAS, with the following results (n = 1265): mean±standard deviation = 5.2±8.3 μg of thallium per 1000 ml of urine, with a range of <0.1-76.5 μg per 1000 ml.

Thallium may be detected in single hair and toenail samples by NAA[16]. Although loss of hair is not always present as an early symptom, it may be a significant sign of overexposure.

2.14. INDICES FOR NICKEL EXPOSURE

Nickel is considered to be an ultratrace element (dietary requirement ≤50 ng) of nutritional significance, the same as arsenic and vanadium[1]. However, spontaneous dificiency states are unlikely in man or animals.

A nickel-containing α_2-macroglobulin called nickeloplasmin has been found in human and rabbit sera[2,3]. The presence of this nickel-containing metalloprotein in sera suggested that nickel may play an essential physiological role. This idea was supported by the finding that chicks that were fed diets containing only 40-80 ppb of nickel showed an apparent nickel deficiency syndrome[4,5]. Other investigators[6,7] did not consider these results to be conclusive.

Spears et al.[8] suggested that nickel may substitute for copper at certain biological sites, sparing copper for some vital functions. In copper-deficient rats nickel has shown a tendency to decrease the copper concentration in certain tissues.

Nickel may also be essential either for the enzymatic formation or for the structural integrity of a molecule involved in the transport of ferric ion (Fe^{3+}).

As is known, ATP is hydrolysed by Na^+-K^+/ATPase (E.C. 3.6.1.3) in the presence of Na^+, K^+, and Mg^{2+}, according to the following reaction:

$$ATP \xrightarrow[\text{enzyme}]{Na^+,\ K^+,\ Mg^{2+}} ADP + P_i$$

Nickel ions (Ni^{2+}) may substitute for Mg^{2+} (required for the activity of the enzyme), in which case the maximum rate of the enzymic reaction is much lower than it is in the presence of Mg^{2+} [9]. The same occurs with Co^{2+}.

Nickel is one of the metals found firmly associated with DNA and RNA[10], and it appears to activate certain enzyme systems in vitro[11,12].

Nickel has been found to act in a similar way to cobalt in regulating haeme metabolism in liver and kidney. It induces haeme oxygenase activity (the rate-limiting enzyme in haeme degradation) in both organs, and causes transient depression of cellular glutathione content followed by increases above normal in liver[13].

Nickel has been shown to depress δ-aminolaevulinate synthetase activity[13], which is the rate-limiting enzyme in haeme synthesis. This would be the way nickel (the same as cobalt) regulates cellular haeme proteins.

When nickel complexes with sulphydryl agents its actions on haeme metabolism are completely blocked.

2.14.1. Interpretation of blood, urine and hair nickel levels

The reader is referred to Chapter 10 for the methodology of the determination of nickel in blood and urine.

2.14.1.1. Nickel in blood

Although detailed opinions vary, it is generally accepted that the determination of nickel in serum, urine and hair can be of value in biological monitoring of industrial exposure, particularly in electroplating works and in the tungsten carbide industry. McNeely et al.[14] consider the measurement of nickel in serum and urine to represent a valid biological index of environmental exposure to nickel. In two groups of people residing in two different U.S. cities, none of whom had occupational exposure to nickel, the following results were obtained for nickel in serum: group A 0.46±0.14 μg per 100 ml and group B 0.26±0.10 μg per 100 ml. The concentrations of nickel in the municipal tap water were 200±43 and 1.1±0.3 μg per 1000 ml for the population groups A and B, respectively.

Nomoto and Sunderman[15] obtained for groups of healthy subjects average values of 0.26±0.08 μg per 100 ml for nickel in serum and 0.48±0.13 μg per 100 ml for nickel in whole blood.

The values obtained by Zachariasen et al.[16] in Norway for healthy persons were 0.60±0.1 and 0.47±0.1 μg per 100 ml in whole blood and plasma, respectively.

The method employed in all cases was AAS.

2.14.1.2. Nickel in urine

A good correlation was found by Tola et al.[17] between the concentration of nickel in air and nickel in the urine of electroplating workers. For concentrations of 100 $\mu g/m^3$ of nickel in the electroplating shop air, the levels of nickel

in urine were about 80 µg per 1000 ml (corrected to a specific gravity of 1.018) in the afternoon of the same day and about 65 µg per 1000 ml (corrected in the same way) the next morning.

The average value found for nickel in the urine of unexposed subjects by Zachariasen et al.[16] was 24±4 µg per 1000 ml. McNeely et al.[14] found mean values in two different cities of 7.9±3.7 and 25±1.4 µg/day. Nomoto and Sunderman[15] found a value of 2.4±1.1 µg/day.

2.15. FINAL REMARKS

The determination of trace elements in biological materials is susceptible to errors, unless special precautions are taken during collection and storage of samples and in the analysis itself. Contamination during collection and processing as well as attenuation of the analyte concentration during storage must be avoided. Otherwise, the results obtained are not valid even though the methods of analysis may be extremely sensitive and accurate. Control procedures should be applied at all stages for ascertaining the sources of error and eliminating them. Developments in methodology and instrumentation for trace-element analysis have been enormous, whereas developments in quality control and reference materials have not experienced such progress.

Various expert committees have emphasized the necessity for quality assurance in trace-element analysis, recommending the development and extensive use of quality control materials, as well as inter- and intralaboratory evaluations at the national and international level, in order to establish whether discrepancies in reported values from various laboratories arise from poor methodology, faulty technique, loss of specimen integrity or other causes. Analytical procedures that are not under control will not give results with the necessary precision. Quality control procedures should also be applied to data processing. It is important to bear in mind that trace-element analysis must meet three main criteria if the results are to be meaningful with respect to health-related studies: specificity, precision and sensitivity.

International recommendations should also be followed for the analysis of enzymes and other biochemical parameters associated with exposure to toxic metals, whenever available.

The Commission of European Communities (CEC), the World Health Organization (WHO) and the United Nations Environment Programme (UNEP) have recently undertaken extensive biological monitoring programmes in the field of environmental health, with the purpose of providing information on exposure levels for both the general population and especifically exposed population groups, with reference to quality control. The CEC programme, limited at the moment to lead in blood de-

terminations, will assess the results in terms of reference values agreed by Member States. The WHO/UNEP programme will include more pollutants, namely lead in blood, cadmium in blood and kidney and mercury in blood and/or hair, among toxic metals.

An International Workshop on The Use of Biological Specimens for the Assessment of Human Exposure to Environmental Pollutants was organized by WHO/UNEP and CEC jointly with the U.S. Environmental Protection Agency (EPA) (Luxembourg, 1977), with the aim of examining the technical feasibility of programmes designed to collect, analyse and store samples, and to develop guidelines for sampling, sample preparation, analytical requirements and storage, in connection with biological monitoring.

Both programmes (WHO/UNEP and CEC) recognize the utmost importance of quality control in view of the serious difficulties that may arise during the analytical processing of biological materials. These organizations understand that close technical cooperation at international level is indispensable if acquired experience in quality control is to be extensively applied in the field of biological monitoring for the prevention of health impairment.

As has been seen in this chapter, advances in biological chemistry and in biochemical toxicology have provided vital tools for the detection and evaluation of minor cellular damage, indicating in some cases only reversible changes that do not have an actual effect on health. Some biochemical parameters with a well established significance have been incorporated in routine studies concerning exposure to toxic metals. Others require further research in order to become available for modern technology in the field of occupational health, but will probably be part of routine practice in the near future.

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CHAPTER 3

INSTRUMENTATION

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CONTENTS

3.1. INTRODUCTION

There is an urgent demand for the reliable and rapid analysis of trace elements in biological materials. The most important are metals and metalloids that are considered as essential, toxic or possibly toxic to man, animals and plants. In conjunction with similar analytical demands from other research branches, this situation has stimulated in recent decades progressively intensified development and progress mainly in the field of instrumentation. Trace analytical methods have become increasingly accessible and indispensable for numerous scientific, medical and legal applications. Manufacturers of analytical instruments have therefore increasingly designed and offered highly advanced systems for trace analytical purposes. This was linked with a rapid change from classical, i.e., predominantly manual, to instrumental, i.e., mechanized, or even completely automated methods.

However, the tremendous increase in detection power and the vast increase in the number of analytical systems on offer has led to the publication of many inconsistent data in the field of trace and ultratrace analysis. The reasons for this situation became obvious as inter-laboratory surveys were increasingly conducted. They disappointingly often showed a wide scatter of results for the concentration of a certain element or compound in a certain homogeneous material[1-7] as well as, typically, erroneously high averages of the lower (i.e., normal) levels[2,5,7]. These facts have different sources: the ability to apply with experience and a broad chemical and trace analytical knowledge the overall analytical procedures from sampling, through sample preparation and pre-treatment to the determination step could not keep up with the tremendous increase in sensitivity attainable with the new generation of analytical instruments. One reason was that these instruments were introduced in numerous laboratories hith-

erto not experienced in analytical chemistry, which then often used a single device in a "black box" manner. This was also the consequence of exaggerated or even unsound but persuasive commercial advertisements. Thus, in addition to error sources stemming mainly from sampling and sample pre-treatment, there were also numerous instruments that could be reliably operated only by the most experienced analysts conducting a comparison with methods based on different physical principles[8]. In those instances, the application of materials with known, preferably low, concentrations of trace metals, that is all types of control, standard, standard reference and certified standard reference materials, would certainly have had improved the situation significantly if there had been more of these materials available. These aspects and the paramount importance of a clear sampling strategy, proper sampling and sample storage have been more extensively treated elsewhere[8-13] and will also be addressed in some detail in the chapters on the elements.

A great challenge for analytical chemists now is therefore to achieve meaningful, i.e., from the present state of the art accurate, data on a more extended scale than before. In order to achieve this target, some change in analytical philosophy by producers and users of trace analytical data is essential. This doubtless applies also to the more experienced laboratories, because even they still cannot always fulfill the required extremely rigid demands on accuracy and precision in all stages of trace analytical procedures. The benefits of the great potential of present highly advanced instrumentation can only be gained if a comprehensive attitude on the analytical problems as a whole is adopted. This also includes a preference for high-quality results in limited numbers over the production of a vast amount of mediocre data. This approach needs, in addition to the use of the most suitable instrumentation for a certain task, the meticulous performance of all steps of an analytical procedure that also requires precise protocols. In this chapter, therefore, we shall discuss critically the present state of the art with respect to sample preparation and analysis from our practical experience in order to provide some advice on the performance of trace analysis on a cost-benefit basis.

There are numerous books, reviews and research papers on the topics treated here. We quote only a selection of them, and those quoted should be considered merely as examples relating to the facts presented rather than as a judgement on these and other publications.

3.2. SAMPLE PREPARATION

The preparatory steps prior to trace metal or species determination are manifold. Numerous workers discuss this aspect from the initial purification of

reagents to the release and loss of elements from and at surfaces during digestion and pre-concentration procedures[8,12,13-25]. These steps may introduce numerous error sources. The evaluation of a reliable digestion procedure, for example, for a certain material and one or more trace metals can be performed successfully only if different and also as far as possible independent methods are applied for the evaluation of optimal conditions and error minimization[8,17,26]. For reference methods and relevant applications, the pre-treatment procedures under consideration should be carefully checked by intra- and inter-laboratory surveys[27].

3.2.1. Homogeneity and weight normalization

Provided that the sampling strategies, sampling and sample storage fulfill all demands, the next steps of an analytical procedure have also to be performed with great care. The reproducibility of the data obtained can depend considerably on the homogeneity of the material. As the level of the statistical homogeneity is a function of the weight of the subsamples taken for an analytical run, the weight itself also has to be considered. Moreover, the weight should be expressed in clearly defined terms.

Homogeneity of a sample is required for a reliable average value. However, materials having a structure that leads to an inhomogeneous distribution of the trace metal to be analysed (e.g., Cd in the kidney and kidney cortex) can be of particular value and require sampling of micro-samples from these structures. Also in that case the degree of inhomogeneity within these structures may be of interest for a reliable overall determination. This is of particular significance if small solid samples [e.g., with graphite furnace atomic-absorption spectrometry (GFAAS) or neutron activation analysis (NAA)] are used, which of course always have to be checked with control materials homogeneous even at the milligram level within the error of the analytical technique applied.

For biological and environmental materials the concentrations are often given for fresh weight. Fresh weight, however, has the disadvantage that, owing to the continuous loss of moisture, even during short-term storage in freezers, the fresh weight reported may decrease considerably depending on the storage time and temperature. Hence, if it is not possible to carryout subsampling and weighing of these subsamples exactly at the time of sampling, the term fresh weight must be replaced by the more practical terms wet weight or actual weight. This, however, needs clarification and we propose to use the following order: fresh weight > wet weight > actual weight. As actual weight can also be used to define the weight of, e.g., a material previously freeze-dried but later again containing some moisture, all of these terms should be clearly defined in the analytical protocol.

From these definitions, it is obvious that the terms wet weight and actual weight mean only that the material still has a considerable moisture content. With a large sample, for example a whole organ, it is evident that the outer parts will contain less moisture than the inner parts. This also has to be considered if subsamples have to be taken from such a sample. Another fact, also mentioned in the chapter on cadmium, is the change in weight and elemental composition of autopsy materials if they could not be taken immediately post mortem[28].

These facts require, whenever possible, weight normalization. It is obvious that terms such as wet weight and actual weight in most instances cannot be used reliably for reporting analytical data. This is the reason why analytical chemists prefer, for biological materials with an initially high moisture content, to report data on a dry weight basis. With a correction factor for fresh weight, which can be determined with real fresh samples, the data normalization to fresh weight, is easily and reliably possible.

This, of course, does not mean that dried materials should always be taken for analysis. There are elements or compounds that may be partially or even completely lost or decomposed during drying. Also, on drying prior to analysis an additional contamination risk may occur at ultratrace levels. From these materials subsamples have to be taken for pre-treatment as well as independently for drying to obtain a normalized weight. As this is common analytical practice it does not need further explanation.

Still under discussion, however, is the mode of dry weight determination or, generally, normalization of analytical data to an, also biologically, sound basis[29,30].

Oven-drying procedures to constant weight at around 100°C doubtless are excellent for robust materials and hence essential in classical analytical chemistry, but they are far from optimal for biological materials. Biological materials may be sensitive and may decompose during thermal treatment. Hence they may not reach a constant weight within a reasonable time period. Therefore, drying at temperatures far below 100°C, drying in a desiccator with P_2O_5, sometimes at slightly elevated temperatures, or freeze-drying is frequently advocated as more reliable. In analytical practice, however, these methods, as also oven drying, can be applied only if they have been carefully checked with a certain material. They commonly provide sufficiently accurate information for weight normalization and at least arbitrary content/weight relationships. If an appropriate and properly standardized drying procedure is applied, errors arising from this source are commonly well below the average error of the determination step.

If biological materials have to be dried prior to analysis, the only method without statistically significant element losses, carefully checked with metabolized radiotracers, is freeze-drying, while oven drying can lead, probably depending on the temperature applied, to losses at least for elements such as Hg, Se, I, Sb and Zn. It should be mentioned, however, that these findings in general are contradictory, indicating that the behaviour appears to be highly dependent on the particular matrix and the species investigated[31-34].

As weight is only one factor in the analysis of biological materials, other factors must also be considered. For example, the application of internal biochemical standards, i.e., the determination of carbon, sulphur or nitrogen, may give additional information. This doubtless should be included in further research efforts towards biologically meaningful data normalization.

3.2.2. Digestion methods

An important, sometimes crucial, step in the analytical procedure is the transformation of a solid or a liquid sample into an analyte solution. This often requires a more or less complete digestion of the materials to be analysed. Because each additional step introduces a new source of errors, there exist numerous methods for the direct analysis of body fluids and even solid materials or slurries by circumventing digestion or by effecting a decomposition inside the analytical detection system, e.g., as in GFAAS, flame AAS and inductively coupled plasma-atomic-emission spectrometry (ICP-AES).

However, homogeneity reasons and serious matrix interferences still necessitate in most instances a complete or at least a partial digestion prior to the determination step. Moreover, most of the reference methods presently applied for the calibration of simpler and faster methods, e.g., the recently published IUPAC reference method for nickel in urine[35], are based on a digestion step. Thus, digestion procedures still play a dominant role in most of the presently applied analytical methodologies[16-19,22,25,27,30,36]. The most frequently applied digestion techniques are critically discussed below with regard to particular advantages and disadvantages, sample throughput and costs. In Table 3.1 the principles of the digestion techniques discussed are summarized together with typical costs (in US $) of commercially available devices.

3.2.2.1. Dry ashing at higher temperatures

If the volatility of the element to be analysed and of its compounds up to at least 823°K (550°C) is negligible, dry ashing in various, and sometimes very sophisticated, types of muffle furnaces, e.g., with temperature programming[37,38] and quartz walls[39], can be the method of choice. This is valid particularly at

TABLE 3.1

DIGESTION METHODS

Method	Features	Price of standard Equipment (US $)	Cost-benefit ratio
Dry ashing, higher temp.	Simple and cheap but experience needed, less recommendable for precision analysis at lower levels. Useful for all methods	$\geq 10^3$	Good
Dry ashing, low temp.	If properly applied extremely low blank, but time consuming. Useful for all methods	$\geq 2 \times 10^4$	Medium
Combustion	Complete ashing of all materials, but sometimes not free from contamination. Useful for all methods	$\geq 5 \times 10^3$	Medium
Wet ashing, open systems	Very simple and inespensive in routine use, but sometimes elemental losses and blank problems if particular precautions are not taken. With a few limitations useful for all methods	$< 10^3$-10^4	Good to excellent
Wet ashing, closed systems under pressure	Method of choice for volatile elements, useful for most methodologies. If properly applied very low to extremely low blanks. Caution: blow-off possible. Incomplete mineralization can pose problems mainly in voltammetry, requiring further treatment. Organometallic compounds of As are incompletely decomposed, requiring further treatment	$< 10^3$-5×10^3	Good
Tissue solubilization with Quaternary ammonium compounds	Rapid, simple and cheap. Not useful for all elements and methods. Considerable blanks due to the reagents still not avoidable	$< 10^3$	Good to excellent

the higher micrograms per gram (ppm) level and if the subsequent analytical step requires a completely ashed material. Further, oven ashing is inexpensive and comparatively easy under the mentioned conditions. If amounts at the lower micrograms or even nanograms per gram (ppb) level are to be analysed, however, dry ashing becomes difficult. Thus, if highly accurate data at the trace and ultratrace level are needed, oven ashing is usually less recommendable and may introduce errors due to contamination from the oven material (walls), dust or ashing aids such as H_2SO_4, HNO_3 or $Mg(NO_3)_2$. Also, substantial and irreproducible losses can occur because of adsorption on the walls of the crucibles used. Despite the fact that some workers have reported the successful application of dry ashing under controlled conditions even for analyses at the nanograms per gram level[39], this needs particular skill and experience. The routine use of dry ashing for sample pre-treatment at nanograms per gram levels is therefore questionable if an inexperienced staff without a substantial knowledge of trace analytical chemistry is involved.

3.2.2.2. Dry ashing at lower temperatures

From the point of view of contamination from the laboratory environment, trace element losses, adsorption and blank minimization, the so-called low-temperature ashing (LTA) in microwave-excited oxygen plasmas, with partial pressures $\leq$ 1 Torr, at average temperatures below 423°K (150°C)[40], is often said to be the most promising approach for trace and ultratrace analysis.

The majority of commercially available instruments, however, show losses of volatile elements such as Hg, As and Se, and sometimes also Cr, but here published papers are contradictory. Systems avoiding these losses by cooling traps have also been designed[41] and are now commercially available.

Some workers advocate the use of low-temperature ashers for biological materials, for example, prior to as well as after neutron, photon and particle activation analysis, prior to X-ray fluorescence[41-43] and prior to spark-source mass spectrometry[44]. Excellent recoveries and dissolution properties from the ash for all elements investigated have been reported.

A commercial low-temperature ashing system was more recently modified for an extremely contamination-free ashing of whole blood samples. Ashing times varied from 6 to 12 h, prior to the voltammetric determination of Pb, Cd and Cu[45].

Limitations of this method are the expensive ashing devices and the usually lengthy procedure. As the excited plasma only reacts at the sample surface, the latter can pose problems in preparing samples for low-temperature ashing. If materials such as bones and plants contain large amounts of inorganic constituents, a complete ashing is extremely time consuming[46].

3.2.2.3. Combustion

Another technique is the combustion of organic material in a pure oxygen stream, which is reported to be especially effective for fatty materials, for example adipose tissue. As the reaction takes place at very high temperatures, volatile compounds have to be trapped in appropriate absorbing solutions, at cooling fingers, on cooled surfaces, etc. Because in these systems high surface areas usually have to be used, methods of this type, despite its excellent ashing potential, were hitherto not very extensively used in trace and ultra-trace analysis.

Burning of a solid material in a quartz dish, a solution or a slurry by direct introduction into an oxygen-hydrogen flame is known as Wickbold combustion. This method can be used successfully prior to the determination of mercury in biological materials[47-49]. A slightly modified Wickbold system was also shown to be useful prior to the analysis of other elements[50]. Recently a system was described and made commercially available for the combustion of up to 20 g of wet material in a stainless-steel bomb under an oxygen pressure of ≥30 bar[51].

An improved all-quartz combustion system with the volume minimized as much as possible to obtain a small reactive surface developed earlier is now commercially available in a slightly modified version. This device is applicable for micrograms to nanogram amounts of elements, as was demonstrated recently by the application of different analytical principles[52].

A limitation of combustion methods is the comparatively low sample throughput. Methods of this kind, however, are useful within the concept of the application of independent analytical principles, and also of different alternative ashing procedures, to confirm independently trace analytical results[3,8,13,17,25,27,32,52].

3.2.2.4. Wet ashing, open systems

Wet digestion of biological materials, frequently with a single acid, mainly HNO_3, with the aid of UV irradiation[53], and also with acid mixtures such as HNO_3-$HClO_4$, HNO_3-$HClO_3$-$HClO_4$, HNO_3-$HClO_4$-H_2SO_4, HNO_3-H_2SO_4, H_2SO_4-H_2O_2 and H_2O_2-HNO_3 has been extensively treated in the literature[12,18,19,22,30,35,36,53-55]. Another, slightly different technique for the oxidative degradation of organic matter is Fenton's reaction. Here free hydroxyl radicals are generated by the action of catalytic amounts of Fe(II) on H_2O_2 at low temperatures above 80-90°C[12,56,57]. This approach probably is preferable to other methods in which H_2O_2 is used in connection with acids, particularly if volatile elements have to be determined[12].

Wet digestion is still the most frequently used digestion technique prior to all trace analytical determination methods. The reason is the simplicity and

adaptability of wet digestion procedures to nearly every analytical task. Also, a high sample throughput can be achieved. Sample weights can amount to several grams, and for the Fenton method even up to 100 g or more[12,56].

Series of samples can be processed in sand or quartz sand-baths[58], on heating plates[56,59,60], in heating blocks, in microwave devices and sometimes also in a flow-through manner, ranging from simple to sophisticated mechanized and also programmable devices which are partly commercially available[61-67]. Digestion devices consist of simple vessels of various forms or tubes made from laboratory glass, Pyrex or quartz and of various reflux systems to enhance the digestion potential and to minimize as far as possible reagent comsumption and trace element losses and contamination[59-62,64,66-68]. Disadvantages are the high consumption of acids in conjunction with elevated temperatures (>300°C) if a complete digestion is required and related contamination problems from the walls of the vessels and the reagents. Trace element levels in acids can, of course, be drastically reduced by sub-boiling distillation[16,17,21,24,36]. Solutions from wet digestion may also pose problems, for example, in GFAAS if they contain too much acid, for example, $HClO_4$ or H_2SO_4. In that event evaporation is necessary prior to the determination step.

3.2.2.5. Wet ashing, closed systems under pressure

In ultratrace analysis and particularly the analysis of volatile elements, pressurized decomposition was found to be advantageous in comparison with most open digestion procedures[69-72]. Commonly HNO_3 is used as the main oxidant, but addition of other acids for particular purposes (HF, $CHlO_4$, H_2SO_4) have also been reported[27,36,69]. As the oxidation potential of HNO_3 increases significantly at elevated temperature and pressure[36], the consumption of acid, preferably from sub-boiling distillation, is usually lower than for open wet digestion. This is also advantageous if blanks have to be extremely low.

Various devices, all equipped with safety systems that allow blow-off in cases of overpressure[73] to circumvent a explosion, are commercially available for sample weights up to about 1 g (dry weight). Also systems for the simultaneous decomposition of up to ten samples are available. A few recently introduced systems with higher volumes now make possible also the decomposition of sample amounts above 1 g dry weight.

It should be mentioned that a blow-off can also be hazardous to staff if the systems are not shielded in an appropriate manner. Thus, each material to be digested needs proper programme evaluation and current control in order to avoid a more or less violent blow-off due to overpressure[74,75].

In closed systems, very low blanks similar to those reported for low-temperature ashing can be achieved. The reason, in addition to the minimization of

the amount of acid used, is that the decomposition vessels made from PTFE, glassy carbon[76] or quartz inside PTFE tubes[77] can be very efficiently cleaned with acids[69,76,78] or by "cleaning decomposition" with pure materials[72].

Pressurized decomposition achieves only partial ashing even for relatively simple materials[75] which could be quantitatively mineralized by wet ashing at higher temperatures, e.g., with HNO_3-$HClO_4$ or HNO_3-$HClO_4$-H_2SO_4 mixtures[79]. This problem can be overcome partly by longer decomposition times than the previously advocated 1-3 h, i.e., decomposition for 6-12 h at 423-433°K (150-160°C). The ashing achieved is usually sufficient for subsequent GFAAS or plasma-induced AES but not always for voltammetry. For voltammetry the solutions from pressurized decomposition have to be after-treated by UV irradiation[80,81] or by heating with $HClO_4$ or $HClO_4$-H_2SO_4[27,29,82] or with a mixture of $NaNO_3$ and KNO_3, in the latter instance up to 723°K (450°C) in two steps[83].

In various foodstuffs, mainly of aquatic or marine origin, and also in body fluids, high-molecular-weight compounds of organically bound As occur that cannot be completely digested under pressure even if the digestion is prolonged. Thus, the analyte solution from pressurized decomposition has to be after-treated prior to hydride AAS with H_2SO_4-$HClO_4$ up to about 583°K (310°C)[84] or with $Mg(NO_3)_2$ up to about 723°K (450°C), which is also recommended for Se determination[83].

These additional treatments can increase blanks, clearly demonstrating the limitations of pre-treatment procedures, particularly of pressurized decomposition, in trace and ultratrace analysis. In this context it is emphasized that the after-treatment by UV irradiation[81] adds no additional sources of contamination.

3.2.2.6. Tissue solubilization

For analytical methods that require only a homogeneously distributed and not a completely digested analyte solution, as in principle is the case for radiotracer studies, flame and graphite furnace AAS and ICP-AES, simple tissue solubilization by quaternary ammonium compounds has been reported to be a rapid and reliable method. Tissue and hair samples were digested at low temperatures, preferably overnight, and the digests directly analysed after appropriate dilution[85-88].

Despite the low costs and simplicity of this technique and the impressive results reported, limitations are that for lower concentration levels contamination from the reagents occurs and, particularly for GFAAS, matrix interferences have to be faced. Moreover, this approach can only be used for a limited number of materials and determination methods and thus probably also for a limited number of elements with the present state of knowledge, but should be evaluated further.

3.2.3. Pre-concentration/separation

In the trace and ultratrace analysis of metals and other elements, pre-concentration and/or selective separation before the subsequent determination step is frequently required. The reasons are either a lack of sensitivity of the applied methods or matrix interferences, sometimes both. The corresponding requirements for selective separation and/or pre-concentration may be satisfied by the application of liquid extraction, chromatography and ion exchange or coprecipitation. There is ample information in the literature on general aspects of liquid extraction[16,17,20,22,30,89-91] and also on chromatography and ion exchange[16,22,30,89,92-94]. Thus, only a few particular aspects are discussed here and several references are given to provide a condensed introduction to that field, as details of the procedures can be found in the chapters on the elements.

The partition coefficients between the aqueous and organic phases for numerous metal chelates with ammonium pyrrolidine dithiocarbamate (APDC), diethylammonium or sodium diethyldithiocarbamate (DDDC or NaDDC), hexamethylene ammonium hexamethylene dithiocarbamidate (HMAHMDC), dithizone and many other chelators, under optimal pH conditions, are of the order of 10^2 - >10^3. This allows an effective pre-concentration and also selective elemental or at least a group separation. That can be achieved either directly with body fluids[8,95-100] or with pre-treated analyte solutions[58,59,101-106]. Particular conditions such as pH, addition of complexing agents, treatment with stripping reagents or particular chelators also make possible the selective separation of single metals[59,101-104,107]. For routine applications extraction procedures were also shown to be achievable in an automated manner[108]. Digestion, adjustment of the analyte solution to an appropriate pH and column separation from matrix elements and also selective separation of single elements prior to the determination step are also possible. This was demonstrated by the use of different column materials and columns, partly in an automated arrangement[109,110], which is particularly valuable in radiochemical activation techniques[111-113].

For methods such as X-ray fluorescence spectroscopy, which require uniform thin-layer preparations, coprecipitation is a useful pre-concentration technique. It is, for example, possible using DDDC to coprecipitate Cr, Ni, Fe, Cu, Zn, Cd, Pb, As, Hg and Se with appropriate carriers to obtain the mentioned preparation[114].

The influence of contamination, which is also important in all these pre-treatment procedures, is discussed in the next section.

3.2.4. Contamination and losses

The most serious problems with analyses at the nanograms per gram (or nanograms per millilitre) level and below is contamination from external sources, for example the laboratory atmosphere, reagents and apparatus and also from the analyst. But even at micrograms per gram (or micrograms per millilitre) levels the contamination still cannot be ruled out completely. If improper storage conditions (pH, materials and form of the storage vessels) and/or less suitable digestion procedures are applied, losses are also possible. The influence of improper handling of solid or liquid samples and analyte solutions appears to be more liable to give erroneously high results than the incorrect use of analytical instrumentation or other error sources such as evaluation errors due to interferences. This is particularly evident for the values published during the last two decades for concentrations supposed to be normal for essential and toxic elements in body fluids. That was recently discussed for Al, Sb, As, Br, Cs, Cr, Co, Mn, Hg, Mo, Ni, As, V and Zn in blood plasma and serum[115]. Within a short time span the reported levels have decreased significantly, for some elements even by an order of magnitude or more. This was probably due to the introduction of, as far as possible, contamination-controlled laboratories or at least working conditions[15-17,116,117]. The levels reported at present for most of these elements in plasma or serum are typically of the order of 1 ng/ml or even lower. This indeed is now the domain of well equipped laboratories with broad experience in ultratrace analysis. A reliable determination at normal levels for most of the mentioned elements requires particular skill, suitable laboratory facilities such as clean workplaces, the best instrumentation and a rigid working discipline for the technical staff. In this kind of high-quality analysis, the number of measurements made in the preparatory steps, in contamination and quality control frequently balance or even exceed the number of measurements made on the analyte solutions of the materials to be analysed.

Typical sources of Pb blanks and procedures for blank minimization have been well evaluated, or example, for Pb in biological material and natural water[118,119] and in blood plasma[120], for nickel in urine and serum[35,58,59,121], for Cd in whole blood[45,100,122] and urine[60] and for Pb in whole blood[45,123]. Careful studies on sources of systematic errors due to contamination have been performed for Cu and Cd in small biological samples[124] and for Mg and Zn in various analyte solutions[78]. The authors of the last study pointed out that sources of blanks mainly determine the actual detection limits of solution procedures, which can differ significantly from idealized data reported in the literature.

The utilization of materials from different types of plastic to minimize contamination has been amply discussed together with appropriate cleaning pro-

cedures[8,16,17,21,24,36,45,60,69,76,78,82,100,105,116,122,123,125-127]. However, it was also noticed that, for example, beakers made from PTFE-FEP may be a consistent source of Fe, Cr and Mn in comparison with superior properties found for beakers made from polyethylene or TPX[125]. Severe contamination for Cd[100,128] and Cu[129] has been observed if particular polypropylene tips were used for the dosage of analyte solutions. Plastic bottles, however, should be avoided in Hg analysis because of diffusion of elemental Hg from the laboratory environment through the walls into the bottles[130]. Thus, for Hg only glass or quartz bottles, preferably also with stoppers made from the same materials, should be used. Labcratory ware made from quartz, despite its considerable cost, doubtless is best suited for most applications in ultratrace analysis, particularly at higher temperatures and also if surface adsorption is considered[8,12,15-19,24,27,36,41,45,49,52,58,60,61,80,116,121]. In particular instances, as was reported for contamination control in ultratrace analysis of aluminium[131], a coating of borosilicate glass may also be useful to minimize contamination and losses.

As extraction/separation procedures frequently require pH adjustment, which can introduce contamination from the reagents used, application of gaseous reagents considerably minimizes this error source[132].

In order to collect data on individual experience with contamination gained in laboratory practice, IUPAC Commission V.2 (Microchemical Techniques and Trace Analysis) reviewed the status of the contamination problem in trace analysis with the aid of a questionnaire and by literature consultation[133]. From this compilation the probability of contamination evaluated from the answers from 96 laboratories around the world was reported to be greatest during decomposition (65%) followed by separation (55%), sample collection (46%), instrumental measurement (32%), comminution (18%), filtration (18%), desiccation (11%) and sieving (10%). From more recent experinece gained in ultratrace analysis, one may dispute the order of these items. Possibly sample collection is at least equally prone to systematic errors as decomposition[115,120]. The paramount importance of contamination during decomposition and separation/pre-concentration, however, is clearly evident. Thus, much more work should be devoted to the identification of contamination sources and their minimization or even elimination. This may be particularly effective if control and standard materials are available containing minimum amounts of the trace elements sought. This approach should render possible careful studies on error sources of all critical stages of an analytical procedure[5,7,8]. In a special issue of Talanta, entitled "Gains and Losses in Trace Analysis", this aspect will be considered as part of analytical research activities within the Specimen Bank Project in the Federal Republic of Germany[134].

Losses, in comparison with contamination, do not play such a dominant role in analytical practice. There are various possibilities for minimizing losses from analyte solutions, for example by diluting analyte solutions only very shortly before the measuring step, and by acidification or complexation to prevent adsorption of trace elements at the walls[135]. It should be mentioned, however, that losses appearing in dilute calibration solutions lead to an apparent increase in the analytical results. Thus, continuous control and daily dilutions from stock calibration solutions are essential for avoiding systematic errors from this source. This error source, however, has already been amply discussed in the analytical literature.

Owing to the use of radiotracers, reliable decomposition studies could be performed relatively early, clearly indicating difficult steps in an analytical procedure. The pioneering work of Gorsuch, published in 1959[136], was a thoroughly performed radiotracer study on recoveries of Pb, Hg, Zn, As, Cu, Co, Ag, Cd, Sb, Cr, Mo, Sr and Fe after different wet and dry ashing procedures. The experiments were carried out by adding appropriate radiotracers to the model material cocoa. The results showed that for all elements, except Hg, it was wet digestion procedures that gave quantitative recoveries. Under optimal conditions the losses of Hg due to volatilization did not exceed about 10%. Dry ashing in some instances led to significant losses, which except for Hg, could be overcome nearly completely by the addition of ashing aids. Similar studies were performed partly also with metabolized radiotracers (e.g., refs. 27, 58, 61, 137-145). From this work the following can be summarized.

Radiotracer and comparative analytical studies with As, Cd, Cr, I, Mg, Fe, Mn, Cu, Zn, Ni and Hg, applying wet ashing, revealed quantitative recoveries[27,58,61,138,140,141,144,145], which could also be achieved for Hg if carefully controlled conditions were maintained and special ashing devices used[140,145].

In dry ashing, several workers observed losses due to adsorption on the surface of crucibles for Ni, Zn, Fe, Cr, Cd, Co and Fe[58,139,141,144]. In one paper it was described that radioisotopes were biologically taken up by molluscs, the soft parts of which were ashed. Significant losses were observed for all elements studied, viz., Mn, Co, Zn, Ru, Ce and Pa, from 383°K (110°C)[137]. Similar experiments performed later with marine and terrestrial biological materials and Ca, Mg, Cd, Cr, Te, Zn, Sb, Ag, Mn, Cu and Ni at ashing temperatures up to 773°K (500°C) or even higher, however, did not confirm these earlier findings[58,138,139,141,142,144].

The same, in principle, applies to low-temperature ashing. The possible loss of elements such as As, Cd, Se[143] and Cr[146] could either not be observed in other studies with other materials or could be overcome as for Hg by the use of cooling traps[40,41,43,45].

TABLE 3.2

TYPICAL RELATIVE DETECTION LIMITS FOR TRACE METHALS AND METALLOIDS OF TOXICOLOGICAL SIGNIFICANCE: COMPARISON OF THE MOST POWERFUL TRACE ANALYTICAL METHODS

Aqueous, non-interfering analyte solution for AAS, DPSV and ICP-AES, non-interfering matrix for NAA. Values are based on three times the standard deviation of the respective noise or blank level for spectroscopic and NAA methods and given in g/kg x 10^{-6} (i.e., µg/kg or ng/g).

Element	ICP-AES* argon-plasma	Flame	GFAAS**	Cold vapour/ hydride	DPSV***	NAA†
As	100	30	0.3	0.03	0.1	1.0
Ba	0.1	15	1.5	-	-	0.1
Be	0.1	3	0.1	-	-	-
Cd	1	3	0.003	-	0.0005	0.2
Co	1	15	0.15	-	0.01	0.05
Cr	2	4.5	0.3	-	3.0	1.0
Cu	0.5	1.5	0.06	-	0.005	0.5
Fe	0.8	15	0.1	-	30	2.0
Hg	8	300	-	0.015	0.02	0.001
Mn	0.6	3	0.01	-	1.0	0.5
Mo	3	30	0.1	-	100	0.3
Ni	3	3	0.08	-	0.001	200
Pb	30	15	0.06	-	0.001	-
Se	20	150	1.5	0.03	0.05	0.02
Sn	40	30	0.3	-	0.01	2.0
Te	60	75	0.3	-	-	0.1
Tl	10	30	0.3	-	0.01	-
V	1	75	3	-	100	0.1
Zn	0.5	1.5	0.0015	-	0.01	0.1

*ICP-AES, compromise conditions, data after ref. 148; detection limits vary considerably with the conditions applied and the instrumentation used and values given here should be considered as optimal.

**AAS, data from ref. 149 and measurements of the authors; graphite furnace data are calculated for a 50-µl sample, which seems more reasonable than the usual 100-µl based calculation; cold vapour/ hydride for a sample of 10 ml. For Hg a pre-concentration on silver or gold-wool can be carried out and the volume much increased. Thus a detection limit of less than 0.001 µg/kg is achievable.

***The data presented represent determination limits and stem mainly from research work carried out in the laboratory of the authors and usually refer to a PAR 174 or equivalent instrument in connection with hanging mercury drop, mercury film or gold disc working electrodes. Average analyte volume in the cell: 20 ml.

†Values given refer to a sample size of 0.5 g wet weight and a thermal neutron flux of 10^{13} cm^{-1} sec^{-1} (ref. 147).

Thus, it can be concluded that despite some discrepancies in the judgement of possible losses, systematic errors arising from this source, even if the worst case is considered, may not exceed 20% at most. This is considerable if precision analysis is required. However, compared with errors possibly introduced by contamination, this is a minor problem which nevertheless also should be evaluated further.

3.3. ANALYTICAL METHODS

This section deals with analytical methods that are either mainly used or still not in common use but are very promising for trace metal analysis in biological materials. The methods are listed in the order of importance for the mentioned task, briefly introduced and their instrumentation and performance discussed.

In Table 3.2, based on several recent compilations and also the experience of the authors, average detection limits for a number of elements that can be obtained by various methods are listed[25,30,80,147,149-152]. The given data refer to interference-free solutions or matrices. The detection limits, wherever possible, are, normalized to the $3s$ value recommended by IUPAC[153]. These data, however, have to be treated carefully, and with some restrictions because in practical analysis the detection limits, due to, for example, matrix interferences and/or blanks, may be higher by an order of magnitude or even more. The latter reasons were discussed in detail in Section 3.2.4.

In Table 3.3 (see Section 3.3.8), a compilation is given of the costs of instrumentation and the cost-benefit ratio for various task-devoted applications.

3.3.1. Atomic spectroscopy

3.3.1.1. Principles

The basis of atomic spectroscopy is the generation of free atoms and subsequent radiation absorption or emission by distinct transitions of valency (photo)electrons within the outermost shell of the atom. The specific identification of elements is possible by characteristic lines attributed to the atomic structure within a range from approximately 200 to 800 nm[149,154-158].

In atomic-absorption spectroscopy (AAS), the principle that an atom is able to absorb at the same wavelength at which it emits radiation is used for analysis. The transformation in the atomic state is performed by applying thermal energy, then the light absorption of the radiation from an appropriate light source is measured. The atoms involved are predominantly ground-state atoms from

its electronic configuration, the number of which is proportional to the total number of atoms present. As the so-called resonance lines, representing the energy absorption by ground-state electrons, are much less frequent than the emitted lines, the absorption spectrum is simpler than an emission spectrum.

Because of relatively weak light sources and the necessary sequential or simultaneous measurement and subtraction of the transmitted and initial light intensity, the dynamic range is limited. This makes the simultaneous determination of elements occurring at different concentration levels difficult and hence AAS is more or less a single-element approach.

In atomic-emission spectroscopy (AES), the sample is atomized by means of arcs, flames or plasmas, and the atoms generated are excited to emission. As various transitions are possible, the spectra, particularly those of heavier elements, contain numerous lines, the number of which also depends on the temperature applied. The radiation practically depends only on the number of atoms present in the exciting system. The dynamic range of intensity versus concentration is thus high and makes AES an excellent multi-element approach.

In atomic-fluorescence spectroscopy (AFS), the atoms generated as described for AAS absorb characteristic lines from appropriate sources, producing excited states. The decay of these excited atoms by emitting the excitation energy (fluorescence radiation) is not only proportional to the number of atoms present but also to the intensity of the source. Thus, AFS can provide extended dynamic ranges comparable to those of emission spectroscopy combined with the simplicity of atomic-absorption spectra.

2.3.1.2. Atomic absorption - instrumentation and performance

The combination of AAS with flame excitation, first proposed 1955, started a unique expansion[159]. Owing to its sensitivity, specifity, element coverage, speed, precision and the initially inexpensive instrumentation, despite the fact of being a single-element method, flame AAS quickly dominated other established analytical techniques, as is obvious by the vast number of papers published and by the adoption of AAS in numerous laboratories. In 1970 more than 10^4 AAS instruments were in use around the world[160,161]. The neglect of the paramount influence of non-atomic absorption, however, for many years led to some confusion and disappointment. The commercial availability of compensation methods by continuum light sources could partly improve this situation for flame AAS and subsequently also for GFAAS. In 1969, the first commercially available graphite furnaces were introduced. They promised, however, for the price of a considerably lower sample throughput and a poorer precision, detection limits several orders of magnitude lower than with flame AAS. Thus, in principle a simple dissolution-dilution and subsequent injection into a graphite furnace

as well as solid sampling seemed within easy realization. However, as a result of the tremendously increased sensitivity and the use of graphite as the basis for thermal excitation, numerous problems arose, ranging from severe deficiencies in non-specific absorption correction over gas-phase reactions which form signal-suppressing volatile molecules[162] to complex reactions of the matrix and also the elements to be determined with the surface of the graphite tube. The latter also can be considerably affected by random differences in particular tubes or batches of tubes changing the resistance (temperature), the useful analytical lifetime and thus also peak heights drastically[163,164]. Those error sources were not completely understood earlier and in numerous laboratories were not even recognized[165]. If the optimistic forecasts of the manufacturers are considered, this indeed constituted a severe disappointment. In the meantime, however, owing to valuable contributions from both the practical and the theoretical viewpoints, a better insight into physical and physico-chemical mechanisms of interference effects and accuracy deficiencies have been achieved, and new concepts for a better performance have been evaluated[165-171].

From these understandings, some instrumental progress could be achieved. The present state of the art in AAS will therefore be critically discussed, taking into account several improvements and new concepts mainly for commercially available systems.

The scheme of atomic-absorption systems in principle is still very similar to that known for many years. An AAS device consists of a radiation source, an atomizer, a wavelength isolator (monochromator), a radiation detector (photomultiplier) and a readout system. Despite the fact that this has not changed fundamentally within the last decade, particularly within the last 4-5 years, significant progress has been made in the details and components.

Relatively early, emission interferences from atomization devices were minimized by the use of modulated light sources, that is, the so-called a.c. techniques. This was followed by the introduction of more powerful light sources, such as high-intensity and electrodeless discharge lamps (EDLs), to improve signal-to-noise ratios and provide a better detection limit. Also, background correction systems working with continuum lamps were improved, either by modifications to the optical system with respect to lamp position, lenses and mirrors or by the use of a two-source system. The latter consists of a UV (deuterium lamp) and a visible (tungsten halide lamp) source for wavelenths up to 400 and from about 400 to about 700 nm, respectively, also with considerably increased light output[149].

During compensation operation, the signal of the continuum source only corresponding to non-specific absorption but in a distinct "spectral window"[149] is electronically subtracted from the very sharp signal of the line source. The

latter, of course, includes both specific and non-specific absorption. This system, used for years and still predominantly applied, however, has serious limitations. Even under optimal conditions, with the highest possible intensity for the continuum sources and perfect optical and electronic matching of the light beams of the line and continuum source, this correction system can only reliably correct static (i.e., flame) signals up to an absorbance of about 1.0. The compensation ability for dynamic signals (cup, cold vapour-hydride techniques, graphite furnaces) is always much poorer. If the background, however, is structured, a systematic error in principle cannot be avoided, owing to the different principles of the reference and resonance beams. In analytical routine, however, owing to a sometimes less than optimal radiation of the lamps and a mismatch of the reference and line source signals optically and electronically, optimal conditions are seldom attainable. This is of particular importance if the system is operated close to the determination or detection limit. Thus, the performance of an AAS instrument of this design must be checked frequently to avoid serious systematic errors from this source. This can easily be achieved if there is a non-resonant line of the element to be determined close to the analytical line. Application of continuum compensation at that line should result in approaching zero level if compensation is operating correctly.

The commercial introduction of the Zeeman effect-compensated instruments was a significant improvement in background compensation. The Zeeman effect is the splitting of spectral lines of an atom into three (or more) components under the influence of a magnetic field. If splitting is applied to AAS resonance lines, both measurement and correction are possible, applying the different properties of the shifted lines of a single light source[172-175]. This makes possible very efficient compensation for non-specific adsorption at the same wavelength or very close to it without the need for the difficult beam matching, which always is prone to errors as was mentioned above.

Two different designs, each in various modifications, may be applied in instrumental designs, that is, shifting of the source and shifting of the analyte. The latter is also called the inverse Zeeman effect[174]. Both of these principles are now used in commercial instruments.

Source shifting is similar to the application of a non-resonant line of the same element for compensation, as applied in some older instruments[149,155], in that it generates a pair of non-resonant lines close to the resonance line[176]. Source shifting has the advantage of providing more flexibility for the design of atomization devices. This was used to introduce an instrument with a graphite furnace particularly devoted to solid sampling by the manufacturer Gruen[177-179]. Problems may arise, however, with the special light sources needed, which seem to require some additional development.

Analyte shifting utilizes the polarization characteristics of the analyte resonance line to perform background correction and thus can utilize all commercially available light sources. Two instrumental concepts, one of them offered by Hitachi as an improvement of the first successfully applied device[180], also including flame Zeeman compensation, use analyte shifting. The Hitachi system places the sample in a constant (a.c.) magnetic field. By alternately polarizing the incident light parallel and perpendicular to the magnetic field, one sees first background absorption and then background and sample absorption. Thus, the device is a true double-beam spectrometer with both beams traveling the same path length at the same wavelength. The earlier introduced system was shown to compensate effectively non-specific absorption up to about 1.7 absorbance units in the analysis of Pb and Cd in biological materials (e.g., refs. 181-184). Another system, very recently introduced by Perkin-Elmer, is integrated into an already existing, fully computerized device (PE 5000). It applies a modulated (d.c.) magnetic field. In operation, one sees first, if the magnetic field is on, background absorption and, if the field is off, background and sample absorption. The operation mode, as in the other source-shifted design, is a true double-beam mode with compensating abilities claimed to be around 2.0 absorbance units. The appearance of a negative slope of the calibration graph at increasing analyte concentrations, however, as known from a.c. magnetic field systems as a real disadvantage of Zeeman AAS, was less pronounced in the d.c. mode[185,186].

Doubtless, the wide availability of Zeeman AAS, with at present three competing designs, will help to minimize most of the hitherto observed interferences due to molecular absorption. This, however, is valid only if the background does not also possess Zeeman spliting potential. In that case, comparison with continuum source compensation or a physically different approach is required in order to avoid erroneous data.

Some progress could also be achieved with atomization devices. Despite the extraordinary growth of electrothermal methods, particularly true for the analysis of biological materials, flame AAS is still used in numerous laboratories if higher concentrations have to be analysed. Flame AAS is also superior to electrothermal techniques, usually without requiring expensive accessories, if precision, sample throughput and matrix interferences are considered. In comparison with the graphite furnace, with a typical day-to-day reproducibility of ca. 5% (at >0.05 absorbance), the flame attains a typical day-to-day reproducibility of ≤2%. The duration of a single measurement with a flame on average is 10 sec (with integration), whereas electrothermal methods on average require about 100 sec[149,187]. This greatly influences the analytical capacity and also cost/benefit ratios, and should be carefully considered if a decision about the routine application of a particular method has to be made.

There are also techniques for enhancing flame sensitivity. They consist in the improvement of burner design[148,149,155], input into the flame in organic solvents after chelation-solvent extraction[149,156,188] and the boat or cup technique. The last approach uses a boat or cup made from tantalum or nickel, which contains the dried residue of a known amount of a liquid sample. It is inserted into the flame for the determination of easily atomizable elements such as Ag, Pb and Cd[149,156,189]. This inexpensive and rapid technique can compete successfully to some extent with GFAAS, at least for Pb and Cd determinations in body fluids, as described in the chapters on Pb and Cd. Techniques of this kind may have an even more extended renaissance if applied with the Zeeman-compensated flame.

As manual flame operation is cumbersome, mechanized or even automated sample changers were applied relatively early. More recently the automated injection of up to 100-µl aliquots from multi-sample turntables has provided a very economical use of analyte solutions for the sequential analysis of several elements[190]. Sampling tables have also been commercially introduced for processing, e.g., 50[191] or 95 samples[192], in a fully automated mode. This provided an extremely rapid sample throughput in the sequential mode by highly sophisticated instrumentation.

Also in flame operation with different systems the optimal adjustment is important for reliable determinations, as was recently described in some detail[193].

The generation of elemental Hg or hydrides of As, Se, Sn, Bi, Sb, Te, etc., and the introduction of gaseous compounds at ambient or slightly elevated temperatures for Hg or at temperatures around 1173°K (900°C) into the flame or electrothermally heated devices has been known for years. For Hg, pre-concentration techniques based on the amalgamation of noble metals are also common[149,155]. Several commercial systems are available that can be operated in a semi-automated mode as an accessory to most AAS instruments[149,194].

It should be mentioned, however, that these techniques, frequently claimed to be interference-free, can produce serious errors if used without particular experience. These error sources can be eliminated only by the application of procedures that have been evaluated very carefully with the aid of independent methods, as has been demonstrated for Se[195,196].

As already discussed, graphite furnace techniques suffer from serious inherent limitations owing to the basic concept of tube atomization and the properties of the particular graphite used. Some progress could be achieved from the work of numerous experts for commercial systems.

Automated sample injection, now offered for nearly all GFAAS systems, introduced a remarkable increase in precision compared with manual sample introduc-

tion. Thus, a significant reduction of manpower in GFAAS could be obtained. In principle, round-the-clock operation has now become possible[58,100,163].

Problems due to carbide-forming metals or those with high boiling points can be minimized by impregnation of graphite tubes with compounds of Ta and W or a surface coating with pyrolytic carbon[58,149,166,170,197]. This technique is now also applied to commercially available graphite tubes and enhances the analytically useful lifetime of tubes and also the detection power for elements such as Al, Ba, Co, Cr. Mn and Ni. A disadvantage, however, is that owing to an attack of the rather thin coating, a significant decrease in signal height can sometimes occur[197,198]. This may also generate systematic errors if the behaviour of the tubes is not controlled.

L'vov[171] proposed the insertion into the graphite tube for the injected sample a small platform, preferably made from a highly resistent material such as pyrolytic carbon. This is claimed to result in a more uniform thermal environment and thus in more predictable results. This could in principle be confirmed in practical applications, particularly for solid samples. This approach has also been commercialized[179,199,200-203].

Despite the fact that this is a very recently introduced technique, from the still very limited experience it appears that platform applications together with an improved background compensation, the use of appropriate matrix modification where necessary and peak-area rather than peak-height evaluation promise gains in accuracy and precision.

Moreover, remarkable technical and electronic improvements in commercial atomizers have been achieved. They consist, for example, of independently controllable internal and external gas flows, temperature programming and rapid heating within the concept of fully computerized operation, for wavelength, lamp and optics adjustment[204,205]. Attempts have also been made to effect temperature control either in the atomization stage[206] or over the whole temperature range[207]. Systems of that kind, however, are still far from optimal because they permit only relative programming without providing absolute temperature indication and control.

Finally, it should be mentioned that within the last few years significant improvements in all modes of AAS have be achieved. This will now enable the skilled analyst to rely more on AAS results than in the past. Regrettably, these improvements pushed the prices of basic instruments and accessories nearly to the level of other spectroscopic systems, which can provide simultaneous multielement analysis (see Table 3.3).

3.3.1.3. Atomic emission - instrumentation and performance

An instrumental system for emission spectroscopy consists in principle of (1) the atomization-excitation part, (2) a spectrometer, where the emitted radiation is separated into a line spectrum, and (3) appropriate means for recording and sometimes also evaluation of the usually very complex spectra[14,22,23].

Classical AES methods with d.c. arc or a.c. spark for solid samples, despite high instrumental costs, provide relatively poor accuracy and precision. For decades, together with colorimetric methods, they played an important role in trace analysis, because at that time this type of AES was the only available multi-element technique. The appearance of NAA, AAS and, for higher concentrations, radiation counting in XRF later precluded a further significant growth of classical AES, despite some obvious improvements in flame emission spectroscopy as well as in optics and electronics.

The introduction of novel approaches in commercially available systems, however, has changed this situation drastically within the last decade. The use of plasma sources for atomization-excitation [the most frequently applied at present is the inductively coupled plasma (ICP) source] significantly improved detection limits, accuracy and precision. The detection limits for about 40 elements could be decreased to a few nanograms per millilitre or even less[148,157, 208-211] (see also Table 3.2).

Owing to promising accessories such as electronic emission detectors coupled with either a conventional dispersion unit or échelle gratings, there are now a number of different systems commercially available. These systems offer a wide variety of designs, ranging from simple instruments permitting only sequential operation to highly sophisticated devices for the simultaneous multi-element determination of up to 40 elements with background subtraction. These novel approaches have doubtless significantly increased the importance of advanced AES in recent years for trace element analysis. There are now numerous instruments, predominantly in the sequential mode, in operation in all kinds of research and routine laboratories. The costs of the currently available instrumentation range from about that of highly sophisticated AAS systems to a high level for fully computerized AES systems capable of performing simultaneous multi-element determinations with background correction (see Table 3.3).

As plasma sources provide temperatures above 5000°K in contrast to, e.g., all modes of AAS, this will render possible a significant decrease or even elimination of matrix interferences. Moreover, owing to its multi-element potential and superior detection power for various elements and an equal or only slightly inferior precision, plasma AES at present competes strongly with flame AAS. This leads to nearly complete superiority for ICP-AES if multi-element determinations are required, as is also often the case in the analysis of biological materi-

als[210-212]. It has been shown that similarly to the injection technique described for flame AAS (see Section 3.3.1.2), small-volume samples can also be used for ICP-AES[213,214].

Despite numerous obviously very promising properties of plasma AES, it appears that compared with the already advanced high-performance AAS-flame systems now available, some improvement in performance and components is still necessary. That, however, seems to be achievable in the near future[211].

From the present state of the art and/or the principles of this approach, the following disadvantages or limitations are obvious: the occurrence of spectral interferences and precision and signal drift deficiencies due to air conditioning or cooling problems, the influence of analyte solutions (density), introduction of the sample as a slurry into the nebuliser[211,215-217]. Also, the type of plasma used can greatly influence the order of magnitude of particular interferences encountered. Ionization suppression may occur in the presence of sodium[211]. Thus, also in ICP-AES the introduction of organic extracts of trace metals may be useful approach to improve precision and accuracy[218].

Not yet commercially introduced, but simple and particularly promising for those elements which are less sensitive in AAS, is the use of carbon furnaces as excitation source for AES. This may provide in the future routine sequential or oligo-element determinations using systems based mainly on commercially available atomic-absorption components of moderate cost, as recently proposed and demonstrated by several workers[219-222].

3.3.1.4. Atomic fluorescence - instrumentation and performance

Owing to the simple fluorescence spectrum, a setup useful for atomic-fluorescence spectroscopy (AFS) consists of an appropriate source, an atomization device, a monochromator and the detection system[23,155,158]. Compared with AAS there are some advantages. In AAS, for example, the radiation source has to be placed on the optical axis, whereas in AFS the source is usually placed at right-angles to the optical axis. Thus, direct source influences, for instance from other wavelengths, very often a limiting factor in AAS, are almost negligible in AFS. Moreover, the sensitivity attainable, if lower concentrations are considered, depends only on the intensity of the light source.

An advantage deriving from the principle of AFS is that it provides broader dynamic ranges, which, together with more powerful light sources, e.g., lasers for sensitivity enhancement[223], should make possible multi-element or at least oligo-element analysis. For some elements AFS further has superior detection limits to AAS[23,158].

Despite these promising properties and several successful applications, e.g., Cd determinations in body fluids, which also demonstrated the possibility of a reliable background compensation[222], wider routine application has not yet occurred.

It appears that the substantial growth of plasma AES techniques in the last decade and the considerable recent progress in AAS instrumentation (see Sections 3.3.1.2 and 3.3.1.3) have prevented the development and commercial introduction of AFS instruments. From present progress based on important past developments, however, there is still a distinct hope that these instruments will be available in the near future, possibly also using the advantage of Zeeman effect-based background correction[158].

3.3.2. Voltammetry

3.3.2.1. Principles

Many inorganic and organic compounds can be reduced by uptake of electrons or oxidized by release of electrons at an inert test electrode if a potential characteristic of a particular compound is adjusted. Such an electrode process, proceeding in either a cathodic or an anodic direction, can be followed by recording its current-potential curve. The electrode potential is the adjusted parameter and the current the measured signal. The complete or partial recording of this current-potential curve, or its first or second derivative, constitutes the principle of polarographic and voltammetric analytical methods[80,224-229].

The most promising properties of this analytical approach become obvious if the large charge transferred per mole of any polarographic active compound is considered. This charge is the number of electrons taken up or released by the species undergoing the electrode reaction multiplied by 96,500 C (Faraday constant). As the faradaic current its proportional to the concentration of the chemical species of interest in solution, voltammetric methods offer excellent potential for the trace and ultratrace analysis of numerous heavy metals and metalloids that are easily accessible to such electrode reactions.

Applications of voltammetry are based mainly on mercury electrodes, but working electrodes made from other materials, for example various forms of graphite, carbon paste, glassy carbon and gold, are also common.

If the test electrode is a dropping mercury electrode (DME) with a typical drop time of a few seconds, the method is termed polarography. Techniques applying stationary test electrodes are termed voltammetry.

3.3.2.2. Instrumentation and performance

In classical d.c. polarography, the sensitivity is limited, with typical determination limits around 5 x 10^{-6} *M*. Methodological improvements, for example the introduction of pulse and differential pulse polarography and voltammetry[228-230], and the use of stripping techniques[150,229,231-233], provided significant improvements in the determination limits for many heavy metals. In pulse and differential pulse voltammetry or polarography the potential is applied in the form of a train of rectangular pulses. These techniques can attain typical determination limits at the 10^{-8} *M* level whether the electrode reaction is reversible or irreversible.

A further increase in sensitivity can be obtained by electrochemical preconcentration of metals performed by a cathodic deposition followed by anodic stripping of the metal to be analysed. In order to speed up mass transfer, stirring or the use of rotating electrodes is advantageous. The deposition time depends on the concentration level in the analyte solution and the volume of the applied test electrode, but usually lies between 5 and 15 min. It is emphasized that this in situ electrochemical pre-concentration has the significant advantage of adding no contamination risks, in contrast to all chemical preconcentration procedures common in trace metal analysis. Subsequently, in the so-called stripping step the potential of the working electrode is scanned usually in the differential pulse mode in an anodic direction. This most important voltammetric mode for trace metal analysis is termed differential pulse anodic stripping voltammetry (DPASV). The most common test electrode is the hanging mercury drop electrode (HMDE), which can be used at analyte concentrations down to 0.1-0.05 µg/l.

With the mercury film electrode (MFE) on a specially prepared glassy carbon support, determination limits down to less than 10^{-12} mol/l in the analyte can be attained for Pb, Cd, Bi and other metals[234].

Differential pulse anodic stripping voltammetry (DPASV) with a mercury electrode is applicable, but with different sensitivities, to metals and metalloids such as Bi, Cd, Cu, Ga, In, Mn, Ni, Pb, Sb, Tl and Zn. Metals with an oxidation potential anodic to mercury as Hg and arsenic at low concentrations require solid electrodes[81,235,236].

Owing to solubility and amalgamation problems with mercury electrodes, solid electrodes have been shown to be advantageous for other elements, for example, the gold electrode for the simultaneous determination of Hg and Cu.

A recent and powerful principle for the simultaneous determination of Ni and Co was recently introduced in routine trace analysis. It is based on the application of d.c. or differential pulse voltammetry after prior interfacial accumu-

lation by an adsorption layer of a metal chelate at the HMDE. In this case dimethylglyoxime (DMG) is used. This method attains detection limits below 10^{-11} mol/l for Ni and around 10^{-10} mol/l for Co[151].

For a number of metals, the described voltammetric approaches compete favourably with respect to determination power and reliability with other important trace analytical methods such as GFAAS, ICP-AES and NAA. For the simultaneous determination of Cu, Pb, Cd, Zn, Ni and Co in fresh waters, sea water, rain and drinking water, DPASV is now regarded as the method of choice. However, also for the analysis of trace metals in biological materials DPASV has become increasingly a powerful and in many instances superior alternative to the non-electrochemical methods mentioned before[45,60,80,81,150,151,237-241].

A significant advantage of voltammetry is that the instrumentation is far less expensive than that for most other analytical methods (see Table 3.3). Commercially available voltammetric instruments are all of multimode design. Thus, they offer to the analyst the choice of a variety of powerful modes. This allows the application of the technique best suited to the required task. Further, the compactness of modern polarographic equipment and its low vulnerability to mechanical and electronic breakdown make it the most favourable method for field studies with, e.g., mobile laboratories and on board research vessels. Moreover, it should be noted that voltammetry is an oligo-substance method. Thus, it provides the advantage that several metals, e.g., the groups Bi, Cu, Pb, Cd, Zn or Ni, Co or Cu, Hg can be determined simultaneously in the same run.

An earlier disadvantage of voltammetry was the time needed for a single analysis and hence the manpower required in daily routine. The introduction of automated, computer-controlled systems will soon overcome this limitation. This will be possible, of course, without precision and specifity deficiencies. The extended use of microprocessors and dedicated computers, already commercially available, promises significant cost savings even for highly sophisticated voltammetric instruments[343]. Hence, in numerous research fields and for important toxic trace metals such as Bi, Cu, Pb, Cd, Zn, Ni, Co, As and Hg the present preference for the application of various modes of AAS over voltammetric methods is expected to disappear[150]. Particularly if reliable high-performance analytical data for several simultaneously determinable heavy metals are required, voltammetry, despite its more stringent digestion demands, is becoming the determination method of choice (80, 184, 233, 241). Moreover, voltammetry is an oligo-substance method. Thus, it provides the advantage that several metals, e.g., the groups Bi, Cu, Pb, Zn or Ni, Co or Cu, Hg, can be determined simultaneously in the same run. This still seems to be valid if the gains in accuracy in AAS by

the introduction of improved background correction by Zeeman compensation, GFAAS platform, hydride and cold vapour techniques are considered.

A limitation, however, is that only a distinct, although important, number of metals are easily accessible to polarography and voltammetry compared with, for example, atomic spectroscopic methods. As is usually the case for most other non-electrochemical trace analytical methods, also for voltammetry the direct determination of trace metals in biological materials requires a prior digestion for the total destruction of organic matter, as already discussed in detail in Section 3.2.2. The reason is that numerous organic species, owing to their surface activity, often inhibit or even suppress the electrode reaction and their elimination prior to the voltammetric measurement is therefore essential. This digestion step may be prone to contamination errors, which limits to some extent the tremendous determination power attainable in principle for several metals in voltammetry, owing to the remaining blank levels in the 0.05-0.5 μg/l range after application of digestion procedures[45,60,80,81,151,239,241].

In important special cases the usually required mineralization by ashing procedures can be substituted by rapid pre-treatment alternatives. An important example in occupational medicine is the determination of Pb in whole blood and blood serum. The blood sample is treated for several minutes with a high, swamping Ca(II)-Cr(III) concentration (ca. 6 *M*) with swirling. In this manner the Pb is transformed by ion-exchange processes into species readily accessible to subsequent anodic stripping voltammetry at a large-area tubular mercury film electrode on a graphite support[242].

3.3.3. Nuclear activation analysis techniques

Nuclear activation has been applied for decades in the trace and ultratrace analysis of numerous elements as a real multi-element approach. Thus it can be regarded as a classical approach in trace element analysis.

3.3.3.1. Principles

If an atom (isotope) of an element is bombarded (irradiated) with particles or photons, inelastic scattering either raises its nucleus into an excited state or transmutes it into another element or isotope. The latter takes place by absorbing the bombarding particle and forming a compound nucleus. Reactions of this kind, predominantly achieved by slow- or fast-neutron nuclear reactors or, less frequently, by high-energy charged particles and high-energy γ-ray photons from accelerators, are also called "activation" reactions.

Usually, subsequent to the scatter and the intermediate formation of a compound or excited nucleus, a distinct elemental particle or radiation is released

immediately and the resulting nucleus becomes relatively stable. Following this, an excited nucleus can "decay", that is, with emission of particles or quanta it reaches a lower energy state. The decay follows the rules of a first-order reaction as the intensity of the radiation is directly proportional to the number of excited atoms present. Consequently, the number of excited nuclei of a certain element (isotope) is proportional to the absolute number of atoms of that element present in the sample.

A particular activation depends on the type, energy and density (flux) of exciting particles or quanta and the statistical probability of the bombarded nucleus reacting, which is termed its "cross-section" (unit: barn).

The emitted radiation mainly consists of γ-quanta, β-particles and α-particles. Specific and sensitive detection and also quantitative determination are possible from the energy and the half-life of the radionuclide formed, i.e., the time span within which 50% of the initially formed radioisotope has decayed.

Because almost every element can form excited isotopes, the spectra obtained are usually complex, so that for reliable analysis very sophisticated detection systems and/or radiochemical separation procedures prior to counting are required. Activation analytical principles and techniques for biological materials have been extensively treated in detail in various books and reviews (e.g., refs 243-247). There are also tables available listing the properties of each individual radioisotope[248] and practical radiation identification and evaluation sheets[249].

3.3.3.2. Instrumentation and performance

Nuclear radiation detection is achieved in various energy-absorbing media (gases, liquids and solids), which are able to transform the initial energy into ionization and/or light energy. This transformation must be proportional to the initial energy.

For numerous elements and materials it is sufficient to count and evaluate the spectra of an irradiated sample without any further treatment (instrumental neutron activation analysis, INAA). Highly sophisticated instrumentation is available for this purpose at comparatively moderate cost (see Table 3.3). Such instrumentation consists of high-energy resolving Ge(li) semiconductors coupled with multi-channel analysers or separate computerized data evaluation systems. INAA-analysis proceeds in a simple way and thus can frequently be carried out in a completely automated manner with a throughput of large numbers of samples.

If, however, very complex spectra with overlaps in the energy range of interest occur or if very low determination limits are required, a radiochemical group separation or even a separation of single elements becomes necessary[111-113]

(see Section 3.2.3). In such cases the less sensitive Ge(Li) detectors may be replaced by more sensitive NaI detectors, which show, however, poorer energy resolution.

If the nuclide to be determined emits β-particles, which is often the case, β-counting techniques with nearly 100% counting efficiencies by liquid scintillation and gas-filled 4π counters are used. Liquid scintillation counting, for example, permits computer-controlled counting with pre-selected double or triple channel evaluation and the automated processing of up to several hundred samples. The high counting capacity of up to 6×10^6 counts/min that is attainable provides an extremely high dynamic range, which is advantageous for radiotracer studies also.

As natural radioactivity contains, in addition to a distinct γ-background, high-energy particles, for example from cosmic radiation, despite careful shielding there is always a definite background level that influences the detection limits attainable. Thus, extremely low detection limits require special and sometimes expensive arrangements, such as anticoincidence devices.

An advantage of radioactivity counting is that by increasing the counting time, owing to the simple dependence of error on count rate, the determination limits can be improved significantly. The standard deviation depends on the square root of the count rate. The corresponding improvement attainable is, however, only feasible if the half-lives of the radionuclides considered are sufficiently long to afford extended counting times.

In addition to its remarkable potential as a fingerprint method (e.g., ref. 250), a major advantage of activation analysis is that the contamination risk is inherently lower than those of other trace analytical methods, because frequently the samples can be irradiated without pre-treatment, which always may be prone to contamination. Nevertheless, even sample handling prior to irradiation can introduce contamination if not properly performed[115]. If the staff are well trained and all precautions are taken, however, activation analysis can be regarded as a very promising checking method for other analytical approaches that require a particular sample pre-treatment expected always to be affected by a certain contamination risk.

As in other trace analytical methods, activation analysis also is not an absolute method, owing to flux variations and matrix influences. Hence it always needs standardization. The absolute sensitivities attainable for a large number of elements accessible to activation techniques depend on individual cross-sections, on the available flux of activation particles, irradiation and "cooling" periods and the counting efficiency. Sensitivity data can be found in various compilations[22,25,147,152,247,251]. For metals and metalloids of particular tox-

icological interest typical determination limits of various trace analytical methods are compared in Table 3.2.

The precision of NAA in general is comparable to that of GFAAS, i.e., a day-to-day precision of ca. 5% is attainable under optimal conditions with some exceptions in very favourable or also less advantageous cases.

Limitations to the broader routine use of activation methods are the dependence on nuclear research reactors or accelerator facilities and the costs of irradiation, storage and radioanalytical processing in lead-shielded cells. Often the necessary cooling time makes a rapid instrumental determination in a particular matrix difficult or even impossible if the generated interfering activity, such as that of ^{24}Na, is very high. Another disadvantage, particularly of NAA, in application to biological materials is the poor sensitivity for toxic metals such as Pb, Cd and Ni, which are usually not accessible to instrumental techniques. Particle and photon activation provide a superior potential for a few elements of interest but frequently are limited by the restriction to very small (and thin) targets and serious quantification problems. This also limits the routine application of activation techniques in baseline studies and biological monitoring despite there doubtless value for certain research purposes. Hence, in general, the application of nuclear activation analysis will be restricted to specially equipped laboratories and cannot be regarded as an approach for general routine application.

3.3.4. X-Ray fluorescence

3.3.4.1. Principles

If atoms are bombarded with radiation of distinct energy, electrons can be removed from an inner shell, the K, L, M, ..., shells, resulting in excited atoms. This causes an electronic rearrangement in that electrons from outer shells fall into the "holes" or vacancies left by the ejected electrons according to definite transition rules. The electromagnetic radiation thus emitted presents a characteristic, simple and readily predictable spectrum for each excited element: the frequency (ν) of the characteristic lines is proportional to the square of the atomic number (Z). The radiation, after an appropriate wavelength or energy separation, can be used for the non-destructive qualitative detection and quantitative determination of most elements. Roentgen, who discovered the effect in 1895 while experimenting with cathode rays, called this radiation X-rays.

In comparison with optical atomic spectroscopy, where mainly photoelectrons of the outer shell are involved, the transition of outer electrons into inner shell vacancies leads to a comparably high-energy radiation with a range from

about 0.6 up to about 120 keV. Expressed in terms of wavelength, this is equivalent to a range from 2 down to about 10^{-2} nm. The energy of the emitted X-rays increases depending on the atomic structure and hence the wavelength decreases with increasing atomic number.

Characteristic X-rays can be generated in the classical manner with X-ray tubes, with X-rays or γ-rays from radioactive sources, and with particles such as electrons, protons, α-particles or even heavier ions from appropriate accelerators. The use of X-rays as exciting radiation is termed X-ray fluorescence. In particle-induced X-ray emission (PIXE) a high-X-ray intensity is generated by the incoming particles losing almost all of their energy in travelling through the sample. PIXE is advantageous for very thin samples and the excitation of elements of low atomic number.

Fundamental aspects and also applications of X-ray methodology have been treated by various authors (e.g., refs. 23, 252, 253, 255-257).

3.3.4.2. Instrumentation and performance

An X-ray spectrometer consists of (1) an X-ray generating system, (2) the sample compartment, (3) a dispersion/detection system and (4) electronics and readout.

Wavelength-dispersive spectrometry produces the exciting X-ray in a high-voltage tube with high output (high wattage) by electron bombardment of an appropriate target made from, e.g., tungsten. As the generated radiation is not monoenergetic, appropriate filters or secondary targets, excited by the primary tube radiation, are also common. For reliable results the sample must fulfill distinct demands of size, density and surface structure. Liquids are also useful.

The characteristic X-rays generated pass through a collimator and are diffracted by an analysing crystal. The sequential detection of the separated lines is performed by moveable collimator-detector or detector-crystal combinations mounted on a goniometer circle. The counters used are scintillation or proportional (gas) counters. Commercially available arrangements of this kind range from simple to very sophisticated systems with computer evaluation.

If large numbers of samples have to be analysed for several elements, multiple crystal spectrometers and an automated sample supply can be used. These instruments contain a particular crystal and detector for each element to be analysed, and are fully computerized for simultaneous multi-element determination.

Wavelength-dispersive systems have a low overall efficiency, owing to radiation losses at the analysing crystals. Moreover, the proportional counters frequently applied have a low detection efficiency at lower wavelengths, i.e., higher energies. Hence strong radiation sources are needed for satisfactory working conditions but the operation mode allows the handling of a high count rate per energy interval.

In energy-dispersive instruments, excitation is performed by tubes of lower output (low wattage) by secondary targets, by radioactive sources or by charged particles. The excited radiation is detected by Si(Li) semiconductor detectors, which have a poor resolution at low energies but satisfactory resolution at higher energies. As in nuclear radiation evaluation, multi-channel or computer modes can be applied. However, as the signals attributed to all the energies appearing reach the detector simultaneously, the data acquisition rate is limited to a total of about 5×10^4 counts[258], which necessitates computer evaluation by particular approaches such as unfolding[259].

If, instead of a light beam, an electronic beam is used and focused on to a sample using adapted microscope optics, very small areas can be investigated. The resulting X-ray spectra can be evaluated qualitatively, and also quantitatively if the sample is prepared in an appropriate manner, e.g., on filters[260]. Those microprobes use different means of resolution and computer evaluation but are expensive (see Table 3.3).

Advantages of X-ray methods are their comprehensive element coverage from atomic number 6, their non-destructivity and the unique potential for simultaneous multi-element determinations. This makes XRF an ideal fingerprint approach. Disadvantages are that with multi-component samples of more than a critical thickness the intensity of any analytical line in principle becomes matrix dependent. Hence this intensity must be expected to vary with the amount of every element with $Z > 20$[253].

As back-scatter effects from the exciting radiation can also influence spectra and determination limits, the use of certified standard reference materials with a composition as close as possible to the material to be analysed is essential. If such materials are not available, careful analysis by independent methods is required in order to obtain reliable data. Fortunately, if trace metals in light, for example carbon-rich, matrices are to be analysed, simpler calibration approaches, such as dilution techniques, can be applied.

As discussed above (see Section 3.2.3), matrix effects can be overcome by selective separation/precipitation, which also may include group separations.

Conventional techniques in X-ray analysis achieve absolute detection limits in the 10^{-6} g range with, under optimal conditions, a precision of ca. 2%. Hence they are frequently applied in homogeneity studies. The detection power, however, is not sufficient for direct ultratrace determinations in numerous biological materials. Much lower absolute detection limits, down to 10^{-9} g, can be achieved by the already mentioned microprobe technique[260]. The 10^{-11} g level can be reached if energy-dispersive SRF with a totally reflecting sample support and infinitely thin samples is used[261]. These approaches are advantageous, for example, if particular biological materials have to be analysed for trace metals.

The advanced technique using protons as the means of excitation, giving for particular elements absolute detection limits at about the same level, however, has the limitation of depending on expensive accelerator facilities and on extremely small and thin samples, and faces problems with accurate quantification. This makes this technique more promising for metal distribution studies, e.g., in hair[262,263], than for conventional quantitative analysis.

3.3.5. Mass spectrometry

Owing to its comprehensive element coverage with a determination limit of down to 10^{-12} g or even less, mass spectrometry is widely used in fundamental scientific research.

3.3.5.1. Principles

The principle of mass spectrometry (MS) of metals is the generation of gaseous positive ions and their separation and detection according to their mass/charge (m/z) ratio. Ions can be generated thermally, by electron impact, by a high-voltage spark and by ion bombardment. The separation of the ions and/or charged molecular fragments formed can be achieved by energy and mass focusing in strong magnetic fields. The detection of the ions or molecular fragments is performed by photographic or, more recently, electrical detection (direct reading) and allows the precise measurement of isotopes[264,265].

3.3.5.2. Instrumentation and performance

This simultaneous determination of trace elements in widely different materials is performed predominantly by spark-source MS (SSMS) using electrically conducting, e.g., graphite, supports. In the last decade the method has been substantially improved by the introduction of electrical detection techniques.

Limitations, if only the analytical potential is considered and despite the recently improved precision (around 10% under optimal conditions), are the poor accuracy if no appropriate standard reference materials for the correction of matrix interferences are available. Also, the instrumental costs are prohibitively high (see Table 3.3). The use of reliable reference materials, however, can significantly improve accuracy[266]. Separation-concentration procedures can also be used to minimize matrix interferences and to enhance sensitivity in SSMS[267].

Extraordinary precision and accuracy, however, are achieved by the isotope dilution approach[268] using spark-source[23,269,270] or, for more volative elements such as Pb, Cd, Tl, Hg and Zn, thermal ionization or ion-impact techniques[8,265,271-275].

In isotope dilution mass spectroscopy (IDMS), a known amount of an appropriate isotope of the element to be determined is added as a spike to a sample aliquot prior to analysis. Subsequently the mass ratios are determined. As the determination of mass ratios is much more precise than the measurement of a mass/charge ratio signal, this method provides extremely reliable analysis. However, each isotope dilution procedure requires highly skilled staff and very rigid contamination precautions before spiking of the sample aliquot to prevent biased data[118]. If careful operation is maintained, the accuracy and precision are of the order of 1% or less from the milligrams per kilogram down to the micrograms per kilogram level. Hence this approach has been applied with outstanding results as a reference method, but owing to the time needed and the high instrumental costs it cannot be regarded as a routine method. The field of application mainly lied in environmental and toxicological research and also in the very important certification of standard reference materials[9,269-271] or tentative standard materials[8,134]. Particularly for the latter tasks IDMS has proved to be a method of great reliability[265].

The extremely precise determination of isotope ratios makes all modes of MS a unique tool for studies on particular sources of Pb in the environment[275,276]. Also, IDMS is essential for metabolic studies with stable isotopes in man and laboratory animals[277,278].

3.3.6. Other methods for elemental determination

There are some other methods of determination of some value or of probable increasing interest for the analysis of trace elements in biological materials. Colorimetry, catalytic reactions, chemiluminescence and chromatographic techniques are briefly considered below.

3.3.6.1. Colorimetry (spectrophotometry)

The principle of spectrophotometry (optical molecular spectroscopy) is the use of radiation absorption or emission for the determination of particular compounds. If these compounds contain trace elements, this allows their sensitive determination.

In contrast to atomic spectroscopy, characterized by sharp lines due to discrete electron transitions, the absorption or emission of light by molecules is much more complex. Thus, electronic transitions in a molecule corresponding to the exciting radiation are split into various quantized vibrational and rotational energy levels. These different energy levels appear in the vapour phase with minimal molecule-molecule interactions as series of absorption or emission bands. Incidentally, bands of this origin can cause interferences in AAs and AFS.

In the condensed phase, however, solute-solvent interactions transform the sharp lines into much broader bands with characteristic but broad absorption or fluorescence maxima and minima. This is the reason why it is nearly impossible to distinguish a particular compound by a simple measurement at a defined wavelength in a mixture of compounds. Colorimetric trace element determinations, therefore, are feasible in most instances only if, for example, chelating agents and selective separation procedures are applied prior to the measurement[22,23,279].

Instrumental designs for molecular spectroscopy are similar to those for AAS. Because of the broad wavelength maxima, however, the spectral dissolution of the monochromators is at best comparable to those used in AAS. Modern spectrophotometric instruments, frequently designed as double-beam instruments, offer excellent stability, low noise levels and all the advantages of microprocessor control and evaluation such as background correction, integration, automatic changing, scan programming and possibilities for derivative spectroscopy[280]. This also in principle makes possible precision spectrophotometry and the determination of very low amounts of elements, down to the nanogram level, in small sample volumes[281,282]

Molecular spectroscopy is a frequently and, in some analytical branches, particularly in clinical chemistry and biochemical analysis, increasingly used technique. For high levels of toxic elements, for instance in acute poisoning, the method is valuable as a rapid and inexpensive approach for instant medical decisions. In environmental research and for screening purposes, in occupational exposure, however, the importance of spectrophotometry for total metal analysis is declining owing to the further growth of AAS, voltammetry and ICP-AES for routine applications. On the other hand, for some particular tasks in speciation and for the determination of anions there still appears to exist a strong demand for molecular spectroscopic methods[23,283] (see also Section 3.3.7).

3.3.6.2. Catalytic (amplification) reactions

Catalytic analytical methods allow the sensitive determination of trace elements without great expense. However, the selectivity is usually lower than that of other trace analytical methods[284,285]. Sometimes it can be improved by modification of the indicator reaction or by combination with a separation method such as solvent extraction. Thus, it is possible to attain, for example, for Ni, Mo, Fe and Cu, absolute detection limits of a few nanograms per millilitre of analyte[285]. Methods of this kind may be useful where another independent analytical approach is required for testing the accuracy of a routine procedure. At present, however, in the authors' opinion it does not appear that the usage of catalytic reactions will play a significant role in practical analysis.

3.3.6.3. Chemiluminescence

Various chemical transformations produce excited species emitting light quanta to reach the ground state. This effect is called chemiluminescence and can also be used for quantification[286]. Measurement of light emission can be achieved with simple and inexpensive devices and chemiluminescence can reach extremely high sensitivities, e.g., at the low nanogram or even picogram level for elements such as Fe, Hg, Ag, Os, Ru, V, Mn, Cu, Cr and Co. The further study of previously unexploited light-emitting processes together with other aspects as instrumental improvements and specific analytes may significantly increase the potential of this principle. Thus, chemiluminescence in solution and in the gas phase, e.g., for nickel carbonyl[287,288], can be regarded as an analytical approach with positive prospects for a significant increase in importance in the near future.

3.3.6.4. Chromatographic techniques

The principle of analytical chromatography is the different migration rates of compounds through a system of two phases, one of which is mobile. The solid phase usually consists of a supporting material, impregnated with appropriate substances. During analysis the liquid phase passes through this column; thus, the separation of the components of a mixture applied to the top of the column is performed. This process is able to separate numerous components if the column length and properties and the solid phase are properly selected. This procedure leads to distinct fractions containing the desired components producing more or less sharp peaks in the time/concentration evaluation. These peaks can be identified by various types of detectors able to differentiate between the background (mobile phase) and the analyte species[289,290,291-293,299].

In gas chromatography (GC), the mobile phase is in inert gas (e.g., nitrogen, hydrogen, argon). Usually heating is necessary for the volatilization of numerous compounds, which can cause problems with compounds that are not thermally stable[289,290].

In liquid and column chromatography, the method of choice at present is high-performance liquid chromatography (HPLC)[291,292]. The mobile phase is a liquid, hence the temperature is comparatively low and also thermally unstable compounds can be determined.

Modern instrumentation for GC, including automated pressure-temperature-time programming together with numerous accessories, is available at moderate prices (see Table 3.3). The accessories offered range from a wide selection of detectors, such as thermal conductivity, flame-ionization, electron-capture and, more recently, flame and also graphite furnace AAS[297], to very potent dedicated computers for the simultaneous control of several GC units. The costs for HPLC de-

vices are considerably higher than those for GC, owing to the pressurized operation usual in this approach, which requires considerably more complex instrumentation with, e.g., UV, fluorescence[295], Zeeman-AAS[296] and voltammetric detectors[297,298] (see Table 3.3).

One of the most important and fundamental differences between several of the spectroscopic methods, such as AAS, AES, AFS, XRF and MS, and the chromatographic methods is that the latter, as also are voltammetry and spectrophotometry (colorimetry), are sensitive to the chemical species of the metals present, whereas the spectroscopic methods are in principle restricted to the quantification of the total elemental content. This aspect of chromatographic methods will be treated again in more detail in the section on speciation (Section 3.3.7).

As inorganic compounds are frequently not accessible to normal gas chromatography, their prior transformation into uncharged compounds of the metal with organic ligands, for example chelators, is more promising[299]. This renders possible, for example, the simultaneous GC determination of Bi, Pb, Fe, Co, Ni, Hg, Cd, Cu and Zn at the absolute nanogram or even picogram level as di(trifluoroethyl)dithiocarbamates with flame-ionization detection[300]. Co, Ni, Cu and Pb have been simultaneously separated and determined at the nanograms per millilitre level as chelates of tetradentate β-ketoamines using HPLC and UV detection[301]. Further, it has been demonstrated that thin-layer chromatography, as a simple and inexpensive approach, is also useful in combination with, e.g., GFAAS for the identification and quantification of inorganic and organometallic compounds[302].

Techniques of this kind, which at present still cannot be routinely applied, may provide in the near future, in addition to an important role in speciation, also a potential for the simple screening or fingerprinting of various important trace metals even in complex materials. However, as with other trace and ultratrace methods, here also rigid contamination precautions and skilled staff are essential for reliable results.

3.3.7. SPECIES SEPARATION, IDENTIFICATION AND QUANTIFICATION

The currently applied wide variety of separation and determination methods in principle allow the identification and quantification of nearly all species regarded as important in human and environmental toxicology. Doubtless this will be of increasing value for present and future research. In this section, therefore, a few examples will be given of methodological potential and the state of the art in a growing research area.

Provided that total elemental analysis can be performed accurately, for the determination of toxicological significance the identification and quantifica-

tion of valency state(s) is required in numerous instances. This is of particular importance as differing valency states may lead to very different toxic actions, e.g., with Cr, Sb, As and Se.

The determination of the valency state in a biological material, however, is complicated. The problem is that prior to the identification reaction the sample usually has to be pre-treated in such a way that this identification reaction and the subsequent quantification can be performed in a manner that avoids serious interferences from the matrix. Another prerequisite, of course, is that no substantial change in the chemical state of the elements to be characterized should occur during the aforementioned clean-up stage. Clean-up for body fluids, especially urine, is simpler than that for tissues. Clean-up for tissues may even be impossible if valency state quantification is required.

In principle, the identification and determination of valency states of elements from analyte solutions can be achieved by specific operations such as solvent extraction, ion exchange and chromatography with subsequent elemental determinations using various analytical methods[303-307]. A special case is elements accessible to hydride formation with subsequent separation. Owing to the pH-dependent formation of their hydrides, As(III) and As(V), for example, can be easily separated and the proportion of each valency state quantified[308,309].

Organometals and organometalloids play a significant role in the environment. They are produced by either biological or chemical transformation of naturally occurring compounds or by anthropogenic activities[310,311]. As compounds of this kind are either more (e.g., Hg, Pb, Sn) or less (As) toxic for man and mammals than the ionic forms, a clear differentiation is needed.

The most toxic compounds, such as methylmercury and lead alkyls, are simple organometallics. Their analysis down to very low levels is usually performed after appropriate clean-up by chromatographic separation techniques using different detection principles. The procedures applied allow the simultaneous separation and quantification of all relevant species[311-315]. The situation for As, however, is more complex. Inorganic As(III) is significantly more toxic than As(V), followed by low-molecular-weight organic metabolites such as methylarsonic acid and dimethylarsinic acid. The latter compounds are partly produced in man and mammals as metabolic products of As(III) and As(V) ionic forms[316,317] In seafood, which usually contains significant amounts of As at the milligrams per kilogram level[318], this element occurs in stable compounds in some organisms, such as arsenobetaine, which are regarded as much less toxic than the compounds mentioned above. Owing to interferences from As from this source, the determination of total As is meaningless in occupational exposure if consumption of fish takes place. Thus, chromatographic and hydride-forming procedures with AAS and voltammetric detection have been developed and applied to the separation and

TABLE 3.3

COST-BENEFIT RATIO OF COMMERCIALLY AVAILABLE DEVICES FOR INSTRUMENTAL TRACE AND ULTRACE ANALYSIS

Method	Device(s)	Approx. price range (US $ x 10^3)	Properties	Remarks on cost-benefit ratio
AAS	Basic instruments with deuterium compensation and recorder	15-30	Flame only; <20 sec per measurement	Good for single samples, good excellent for series and higher concentrations
	Complete setup with all accessories, deuterium compensation and automated sampling	40-60	All modes possible with microprocessor control; <100 sec for graphite furnace measurement	Poor for single samples, medium for series of normal samples, medium-good for difficult samples, good for 24 h operation
	Complete setup with all accessories, Zeeman compensation and automated sampling	60-80	Not for all instruments. All modes possible but allways with highly sophisticated electronics	Poor for normal single samples, medium for difficult samples, medium-good for series of difficult samples, good for 24 h operation
ICP-AES	Sequential systems, complete unit	40-80	<20 sec per measurement with microprocessor	Poor-medium for normal single samples, medium for difficult samples and series, high gas consumption
	Multiple channel, complete unit	100-200	With background compensation up to 50 elements simultaneously, with complete computer evaluation	Poor for normal single samples, medium for difficult samples, good for series because of high sample thoughput but high gas (argon) consumption
DPP/DPSV	Basic instruments with recorder	8-10	For several elements simultaneously, also species detection	Excellent for single samples, good for series but increasing manpower needed

	Partly computerized setups with all accessories	20-30	Microporcessor control and evaluation	Good-excellent for single samples, excellent for series due to automated operation also for an oligo-element approach and 24 h operation
NAA	Complete system with 4096 channels, computer evaluation and Ge(Li) detector	100-120	Multi-element method; access to nuclear reactor necessary	INAA: medium-poor except for simple series. Radiochemical NAA: poor-very poor owing to high nampower requirement
XRF	Multiple crystal, wavelength dispersive	≤150	Multi-element method with computer evaluation standards needed	Poor for single samples, medium for series, medium-good for 24 h operation
	Energy dispersive	40-60		Poor-medium for single samples, medium for small series, medium-good for larger series, good for 24 h operation
MS	Spark source MS, complete setup with computer evaluation	≥300	30 or more elements simultaneously	Very poor for single samples, poor-medium for small series, medium for larger series, high manpower needed for isotope dilution
	Isotope ratio and isotope dilution with, e.g., thermionic sources	≥200	A few elements simultaneously	Medium for single samples, medium-good for small series owing to outstanding properties (definite method)
Colorimetry	Complete device with microprocessor control	10-25	Second and higher orders, also automated operation	Good for single samples, excellent for series
GC	Complete device with conventional detectors, automated injection and computer control	25-40	Fingerprint method	Medium-good for single samples, good-excellent for series, medium with, e.g., AAS detection
HPLC	Complete with automated injection, UV detector or voltammetric and microprocessor evaluation, conventional detectors	35-60	Fingerprint method, very versatile	Medium for single samples, good for series and with AAS detector, good-excellent with DPSV detector

quantification of low-molecular-weight species[319-323]. The rapid differentiation of these species from the mentioned stable complex As compounds from seafood is possible, however, by a simple reaction with $NaBH_4$, which only transforms the inorganic metabolites[324,325].

Metals in the human body occur only in very low amounts in the form of free ions as they are predominantly bound to the erythrocytes[326,327] and to relatively low-molecular-weight proteins such as metallothionine and similar compounds[328]. Thus, low-molecular-weight proteins able to bind metals are regarded as key compounds in very important investigations on physiological transport and storage mechanisms and with respect to their role in detoxification processes.

Separation, identification and quantification can be performed with techniques suitable for protein separation, that is, all modes of chromatography, particularly also using isoelectric focusing[329,330] coupled with powerful detection systems[331-336]. Particularly advantageous in this context is the application of labelling techniques with radionuclides in the identification of metal binding sites("sinks") in various physiological media[326,327,331,333-338]. Moreover, voltammetry provides a very powerful approach for heavy metal speciation by planktonic exudates and other components of dissolved organic matter in natural waters[232,339-342].

3.3.8. INSTRUMENTAL COSTS, COST-BENEFIT RELATIONSHIPS

The prices of commercially available devices and accessories for instrumental analysis, their sample capacity and estimates of the cost-benefit relationships are given in Table 3.3. This, in principle, is self-explanatory but a few additional comments will be given. If large numbers of similar samples have to be analysed, despite the high investment partly or even completely automated systems for AAS, ICP-AES, DPSV, XRF, etc., are the most suitable for decreasing running costs by saving manpower. The choice and application of a particular method for routine duties, however, depends not only on the problem but also on the experience and knowledge of the laboratory staff, which can greatly influence preferences for a certain method and even for the products a particular manufacturer.

Moreover, it is obvious that highly sophisticated and expensive methodologies such as NAA, PAA, X-ray microprobe, PIXE and all modes of MS in numerous instances are valuable reference methods and, isotope dilution MS a definitive method of high analytical power. Nevertheless, the high investment costs and the expertise required for reliable use commonly exclude their installation in smaller routine and even in many research laboratories. On the other hand, it should be emphasized that with respect to reliability of the analytical data

produced, at least two independent analytical methods should be regarded as minimal for routine and research laboratories working on trace metal analysis of biological materials in occupational medicine and toxicology, environmental surveillance, food control or clinical chemistry. At present the two most commonly used methods are AAS and voltammetry.

3.4. FUTURE PROSPECTS

The most desirable progress doubtless would be the adoption of trace analytical chemistry as an overall approach, as already mentioned above, in a steadily increasing number of laboratories. This could be achieved first by gaining the necessary experience and skill while performing practical trace and ultratrace analyses and second by introducing improved conditions such as clean workplace methods, the use of independent analytical approaches, wherever possible in the same laboratory, and the use of a good selection of appropriate control, standard reference and certified standard reference materials. There are good reasons to believe that such progress could be achieved within a few years.

If the state of the art of instrumental techniques is reviewed, it is obvious that also here considerable progress is possible. AAS has increased its reliability by the recent commercial introduction of significant improvements such as Zeeman background compensation and platform techniques for graphite furnaces. This should make possible, for numerous materials and for a number of elements, much simpler sample pre-treatment or at least at elevated trace element levels the direct analysis of liquid or even solid samples, which can considerably reduce the need for contamination precautions. Rapid signal recording and a effective temperature control for all stages of furnace programmes coupled with a steady improvement in furnace materials and furnace techniques can be expected in the near future for routine use. This should further improve the precision and accuracy down to the picogram per gram or picogram per millilitre level.

Plasma AES, owing to steady progress in all components of commercial devices and despite its high investment costs, will compete successfully with flame AAS if simultaneous multi-element analysis is required. The latter is advantageous in all types of environmental and biochemical research because of a lack of data on elemental correlations, a task which highly favours multi-element methods.

Despite the progress in GFAAS and plasma AES techniques, there seems still to be a place for AFS, which possibly will lead to the production of commercial flame AFS instruments in the near future.

Voltammetry at present has unique potential as a simple but extremely versatile and sensitive oligo-element approach. However, there are still some instrumental demands that need to be satisfied in order to offer the user of commercial instruments all the advantages of this approach, i.e., computer control and evaluation and wide selection of commercial working electrodes. Also, simpler evaluation methods and an extension to nearly direct methods, at least in simpler matrices or for screening purposes in environmental chemistry or occupational exposure, can be expected. This may again initiate a significant growth of applications in all trace analytical branches, because even highly sophisticated and versatile instrumentation would be still available at reasonable prices.

Whereas other methods indispensable for a variety of trace and ultratrace analytical tasks, such as NAA, XRF and MS, will still hold their position without exceptional growth, chemiluminescence can be expected to gain influence owing to its simplicity, specifity and sensitivity, at least for particular elements and when the still necessary basic research has been completed.

Also a gain in application and significance for species identification and quantification can be forecast, as interest will swing more towards methodologies that are able to answer particular questions on species distribution and fate for a better insight into the mechanisms involved. This may be coupled also with the critical but increasing use of computer techniques in all analytical branches dealing with trace element distribution and correlation in biological and environmental materials.

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CHAPTER 4

LEAD

PHILIPPE GRANDJEAN and NIELS BERG OLSEN

4.1. INTRODUCTION

Several classical writers have described the toxic properties of lead and its compounds[1] and high lead exposures in previous time periods have been documented[2]. Although the hazards associated with lead intake are now commonly known and the exposure levels are subject to regulation, cases of lead poisoning still occur.

Lead and its compounds have very useful physical and chemical properties, and lead is probably the most widely used non-ferrous metal[3]. Available figures from the U.S.A., Finland and Denmark suggest that about 1% of the working population has a significant occupational exposure to lead[4]. Children may ingest dangerous amounts of lead by eating or mouthing flakes of lead paint ("pica"), and in some U.S. cities several percent of young children have high blood lead levels[5]. Hence there is a considerable need for lead analyses as a monitor of lead exposure. Indeed, blood lead determination has become a routine procedure in a large number of laboratories.

More than 100 years ago it was discovered that lead is present as a ubiquitous element even in human tissues[6]. The analytical method of that time was precipitation with hydrogen sulphide and weighing the black precipitate. Although not specific for lead, this method was the standard choice until the beginning of this century. Then, for about four decades, chelation with dithizone and polarographic detection of the lead complex was the method of choice. Expensive emission spectrographs came in use for lead analyses more than 20 years ago, but the major step forward was the atomic-absorption spectrophotometer, which was later improved with graphite tubes for flameless determinations. Atomic absorption is at present the most widely used method for the determination of lead in blood and other biological samples. During the last few years, anodic stripping voltammetry (ASV) has been developed and improved to a degree that makes it comparable to atomic absorption. Other recent developments include some specialized methods that need very costly instrumentation. Proton-induced X-ray emission (PIXE) can only be used for small, thin samples. Multi-element analysis by X-ray fluorescence may become one of the most powerful analytical methods in the future; present-day instruments are not very sensitive to lead, but a portable model has been developed to measure lead in calcified tissues in vivo[7]. Fast-neutron activation analysis is possible but has not been extensively used[8].

As analytical capabilities improved, contamination problems arose. A survey of published results on blood lead levels in the general population has shown that the levels have been decreasing[9]. This is primarily due to better analytical accuracy and more stringent contamination control. Some of the traditional dithizone methods involved several steps and addition of chemicals that were not necessarily lead-free. Especially when lead levels are low, such factors become a problem. Reliable results at low lead concentrations can be obtained by isotopic dilution and mass spectrometry under clean laboratory conditions, but few need such sophisticated methodology for routine lead analyses.

As lead determination was a cumbersome process until a decade or two ago, other biological tests of lead exposure came into use. For many years the basophilic stippling of the erythrocytes was used as an indicator of lead exposure[8]. Later, urinary excretion of coproporphyrin IV and δ-aminolaevulinate (ALAU) were introduced[10]. During the last decade an increasing number of laboratories have performed determinations of aminolaevulinate dehydratase (ALAD) activity and zinc protoporphyrin (ZPP) concentration in erythrocytes. These biochemical tests show a biological effect of the lead exposure, not just a temporary exposure level. Not all tests are, however, specific for lead. Some of the most useful measurements will be discussed in a later part of this chapter.

4.2. SAMPLING AND STORAGE

No analytical technique can provide results that are better than the sample presented to it. As the development of new methods has permitted the detection of trace metals in the ppb (µg/kg) and sub-ppb range, measurements at such low levels generate unique problems that do not confront workers concerned with analyses of concentrated samples. Owing to ubiquitous lead pollution, extreme caution must be taken to avoid contamination of biological specimens. Almost every step from sample collection to final lead detection is susceptible to the introduction of exogenous lead. Dust may contain more than 1% of lead, and a few particles can increase the lead content considerably. Further, hairs and shed skin cells of laboratory personnel often contain much higher lead concentrations than the samples being analysed. Blank samples should always be incorporated in each batch, but this procedure does not protect against contamination. In general, the simpler the analytical method, the smaller is the risk of contamination. Acids must be of ultra-pure quality, and all equipment must be cleaned with dilute nitric acid (1%) or chelating solutions. In special cases, procedures have to be carried out in a laminar air flow bench or in a "clean room"[11]. Generally, more rigorous contamination control is needed when the lead levels in the specimens are low.

Most blood samples are obtained by venepuncture, and low-lead Vacutainers are available (Nos. 4610 and 6527 from Becton-Dickinson, Rutherford, NJ, U.S.A.). As the blood runs directly from the vein into the tube, contamination problems during sampling are very rare. If other techniques are used it becomes important to check the lead leakage from needle, tube and stopper, and if necessary to wash the equipment with dilute acid or detergent before use. Micro-sampling of blood from a skin puncture has been used extensively, because some individuals, especially children, may not accept venepuncture. The puncture is usually performed on a finger-tip in the U.S.A., while ear-lobe puncture is commonplace in Europe. Commercially available lancets can be used for this purpose, but vigorous cleaning of the skin is necessary, preferably with both soap and 70% alcohol[12]. During this procedure, the tissue becomes filled with blood, thus making the sampling easier. To avoid contamination from dirt on the fingers, collodion spray can be used to cover the skin before finger puncture. The blood is usually sampled in a 50-100-µl large-base capillary tube.

The choice of anticoagulant may be important. Heparin is most commonly used, but concentrations of heparin greater than twice the recommended concentration can cause interference with lead absorption in some atomic-absorption spectrometric (AAS) procedures. Addition of EDTA may interfere with complex formation in some methods. As whole blood is the specimen analysed, haemolysis does not

interfere with the determination, but the presence of micro-coagulates can make the transfer of a representative sub-sample difficult. Extraction of lead from the coagulates may be necessary[13]. Blood samples can be stored for only a limited time in a refrigerator, and it is recommended to deep-freeze samples.

A fundamental problem is the gain or loss of lead during sample storage, observed both for aqueous solutions and for blood. For Pyrex and Kimax containers a loss of 30% of the lead was observed in a 400 ppb aqueous solution within 5 min and a loss of 50% after 1 h[14]. The loss in polyethylene containers was smaller, being 10% after 15 min. Adding 1 ml of either concentrated HNO_3 or H_2O_2 to 100 ml of solution changed the results dramatically, and no loss was observed after a storage for 1 week[14]. Other experiments indicated a loss of about 50% of lead in aqueous solution for both Pyrex and polyethylene; a 3% H_2O_2 solution seemed sufficient to prevent the loss of lead by wall adsorption[15]. In our experience, however, HNO_3 is superior to H_2O_2 as an additive to aqueous solutions for long-term storage. However, a lead contamination of 0-33 µg/ml in tubes filled with 1% HNO_3 during 14 days has been reported[16]. For blood samples the results were more variable as both losses and gains of as much as 15 µg of lead per 100 ml was observed (the lead lost could be quantitatively recovered from the walls by washing with 1% HNO_3). Nackowski et al.[16] "strongly recommend that several of the evacuated blood tubes from each lot ... be leach tested prior to use with blood or plasma and the eluate analysed for potential trace metal contamination problems".

4.3. BLOOD LEAD ANALYSIS

The lead concentration in blood has hitherto been regarded as the best estimator of the risk of lead poisoning[8]. Because of the great need for blood lead measurements, a wide variety of analytical methods has been described, and improvements are constantly being reported. Popular methods in the past were the colorimetric dithizone method[17] and the extraction method after acidic protein precipitation followed by flame AAS[18]. At present, in our opinion, flameless AAS, preferably after acidic ashing, and anodic stripping voltammetry (ASV) seem to be the methods of choice. These methods, and useful modifications, will be discussed in detail below.

One analytical method is superior to all other methods in accuracy and sensitivity: isotope dilution and detection by mass spectrometry. Although this technique is probably the most reliable reference method available for lead, it is too slow and expensive to find application in the routine determination of lead in blood.

4.3.1. Atomic-absorption spectrometry

In this technique the pre-treated sample is atomised in a flame or on electrothermally heated metal or carbon. Light of the characteristic wavelength from a well defined source is absorbed by the atoms in the ground state. Re-emission of photons is isotropic, resulting in a decreased intensity of the signal. Complete atomization is necessary and is probably achieved via the oxide. During pre-treatment with low-temperature ashing (with radio field excited oxygen) the oxide is formed directly. In acidic ashing methods, e.g., with HNO_3, the oxide is formed during the ashing procedure. HNO_3 is excellent also for deproteinization and matrix modification. The nitrate reacts through three steps, the overall process being $Pb(NO_3)_2 \rightleftarrows PbO + 2NO_2 + \frac{1}{2}O_2$. Sturgeon et al.[19] showed that the carbon of the walls of a graphite tube atomizer reacted with PbO, thus aiding the atomization process, which seemed to occur in two steps (at the stated temperatures):

$$Pb(NO_3)_2 \xrightarrow{650^{\circ}C} PbO(s) \xrightarrow[\text{reduction at carbon}]{770^{\circ}C} Pb(l) \rightleftarrows Pb_2(g)$$

$$\downarrow \quad \uparrow$$

$$Pb(g)$$

where (s) = solid, (l) = liquid and (g) = gaseous state. Lead chloride, which is formed after HCl digestion, is hydrolysed to the oxide which then follows the same reaction scheme. Application of an inert purge gas (usually argon) protects the graphite tube against combustion, removes sample vapours and fumes formed during the drying and ashing stages and prevents oxidation of the atomized lead. During the atomization step (with temperatures typically reaching 2000°C) an external gas stream will prevent intrusion of oxygen into the analytical volume of lead atoms. The introduction of such carbon tubes[20] and equivalent carbon rods improved the signal-to-noise ratio and the detection limit compared with flame atomization methods with tantalum or carbon filaments.

During the past decade a number of methods have been in use for pre-treatment. Among these, four major groups can be outlined: (1) acidic ashing, (2) haemolysis with detergents or by simple dilution with water, (3) drying and ashing with H_2O_2 in a Delves cup and (4) drying blood spotted on filter-paper according to Cernik. Of these, (1) and (2) are now the most popular, as availability of autosamplers has solved the pipetting problem.

Acidic ashing methods seem to be superior to other pre-treatments. Garnys and Matousek[21] ashed 2 µl of blood with 1 µl of conc. HNO_3 in a carbon cup with a three-step temperature programme. The precision was 6.7 and 4.5% at 14 and

20 µg per 100 ml, respectively. HNO_3 was shown to be very efficient at eliminating the background signal. Dilution with Triton X-100 was less efficient. Measurement with a graphite tube of the supernatant from blood ashed at 65-70°C for 20 min with HNO_3 gave a coefficient of variation (CV) of 3.5%. Posma et al.[22] placed 5 µl of blood directly in the graphite tube followed by 10 µl of conc. HNO_3. Their detection limit was 1.5 µg per 100 ml with C.V.=15% at 25 µg per 100 ml. Later work using this method gave C.V. = 45, 14 and 6% at 4, 12 and 28 µg per 100 ml, respectively[23]. Castilho and Herber[24] diluted 50 µl of blood with 950 µl of water and 5 µl of concentrated HNO_3 and injected 5 µl of the mixture into a graphite tube. The detection limit was 0.1 µg per 100 ml with C.V. = 4% at 15 µg per 100 ml.

Graphite tube AAS has been perfected by the group at KFA Jülich[25-29]. An auto-sampler eliminating personal errors in pipetting the analytical sample of 10 µl has been developed in collaboration with Perkin-Elmer. The volume delivered had a precision of C.V.=0.15%. Dahl and Stoeppler[25] showed that the precision of determination of lead in acidic solutions was better with HNO_3 and HCl (C.V.=2%) than with $HClO_4$ (C.V.=4-5%), the largest signal being obtained with HNO_3. The signal height and the lifetime of the tube decreased with increasing acidity. The lowest possible concentration of HNO_3 allowing efficient ashing (deproteinization and matrix modification) should therefore be applied. The authors recommended mixing 2 *M* HNO_3, blood and water (3:2:3) for 30 sec. The supernatant was then autosampled into the graphite tube, dried at 100°C, ashed at 600°C and atomized at 2200°C. The C.V. was 8.4, 5.0, 4.5 and 3.5% at 10, 17.5, 23.3 and 48 µg per 100 ml, respectively. The precision was higher with the signal read as its maximum height, i.e., "peak mode", than with the signal calculated as the integral of the curve, i.e., "area mode". The increase in precision was higher (18%) with standard graphite tubes than with tubes pyrolytically treated (8%). Correlation with an ASV method was good.

Koizumi and co-workers[30,31] have described a unique method for elimination of the problem with uplitting of the analytical and the background compensating light beams by application of a steady magnetic field splitting the wavelength, according to the Zeeman effect, in components with different polarization characteristics. Applying polarization modulation made it possible to compute the difference between a component proportional to the sum of analytical and background and one giving the background alone. The only sample preparation was dilution with water, and measurement was made at constant temperature (1800°C). The detection limit was 0.2 µg per 100 ml. This Zeeman effect-based background compensation seems very promising, and its use should be encouraged.

Other popular methods of preparation have been haemolysis with detergents[32] or drying on filter-paper[33] or in a nickel crucible followed by ashing with H_2O_2[34].

Hessel[32] released erythrocyte-bound lead by haemolysis with the detergent Triton X-100 [a salt of the sulphate ester of alkylphenoxypoly(ethyleneoxy) ethanol]. In the original method the lead was then extracted with ammonium pyrrolidine-1-dithioate (erroneously named ammonium pyrrolidine dithiocarbamate, APDC) into 4-methyl-2-pentanone (methyl isobutyl ketone, MIBK) and measured by flame AAS. Modifications using flameless lead detection are currently used[35,36]. A special chelate solution was developed by Kon and Sarkozi[13].

Knutti et al.[37] obtained good precision even at low lead levels (C.V.=3.5% at 5 µg per 100 ml) by adding HN_4NO_3 to the sample in an attempt to remove chloride as NH_4Cl during the ashing phase, thereby eliminating the interference with lead from NaCl druing the atomization phase.

Delves[34] developed the first micromethod used in mass screening by drying and ashing (with H_2O_2) 10 µl of blood in a specially designed nickel crucible now called a Delves cup. He obtained a detection limit of 1.2 µg per 100 ml and C.V.=15% at 10 µg per 100 ml and C.V.=4% at 30 µg per 100 ml. In Ediger and Coleman's[38] modification the sample was ashed by igniting it near the flame of the AAS instrument. The C.V. was 6% and correlation with the H_2O_2-ashing method was good.

Cernik and Sayers[33] replaced pipetting of the blood microsample with spotting the blood on to filter-paper, drying it in the air and placing a punched-out disc in a nickel crucible for flame AAS analysis. Blood spiked with ^{51}Cr showed that fluctuations in blood density across the spot could be corrected for by using discs larger than 4 mm. The precision was C.V.=4%. Low haemoglobin concentrations may, however, result in abnormal and variable spreads[39].

The methods described in this section are all applicable and useful for blood lead measurements. As operator skill is an important factor, although less so with autosamplers being available, it is important to avoid changing techniques. Pre-treatment with HNO_3, or other simple methods, should be preferred. Interlaboratory comparison programmes, however, have not shown any major differences between the performances of the various AAS methods[40,41].

4.3.2. Anodic-stripping voltammetry

Classical polarography is now out of date as improvements in sensitivity and precision have been obtained by using carbon electrodes and ASV. The modification differential pulse ASV (DPASV) has the lowest detection limit. In ASV the metal is plated by electroposition on a mercury drop or on a mercury film on a carbon electrode. After this plating phase, the current is reversed and strips the metals from the electrode at more positive, i.e., anodic, potentials that

are specific for each element. The peak current is proportional to the amount of metal in the mercury and also to the concentration in the solution.

Duic et al.[42] used DPASV after ashing with H_2SO_4-$CHIO_4$ of 200 µl of blood with C.V.=5%. Morrell and Giridhar[43] used a commercially available chromium-calcium-mercury reagent, Metexchange, to release erythrocyte-bound lead in 100 ml of blood with C.V.=8% at 35 µg per 100 ml and 25% at 11 µg per 100 ml. From their data the detection limit can be calculated to be ca. 5 µg per 100 ml.

DeAngelis et al.[44] used a thin-layer method in a micro-cell with 50 µl of solution. Blood was ashed with acid (H_2SO_4, HNO_3, $HClO_4$). Oxygen was reduced to water during the plating phase, thereby making the degassing with nitrogen, typically lasting 15 min, unnecessary. We suggest that the possibilities inherent in the (1) small volume and (2) short plating time (60 sec), (3) without degassing, be exploited further, perhaps with wet ashing substituted by low-temperature ashing of blood sampled directly into the electrochemical cell from ear lobe or finger puncture.

The most reliable routine method for blood lead determination is that described by Valenta et al.[45], who used low-temperature ashing and DPASV with excellent results. The detection limit was ca. 0.4 µg per 100 ml with C.V.=2%. Correlation with AAS after acidic ashing according to Stoeppler et al.[29] was excellent.

At low blood lead levels many AAS methods are probably still superior to ASV techniques. Both are, however, useful at higher levels. ASV offers the additional possibility of concomitant measurement of concentrations of cadmium and other metals. Portable instruments for blood lead determination have been designed. Thus, in some circumstances, ASV may be the method of choice.

4.3.3. Additional detection methods

Visible fluorescence was recommended by Amos et al.[46] and Human and Norval[47], who found a detection limit of 0.1-0.5 µg per 100 ml and C.V.=5%. The method has not, however, been used on a larger scale.

Radioisotope-induced X-ray fluorescence was applied by Laurer and Kneip[48], who excited elements in a dried blood sample with a ^{238}Pu source. Correction for differences in mass (Hb content) was possible by simultaneously registering fluorescence from Pb and Fe, the latter being proportional to the Hb content. Correlation with AAS was good. The possibility of activation with fast neutrons has been mentioned[8,40]. The sensitivity for lead is low, however. Proton-induced X-ray fluorescence may be possible, especially if a calibration method such as that of Laurer and Kneip[48] is used. As AAS and ASV instruments with excellent

performance are commercially available at relatively low prices, the other methods mentioned here are of limited interest at present.

4.4. STANDARDS AND CALIBRATION

Reference materials are available from National Bureau of Standards (U.S. Department of Commerce, Washington, DC, U.S.A.). Most relevant is bovine liver (Standard Reference Material No. 1577) with 34 ± 8 μg of lead per 100 g. This concentration was measured by isotope dilution mass spectrometry. Matrix characteristics may vary among different species and different tissues, but in spite of the relatively high lead levels in the reference material it should preferably be analysed regularly by laboratories with current lead analysis programmes.

Aqueous standards of 1000 μg/l are available from several companies and can be used as primary calibration material.

Secondary calibration materials can be made in the laboratory from lead acetate or nitrate (analytical-reagent grade) dissolved in redistilled water. As discussed above (see Section 4.2), it is necessary to add HNO_3 to aqueous standards.

The matrix effect of blood in AAS is obvious when comparing plots of standard addition curves for aqueous solutions and for blood. Eller and Haartz[49] found the slope of the former to be about twice that of the latter, and this is in agreement with our experience. This effect is due to initial variations in atom formation, escape of the analyte in molecular form and formation of non-volatile compounds. The use of blood standards is therefore highly recommended. Such control materials can be prepared by enriching specimens of a large blood sample with different amounts of lead. Although the lead will rapidly be bound to erythrocytes, some variation may occur owing to precipitation or adsorption to the walls of the container. We have obtained better results by mixing various proportions of blood form an individual with heavy occupational lead exposure with blood of the same blood type from an unexposed individual.

The proficiency-testing programme of the Center for Disease Control of the U.S. Department of Health, Education and Welfare has used isotope dilution mass spectrometry as the reference method[41]. If a reference method, or material, is not available, inter-laboratory comparisons will be necessary. Mostly, such studies are group mean comparisons, and it is assumed that the mean or median, i.e., the concensus, provides the best answer. Specimens from a comparison programme are probably treated in a manner different from that used for routine samples, and the results may not represent the average performance of the laboratory. Apart from these limitations, inter-comparison programs are very useful.

In a review of the state of the art in 1975, Pierce et al.[40] concluded that the findings of 30% of the laboratories engaged in routine analysis for lead in blood differed by more than 15% from the "true" value. They suggested that the state of the art of blood lead analysis was probably improving at the 40 µg per 100 ml level and above, but still many erroneous values were being reported and much improvement was needed. With regard to low lead (20-30 µg per 100 ml levels) the situation was "disastrous"[40].

In the previous study by Keppler et al.[50], the reported values for one blood sample (known to contain ca. 40 µg per 100 ml) ranged from 0 to 29,200 µg per 100 ml! The report on a similar study by Lauwerys et al.[51] was less discouraging, although systematic errors were found to be responsible for a high inter-laboratory variation of about C.V. = 50%. Only half of the laboratories had acceptable precision in this study. The differences among values obtained by laboratories either with different methods or with different degrees of experience were small[51].

Few laboratories comply with the conditions for contamination control recommended by Patterson and Settle[11]. Participation in intercomparison programs and increased experience seem, however, to have improved the performance of many laboratories[41]. We are aware of recent unpublished comparisons that have revealed excellent results with C.V. <15% even at low blood lead levels. In skilled, but cautious, hands, blood lead analyses are, therefore, reliable and useful in most instances.

4.5. ANALYSIS OF OTHER BIOLOGICAL SAMPLES

In the past, determination of urinary lead excretion has been very popular. The unavailability of 24-h urine samples and difficulties associated with corrections according to creatinine excretion decreased the popularity of urinary lead determinations. Moreover, day-to-day variations were large. Reduced glomerular filtration rate after long-term lead exposure[52] may result in decreased urinary lead excretion. Should a urinary lead analysis be convenient, however, virtually all analytical methods for blood lead are applicable with little change needed. As for blood lead, large inter-laboratory variations have been documented in urinary lead analysis[51].

Tissue samples from biopsies or autopsies have been analysed in the past and provided much information on the metabolism of lead. Most lead is stored in the bones and teeth. Both tissues may be dissolved directly in concentrated HNO_3, and the diluted solution can be used for AAS[53]. Wet ashing with $HClO_4$ may be used before ASV determination[54]. As most tooth lead is in the secondary dentine,

this tissue can, preferably, be isolated by the elegant methods devised by Shapiro and co-workers[55,56]. The hard tissues are especially suitable for non-destructive methods such as X-ray fluorescence[7].

Soft tissues are not always suitable for wet ashing. Dry ashing in a kiln always carries with it a risk of loss of lead, especially at temperatures above 450°C[57]. Yeager et al.[58] recommended a combination of dry and wet ashing. A sophisticated instrument has been developed to ash tissues at low temperatures by means of activated oxygen. This low-temperature asher has proved very useful for tissue analyses[59,60].

Hair has been used regularly for monitoring lead exposure. Different washing methods to remove external contamination, variable sampling schemes and the possible influence of hair colour and age on the lead level have made comparisons between different studies difficult. As contamination increases along the hair shaft[61,12], only the first 1-cm segment or so close to the hair root should be analysed. Simple washing in an ultrasonic bath for 30 sec or 1 min with acetone or Freon TF is an easy and adequate method to remove surface contamination[62,12]. Hair lead can be determined directly by flameless AAS[61,12], but traditional ashing and detection as described above are also feasible.

4.6. ORGANIC LEAD

Organic lead compounds, e.g., tetraethyl- and tetramethyllead, differ from other lead compounds in physico-chemical properties and toxicological characteristics. Thus, when organolead exposure is suspected, the blood lead level may not be very high. However, the lead concentration in the lipid fraction of the blood is usually increased[63]. Further, urinary lead excretion is high, while the haem biosynthesis may not be much affected[64]. The source of exposure is often occupational, but gasoline sniffing and accidental inhalation or ingestion may cause poisoning[64]. Although organic lead compounds may be determined by gas chromatography-mass spectrometry, analytical techniques are not yet available for routine determinations of low concentrations in biological samples.

4.7. ASSESSMENT OF LEAD TOXICITY

The toxic effects of lead have been reviewed in detail elsewhere[3,65-67]. The main interest for diagnostic and screening purposes is focused on two biochemical assays of effects on haem biosynthesis. Both tests are inexpensive and may, therefore, in many instances replace more complicated determinations of lead in blood and other biological samples.

Since Hernberg and Nikkanen's[68] original demonstration that the enzyme aminolaevulinate dehydratase (ALAD) is inhibited even at low enrironmental lead exposures, this enzyme assay has been very popular. The correlation with blood lead is excellent, and the enzyme activity decreases exponentially with linear increases in blood lead[66]. This assay is especially useful in the blood lead range from 10 µg per 100 ml, where inhibition begins, to about 60 µg per 100 ml, where inhibition is almost complete. A European standard method for this assay has been described[69]. Recent research indicates that the ratio between actual enzyme activity and reactivated activity is a more exact measure of lead toxicity[66]. Although the assay is simple and fast, a slight disadvantage is that the blood sample must be assayed a few hours after collection, or frozen in liquid nitrogen until assay.

Another biochemical assay is analysis of zinc-protoporphyrin (ZPP) in erythrocytes. This compound is accumulated and bound to the haem-binding sites in haemoglobin when lead inhibits ferrochelatase activity[70]. As ZPP is stable and is retained in the erythrocytes during their lifetime (ca. 3-4 months) the ZPP level in blood is an average measure of lead toxicity during the previous 3-4 months. ZPP can now be measured in a few seconds by means of an important development, the haematofluorimeter[71]. ZPP levels correlate well with blood lead levels, especially above 30 µg per 100 ml[4,72,73], and they are useful predictors of lead toxicity[74,67]. Bilirubin and other substances in the blood may interfere with the measurement[75,73], and ZPP may increase as a result of iron deficiency[76]. This, however, is uncommon in males but may be encountered more often in females and children. The haematofluorimeter is a powerful instrument with important applications for screening and diagnostic purposes[72,4].

4.8. INTERPRETATION OF RESULTS

The analytical result must first be evaluated with regard to the validity of the analytical method. Poor analytical quality may lead to false conclusions[77].

Some controversy exists concerning the evaluation of blood lead levels. An international working group reached a compromise, that a blood lead level of 60 µg per 100 ml should be regarded as the permissible limit for occupational lead exposure[78]. However, the experts recognized that some manifestations of lead toxicity develop at lower blood lead values, and that females may be more susceptible than males. Subsequently, the Occupational Safety and Health Administration in the U.S.A. decided on a blood lead limit of 40 µg per 100 ml[79]. Although the first mentioned limit may protect against gross clinical lead poisoning, insidious effects on nervous system, kidney and reproductive functions may occur at levels between 40 and 60 µg per 100 ml (see Table 4.1). The

TABLE 4.1

APPARENT BLOOD LEAD "THRESHOLDS" FOR ADVERSE EFFECTS

Blood lead level (µg per 100 ml)	Effect
10	ALAD inhibition
15	ZPP accumulation (children)
25	ZPP accumulation (women)
35	ZPP accumulation (men)
35	CNS dysfunction (children)
40	ALA excretion in urine increased
40	CNS dysfunction (adults)
40	Decreased nerve conduction velocity
50	Decreased haemoglobin concentrations
60	Colic and other clinical effects

Commission of the European Communities recommends that the median blood lead level of the general population should not exceed 20 µg per 100 ml, and that virtually all (98%) results should be below 35 µg per 100 ml[80].

Children are more susceptible than adults (Table 4.1). Thus, they can develop encephalophathy at lower blood lead levels. Also, the haem synthetic pathway in children is more sensitive to lead. Therefore, the U.S. Environmental Protection Agency has proposed that mean blood lead levels in children be kept below 15 µg per 100 ml and almost all below 30 µg per 100 ml[81].

"Natural" lead exposure is probably only about 1% of present-day levels in industralized countries[53]. However, dietary lead intake varies geographically[8]. The blood lead level is related to occupation, smoking, alcohol consumption, diet and other factors[82]. In most countries, average blood lead values are between 10 and 20 µg per 100 ml. A result above 30 µg per 100 ml is considered elevated. The source of lead exposure should be sought and eliminated if the blood lead level is above 40 µg per 100 ml (adults) or 30 µg per 100 ml (children). If symptoms occur, and the blood lead level is above 60 µg per 100 ml, chelation therapy may be considered.

Recent studies have indicated that the blood lead levels is not always the best risk estimator[67]. Other tests may be more practical, too. The correlation among some of these tests is shown in Table 4.2.

Some confusion exists as to the units of measurement. While the Système International (SI) recommendations have been widely accepted in European clinical chemistry, blood lead levels are still mostly reported in µg-%, i.e., µg per 100 ml or µg per 100 g (the latter unit is ca. 6% greater than the former).

TABLE 4.2

APPROXIMATE LEVELS OF OTHER DIAGNOSTIC TESTS AT TWO BLOOD LEAD LEVELS (ADULT MALES)

Other level	Blood lead (µg per 100 ml)	
	40	60
Urine lead (µg/l)	70	100
Urine ALA (mg/l)	5	10
Hair lead (µg/g)	20	70
ZPP (µg per 100 ml)	70	180

It seems, however, that the molar concentrations will become more popular in the future. Blood lead concentrations should, therefore be given in µmol/l, where 1 µmol/l = 20.7 µg per 100 ml or 19.5 µg per 100 g of blood.

4.9. FUTURE DEVELOPMENTS

AAS will continue to be the preferred routine method for measuring lead in blood and other biological samples, and some of the techniques will probably be further refined. ASV will continue to be a useful alternative, especially for blood lead. Further developments in X-ray fluorescence techniques are expected. Such methods will allow multi-element determinations of hard and, possibly, soft tissues even at low levels in vivo and will, therefore, become important diagnostic tools. Compared with the fluorimetric measurement of protoporphyrin, blood lead analysis is expensive and cumbersome. For screening of large population groups the haematofluorimeter will probably become increasingly popular. As epidemiological studies tend to show toxic effects of lead at lower and lower levels, the need for blood lead and other analyses will continue, even though some lead exposure sources are being controlled. The ultimate goal of complete prevention of undue lead exposure would make most lead analyses unnecessary, but this goal is not in sight.

4.10. ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

Since this chapter was finished in 1979, Zeeman background correction equipment has become commercially available with AAS instruments. New developments have also occurred with inductively-coupled plasma (ICP) emission spectrometry, although the detection limit for lead is not yet quite satisfactory. Useful reference materials are becoming available from the International Atomic Energy Agency and soon through the EEC Community Bureau of Reference. New information has suggested lower thresholds for lead toxicity, especially for children, and a blood lead level above 25 μg per 100 ml in a child is no longer considered safe or innocuous. The interested reader is referred to M. Rutter and R.R. Jones (Editors), Lead versus Health, Wiley, Chichester, 1983; and Royal Commission on Environmental Pollution, Ninth Report, Lead in the Environment, H.M. Stationary Office, London, 1983.

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CHAPTER 5

MERCURY

LASZLO MAGOS

CONTENTS

5.1. INTRODUCTION

Development in the analysis of mercury in biological samples has been both the cause and the consequence of changes in our views on mercury as a potential environmental health hazard. While the need for higher sensitivity and precision stimulated the development of new analytical techniques, improved methodology has been instrumental in studying the epidemiological implications and behaviour of environmental mercury. Cases of homicide and suicide, when the use of corrosive sublimate ($HgCl_2$) was suspected, required crude analytical techniques such as Reinsch's test[1]. The effective control of occupational mercury exposure advanced the need for more sensitive methods[2]. The drive for higher sensitivity received a powerful impetus when the causative factor in the so-called Minamata epidemic was tracted back to methylmercury in fish[3] and it was discovered that methylmercury is synthesized in aquatic ecosystems and accumulates in the food chain[4,5].

Mercury as an environmental hazard presented a need not only for increased sensitivity, but also for methods that make possible determinations on a large scale. The importance of the suitability of a method for large numbers of determinations is illustrated by the fact that nearly 150,000 determinations were made within a few years with a modified version of the selective atomic-absorption analysis of Magos[6] by the Medical Services Branch, National Health and Welfare Canada[7]. Moreover, new methodology extended the scope and possibilities of experimental toxicology. In this field the most important step was the use of ^{203}Hg-labelled mercury, which made it possible to determine, without any processing, mercury concentrations in whole organs[8] or to follow mercury clearance in the whole animal[9] or in man[10].

The selection of the analytical method and frequently the target of analysis are influenced by the availability of laboratory facilities: instrumentation and time. Thus before the method is selected the analytical aim must be clear: is the determination of total mercury satisfactory, is there a meed to make a distinction between inorganic and organic mercury or should the organomercurial be identified? The aim of the analysis influences also the requirement for accuracy, precision and detection limit.

Atomic-absorption spectrometry, gas chromatography or neutron activation analysis usually satisfy the requirements for sensitivity, accuracy and precision and at the same time increase the possible number of analyses per working day. In the absence of such facilities, the remaining choice is colorimetry.

5.2. SURVEY OF METHODS

5.2.1. Total and inorganic mercury determinations

5.2.1.1. Colorimetry

The most popular forms of colorimetric methods use the orange colour of the complex of Hg^{2+} with dithizone ($C_6H_5N{=}NCSNHNHC_6H_5$). This complex is soluble in chloroform and is usually extracted from an acid digest of the sample. The rate of analysis with the dithizone method is 3-6 samples per working day and recovery is about 80% with a relative standard deviation from 5.0[11] to 10-20%[12] for 5 µg of Hg per 50 g sample. As colorimetric determinations need large amounts of reagents, the blank can be as high as 1.0 µg per sample[11,12], which naturally affects the limit of detection. Moreover, large reagent volumes and a long processing time increase the possibility of error by contamination not only with mercury but also with other metals or elements that interfere with colour development. Error at best can be suspected only at the very end of the analytical procedure and most frequently only when determination is repeated with a more reliable method. Thus in the Minamata epidemic, determination of mercury in autopsy materials by atomic-absorption spectrometry revealed that the dithizone method frequently underestimated mercury concentrations by a factor of three and in one in ten cases by a factor of 12.7[13].

5.2.1.2. Atomic-absorption spectrometry

Atomic-absorption determination at first only replaced the final step in mercury determination that followed digestion and extraction. Mercury from its dithizonate was released by heat and moved by aeration through the gas cell of an atomic-absorption spectrophotometer where absorption at 253.9 nm was measured in a quartz-windowed gas cell[14,15]. This method showed improved sensitivity compared with colorimetric determination but did not reduce the processing time.

Extraction and evaporation are eliminated when, as in the atomic-absorption method of Hatch and Ott[16] adapted to biological samples, mercury is reduced to atomic mercury by tin(II) or tin(II) plus hydroxylamine in the acid digest[17-22]. The processing time depends mainly on the method of digestion, which with cereals, vegetables or tinned tuna fish is long[22], whereas with urine simple acidification of the sample is satisfactory[19,23].

The sensitivity, precision and accuracy of atomic-absorption determinations are good. In the most widely used flow-through methods the peak absorbance can be changed by such simple measures as adjustments of the aeration flow-rate, solution volume in reduction vessel, dead space between reaction mixture and gas cell, gas cell dimensions and the type of ultraviolet radiation source in the spectrophotometer[24]. Reduction by $SnCl_2$ linked to the alkalinization of the acidified urine[23] eliminates interference by iodide or bromide[25] and duplicate determinations require less than 5 min. About 95% of the mercury is volatilized from samples and mercury added in saline or in urine gives identical peak heights, which makes unnecessary the use of internal standards. Results obtained with the $SnCl_2$-alkalinization method of Magos and Cernik[23] were in good agreement with those of the colorimetric ion-exchange method of Kopp and Kennan[26]. The detection limit is less than 5 ng/ml in urine with a maximum deviation of 13% from the mean; at 10.0 ng/ml the maximum deviation is 10.0%[23]. The matrix effect influences the rate of release and consequently the peak height from blood, and thus its analysis by this method requires the use of internal standards. Moreover, the method of Magos and Cernik[23], unlike the atomic-absorption determination of digested samples, determines only inorganic mercury. This can be an advantage when only inorganic mercury concentrations are required or when values given with or without oxidative digestions are compared to calculate the concentration of organomercurials[27] or a disadvantage when only total mercury concentration is required. The methods of Lindstedt[18] and Skare[28] satisfied the latter requirement, have similar precision, accuracy and sensitivity and are suitable for automation[25,28]. Another semi-automated method for digested samples was described by Armstrong and Uthe[29].

The selection of digestion methods with the aim of converting all forms of mercury into inorganic mercury and making it accessible to the reducing agent depends on the sample. Digestion generally involves acid treatment with or without heat or an oxidizing agent[30]. The conventional digestion can be replaced by oxygen bomb combustion[31] or decomposition in a closed-system Teflon-lined pressure chamber[32,33]. Losses during digestion with heating can be minimezed by the use of a reflux condenser[34] and losses on opening the bomb or decomposition chamber can be corrected by the addition of ^{203}Hg to the sample followed by the determination of the recovery by gamma counting[31]. Digestion can be avoided with

urine by the injection of the sample into the flame of an atomizer burner and by passing the exhaust gases from the combustion chamber through condensers and filters before atomic-absorption determination[35]. The difficulty of the complete removal of interfering substances explains why this simple method has not gained popularity. The combination of cold vapour atomic-absorption spectrometry with direct combustion in oxygen requires an elaborate filter system and the absorption of mercury vapour on gold, before its subsequent release by heat for atomic-absorption measurement[36]. Atomic-absorption analysis with a graphite furnace requires matrix modification in order to increase the volatility of the matrix and promote its removal before atomization and to make mercury non-volatile during charring[37].

Although predigestion decreases the sample effect on the rate of mercury vapour release in the flow-through system, it does not completely eliminate the matrix effect. This naturally affects the maximum deflection on the recorder attached to the atomic-absorption spectrophotometer. The use of the area under the peak helps to overcome this difficulty, but it increases the determination time and complicates calculation. Other investigators attempted to achieve the same aim by modifying the introduction of mercury vapour into the gas cell of the atomic-absorption spectrophotometer. In one method the sample is stirred with a magnetic stirrer and air is cycled through the gas cell and reaction mixture by a peristaltic pump until a steady concentration is recorded[38]. The advantage must be set against a longer reading time and the possibility of surface absorption within the system with possible memory effects. In another method an exact amount of air is withdrawn with a hypodermic syringe from the closed air space above vigorously shaken reaction mixture and injected into the sealed gas cell[39]. In a third method the sample is stirred with a Vortex mixer and, 10 sec after the addition of $SnCl_2$, mercury released from the reaction mixture into the air phase by partitioning is forced into the gas cell by filling the reaction vessel with tap water[40]. Absorption of mercury vapour on a gold filter[41], cadmium sulphide[42] or charcoal[43] followed by volatilization by heat into the gas cell of an ultraviolet spectrophotometer are other attractive ways of overcoming the matrix effect of the sample.

5.2.1.3. Fluorescence, X-ray and emission methods

Atomic-fluorescence spectrometry, X-ray spectrometry, X-ray fluorescence spectroscopy and emission spectrography[44-46] differ from atomic-absorption spectrometry in the physical principles used for final evaluation but they encounter the same problems in sample preparation and interferences by sample components. Their sensitivity is not higher than that of atomic-absorption spectrometry and, in the case of X-ray methods, is considerably lower[46].

5.2.1.4. Neutron activation analysis

Neutron activation analysis is usually accepted as a standard with which all other methods of mercury analysis can be compared, although comparative studies seem to indicate that in the neutron activation analysis of mercury "the art of analysis" still plays an important role[47,48]. Neutron activation analysis is as sensitive to loss, contamination or interference as any other method. In the most frequently used procedures mercury must be separated either before or after irradiation from other sources of radiation. Losses of mercury may occur during radiation, on opening the container in which the sample was irradiated or during digestion and separation. Although there are non-destructive methods, these require a longer irradiation time, a long waiting period after irradiation and more selective and less sensitive detectors than the common NaI detector[46]. The detection limit for a non-destructive method is 3.5 ng/g sample[49]. In an interlaboratory study two of the three laboratories that used non-destructive techniques gave absurd values and only one gave a mean reasonably near to the overall mean estimated by destructive analysis in 13 laboratories. The accuracy of destructive techniques in the same study was better; 4 of the 13 laboratories had means within ±10% and 10 within ±30% of the overall mean of 4.6 μg/g. The result given by one of the laboratories deviated 50-fold from the overall mean[50].

However, the accuracy of destructive neutron activation analysis can be very much higher. The accuracy was ±2% in another study in which seven laboratories using the method of Sjöstrand[51] collaborated[50]. The precision for the following method of Pillay et al.[48] was ca. 7% at the 1.5 ppm level. In the first step the homogenized sample sealed in a thick polyethylene bag is irradiated at a thermal neutron flux of about 5×10^{10} neutrons cm^{-2} sec^{-1} for 2 h. In the second step the polyethylene bag is trimmed around the sample and the remaining bag with sample is digested in a nitric acid-sulphuric acid-perchloric acid mixture. In the third step mercury in the condensate is precipitated as sulphide and washed with nitric acid to separate it from other sulphides. In the fourth step mercury is electrochemically deposited on gold foil and finally its gamma and X-ray emission from ^{197}Hg are counted using a sodium iodide detector with a beryllium window. The recovery is increased by adding carrier mercury to the sample before digestion.

5.2.1.5. Isotope exchange method

A radiochemical method based on an isotope exchange reaction previously used to determine the rate of cleavage of mercury from organic mercurial diuretics[52] was adopted for the determination of inorganic mercury derived from the decomposition of labelled methylmercury[53]. The isotope exchange is based on three principles: (1) aqueous solutions of mercuric salts are capable of undergoing isotope exchange with mercury vapour in contact with the solution; (2) after the

isotope has exchanged into a vapour state, it is volatile and can diffuse from one liquid phase into another; (3) mercury bound covalently to a carbon atom in organic mercurial compounds either does not undergo isotopic exchange or does so much more slowly than inorganic mercury. In the method described by Norseth and Clarkson[53], homogenate or biological fluid is placed in the outer well of a Conway microdiffusion unit and the sample is mixed with cysteine and NaOH. The inner well contains 0.1 ml of metallic mercury. After incubation for 4 h at 40°C, all of the radioactivity (^{203}Hg) in the inorganic mercury form diffused from the homogenate into the metallic mercury globule. The inorganic mercury content of the sample can be calculated from the specific activity of the injected methylmercury corrected to decay and the radioactivity in the mercury pellet corrected to self-absorption. A reversal of this radioactive technique is when unlabelled mercury in the sample is exchanged to ^{203}Hg labelled mercury. This isotope exchange technique, first used to determine mercury concentrations in air[54], was applied by Clarkson and Greenwood[55] to biological samples. Their method avoids difficulties presented by digestion and extraction. Sufficient amounts of cysteine are added to the biological sample (urine, blood, homogenates) to complex all the mercury present and trace amounts of radioactive ^{203}Hg are added to label all mercury in the cysteine complex. Air containing mercury vapour of known concentration is bubbled through the impinger containing the sample, and the released radiactive mercury is absorbed on to a Hopcalite filter. The filter is inserted into a sodium iodide crystal well counter to record ^{203}Hg activity. The experimental conditions are adjusted so that the half-time of the exchange process is directly proportional to the concentration of mercury in the sample.

5.2.2. *Selective mercury determinations*

5.2.2.1. *Combination of total and inorganic mercury determinations*

Theoretically, every method is suitable for differentiating between various forms of mercury or between inorganic and organic mercury if these forms are separated before analysis and sensitivity permits determination at concentrations below the concentration of total mercury. Thus, in the method of Gage[56] phenylmercury or methylmercury is extracted from the strongly acidified sample with benzene, and after oxidation with acidic permanganate, mercury is determined with a titrimetric dithizone method.

Separation can be avoided when the determination of total mercury is supplemented with methods that estimate only inorganic mercury, as in the methods of Clarkson and Greenwood[55] and Magos and Cernik[23]. The difference between total and inorganic mercury concentrations will give the concentration of organic

mercury. This approach led to the method for the selective atomic-absorption determination of inorganic and organic mercury[6,57].

5.2.2.2. Selective atomic-absorption determinations

The atomic-absorption determination of mercury vapour is simple, fast and reliable, but when this method follows acid digestion, there seems to be an imbalance between the actual measurement and preceding work. The determination of mercury in undigested samples by Magos[6] resolved this imbalance for inorganic mercury. However, the selective atomic-absorption determination of inorganic and organic mercury requires two conditions: first, a reagent that can break the covalent mercury-carbon bond and reduce all mercury to mercury vapour, and second, that depending on the choice of reagents, in one run only inorganic and in another run organic or total mercury could be converted to mercury vapour in otherwise identically treated samples. The discovery that $SnCl_2$ in the presence of $CdCl_2$ can reduce organic mercury to mercury vapour satisfied the first condition and the timing of the reduction with the alkalinization of the cysteine-supplemented acidified sample satisfied the second[6]. The reagent introduced by Magos[6], which can break the covalent mercury-carbon bond without oxidative digestion, contained 500 mg of $SnCl_2$ and 100 mg of $CdCl_2$ per sample, while inorganic mercury can be reduced to the vapour state by 100 mg of $SnCl_2$. The partial disruption of the mercury-carbon bond in ethylmercury by tin(II) makes this method unsuitable for the quantitative determination of ethylmercury. As in biological samples not directly contaminated with other organomercurials the organic form of mercury is always methyl, the selective atomic-absorption method gives the concentration of methylmercury in all biological samples. When inorganic mercury is reduced and removed from the sample by aeration, in the re-acidified sample the remaining organic mercury can be converted to mercury vapour by the $SnCl_2$-$CdCl_2$ reagent. With the combination of inorganic and organic standards added to a sample aliquot (inner standards), the inorganic and organic mercury concentration in the sample can be calculated[6]. This single sample determination method was applied by Kacprzak and Chvojka[56] to determine inorganic and methylmercury in fish. However, in most laboratories that use the $SnCl_2$-$CdCl_2$ reagent, total mercury and inorganic mercury are determined separately, and the methylmercury concentration is calculated from the difference.

In the first description of the selective atomic-absorption determination of mercury with a simple mercury vapour detector[6] and in the second[57] with the much more sensitive LDC UV Monitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) the procedure is as follows.

A 1-ml or greater volume of sample is mixed in the reaction vessel with cysteine, saline, sulphuric acid and 1 ml of $SnCl_2$ or $SnCl_2$-$CdCl_2$ reagent, fol-

lowed by alkalinization with sodium hydroxide. By linking up the reaction vessel with the flow-through system (ca. 2 l/min), mercury is flushed through the gas cell of the atomic-absorption spectrophotometer. Peak deflection on a recorder or digital voltmeter attached to a UV monitor is read and the blank is deducted. The use of an internal standard cannot be avoided with the selective atomic absorption of total, inorganic and methylmercury with the flow-through system and peak deflection measurement, and therefore determinations must be repeated with internal standards. As methylmercury and inorganic mercury standards give identical readings with the $SnCl_2$-$CdCl_2$ reagent, the use of an inorganic mercury standard is satisfactory both for inorganic and total mercury determinations. Inorganic mercury standards prepared with EDTA-cysteine-NaCl are stable for at least 6 months[6].

The procedure described is suitable for blood, urine, tissue and homogenates, but fishmeal must be solubilized. This can be done in a test-tube where 0.5 g of fishmeal mixed with 1 ml of 1% cysteine, 1 ml of 20% NaCl and 1 ml of 45% NaOH are heated just to the boiling point[6]. A strongly alkaline solution can solubilize crushed grain[59] or hair[60,61].

The sensitivity of the selective atomic-absorption determination depends on the reagent blank, the diameter of the reaction vessel and the diameter and length of the gas cell. The cause of a high reagent blank is either contaminated glassware or impure sodium hydroxide. Standard analytical-reagent grade reagents occasionally must be demercurized. $SnCl_2$ can be added to the NaOH solution to help to remove mercury whilst bubbling through nitrogen or CO_2-free and mercury-free air[57]. A better result can be achieved when $SnCl_2$ is added during the preparation of the solution and nitrogen is bubbled through until the solution is cooled to room temperature[62]. Of NaOH from different sources, Riedel-de Haen (Seelze-Hannover, G.F.R.), Reag. ACS, DAB 7 (R 319) material gave the lowest blank. The blank can also be lowered by decreasing the amounts of NaOH and H_2SO_4 used with a corresponding decrease in the diameter of the reaction vessel[63]. Interference by water vapour is prevented by the insertion of an impinger that contains ice-cooled water between the reaction vessel and gas cell. This avoids the problem of temporary retention of mercury vapour with a memory effect that occurs with solid absorbers.

Some samples, mainly urine and fish pre-treated with NaOH-cysteine, can release white fumes during the reaction. These fumes are not observed with properly preserved blood, fresh rat tissue homogenates or NaOH-cysteine pre-treated hair samples. When present, the fumes are visible to the naked eye and move more slowly than the mercury vapour, but can cause a large difference between parallel determinations. Fume formation can be decreased by bubbling air through the acidified sample before the reducing agent is added and/or by starting aeration a few seconds after sodium hydroxide is mixed with the

acidified sample. Fume formation is also decreased by the reduction of the volume of chemicals as in the modification by Farant et al.[63]. Work carried out in our laboratory has shown that interference by fumes is completely prevented by the insertion of a thin cotton-wool layer in the tubing just after the reaction vessel. For the elimination of other possible gaseous ultraviolet-absorbing substances Toribara and Clarkson[64] made use of the twin cells of the LDC UV Monitor. The air flow is divided into two halves before reaching the gas cells and both air flows pass through glass-wool, that of the reference cell being impregnated with palladium chloride as described by James and Webb[65].

Selenium in certain circumstances can interfere with the selective atomic-absorption determination of mercury. This interference depends on the chemical form in which the mercury is ingested. Thus no interference was observed after the administration of methylmercury, although selenite given to mice with equimolar doses of $HgCl_2$ affected both total and inorganic mercury determinations[66]. This effect could be prevented by heating blood or homogenates with equal volumes of 45% NaOH and 1% cysteine[67]. Interference seems to be linked to the metabolism of selenite in blood, as in vitro added selenite interfered with mercury determination in blood but not in liver homogenate[67]. The forms of selenium and mercury in food may explain why in a wide variety of biological samples interference was absent, as judged from agreement between the selective atomic-absorption and other types of determinations.

Using the LDC UV Monitor for the analysis of 1-ml blood samples, Magos and Clarkson[57] reported a detection limit of 0.5 ng/ml of mercury with 3.5-3.0-ng blanks. The reproductibility was 4.1% (relative standard deviation) for total mercury, 8.7% for inorganic mercury and 5.6% for organic mercury with blood containing 7 ng/ml of mercury. The recovery was 97% for both inorganic and organic mercury with relative standard deviations of 6 and 12%, respectively. The main deviation between two sets of results was 3.6% when five freeze-dried blood samples were analysed by neutron activation analysis and selective atomic-absorption spectrometry[57]. Inter-laboratory comparison of mercury concentrations in blood obtained in two laboratories gave a ratio of 1.01 with a standard deviation of 0.22 for total mercury[61].

The reliability was confirmed by other investigators who compared selective atomic-absorption spectrometry with neutron activation analysis[68], digestion followed by atomic-absorption spectrometry for total mercury[22] and gas chromatography[22,69,70] and graphite furnace atomic-absorption spectrometry for methylmercury[71].

Modifications of the original selective atomic-absorption determination of inorganic and total mercury retain the $SnCl_2$-$CdCl_2$ reagent of Magos[6] and fall into the following categories:

(1) The volume of chemicals and numbers of steps are reduced. The reducing reagents plus cysteine and saline are added with sulphuric acid in one solution[63].

(2) Hair, fishmeal and some other samples are digested either in sodium hydroxide-cysteine[58,63] or sulphuric acid-saline-cysteine[72,73].

(3) One step is omitted. In one version the sample is not acidified, but alkalinized before the addition of $SnCl_2$ for the release of inorganic mercury. Subsequently added $SnCl_2$-$CdCl_2$ releases the organic form of mercury from the same sample[74]. In a third variation of the original method, inorganic mercury is reduced from the acidified sample by $SnCl_2$-$CdCl_2$, which in this medium can reduce only inorganic mercury. Organic mercury is released after inorganic mercury determination by alkalinization with NaOH[75].

(4) The modification used in the Ottawa River Project[72] reduces the amount of $SnCl_2$ from 100 to 50 mg per sample, but supplements it with hydroxylamine. This reagent inhibits the decomposition of methylmercury during an extended period before mercury vapour is flushed out with nitrogen. The aim of this modification is to avoid the need for analysis with internal standards[72,73].

Hydroxylamine and the predigestion of urine and fish increase the possibility of white fume formation. Digestion certainly decreases, but might not completely eliminate, the effect of sample composition on peak deflection. Thus the identical height of peak deflections caused by the aliquots of standard with or without sample must be demonstrated before the calibration graph is used for calculating mercury concentrations. Methods that are based on the separate release of inorganic and methylmercury from the same aliquot[6,58,74,75] must be tested with inorganic and methylmercury standards for peak deflections.

In a modification designed to measure only urinary mercury concentrations in presonnel exposed to mercury vapour, urine is acidified only for the total mercury determination and the volume of chemicals is decreased[76]. However, this method has no advantage over the method of Magos and Cernik[23] for inorganic mercury as mercury vapour exposure increases only the excretion of this type of mercury. A modification of the method of Magos and Cernik[23] by Gage and Warren[77] extends the method to distinguish between the various groups of organomercurials based on their varying lability in the presence of acidic cysteine.

5.2.2.3. Gas-liquid chromatography

The gas chromatographic analysis of organomercurials is based on their separation from inorganic mercury by extraction into an appropriate solvent and their different retention times in the gas chromatograph. Extraction is effected in multiple steps in order to eliminate interferences, but losses must be kept to the minimum.

In the modification of Gage's[56] extraction procedure for the gas chromatographic determination by Westöö[78], methylmercury is extracted from HCl-treated

homogenates as MeHgCl into benzene, followed by back-extraction into alkaline sodium sulphite. Acidification of this aqueous phase with HCl and re-extraction of the generated MeHgCl into benzene completes the separation and purification procedure. Extraction both from the homogenate into benzene and from benzene into alkaline sodium sulphite is incomplete, with ca. 70% recoveries. The introduction of cysteine for the extraction of methylmercury as the methylmercury-cysteine complex from benzene into alkaline sulphite[79] and the addition of copper(II)[22,80] and urea to homogenates[70] increased recoveries. Correction for loss of methylmercury can be made by the use of partition coefficients[81] or by measuring the loss of small amounts of ^{203}Hg-labelled methylmercury added to the homogenate[70]. To avoid over-correction, utmost care must be taken to remove any inorganic mercury contamination from the labelled MeHgCl. In the method of Goolvard[82] the identical extractibility of methylmercury and ethylmercury is urilized. The unknown methylmercury concentration is calculated by the ratio of the area under the methylmercury peak to the area under a separate peak given by the ethylmercury internal standard. Modifications include the use of hydrobromic acid instead of hydrochloric acid[83] and toluene instead of benzene[22,84] and the use of a combined gas chromatograph-mass spectrometer instead of a standard gas chromatograph with an electron-capture detector[85].

The recovery of methylmercury in the extract without the presence of interfering substances is only one factor in achieving high sensitivity, accuracy and precision in the gas chromatographic determination of methylmercury. Column packing and instrumental conditions are the other factors. The sensitivity is 1 ng of mercury[50], accuracy and precision is good and deviation from the mean or from values given by other accepted methods is less than 5%[67,70,80].

A gas chromatographic method with claimed higher sensitivity uses a completely different separation procedure. In the method of Zelenko and Kosta[86] the sample is placed in the inner compartment of a standard Conway cell and mixed with potassium hexacyanoferrate(II) and sulphuric acid. After closing the cell, cysteine buffer-impregnated paper placed in the outer compartment absorbs volatile methylmercury cyanide. After incubation for 14 h at 75°C, the paper is removed and dropped into hydrochloric acid, from which it is extracted into benzene for gas chromatographic determination.

When the total mercury concentration is required, gas chromatography is usually complemented either with the atomic-absorption determination of total mercury in the digested samples, or with neutron activation analysis. However, inorganic mercury can be methylated and determined gas chromatographically in this form. In the method of Zarnegar and Mushak[87], inorganic mercury is converted into methylmercury in an acidic medium by pentacyanoalkylcobaltate(III). One aliquot treated with pentacyanomethylcobaltate(III) and another with deionized water before extraction permits the determination of total and methyl-

mercury with the same gas chromatographic method. In the method of Cappon and Smith[70] inorganic mercury is methylated in the aqueous layer of the initial benzene extract with methanolic tetramethyltin. Recovery is followed by the addition of a small amount of ^{203}Hg-labelled $HgCl_2$ to the sample before methylation.

5.2.2.4. Molecular activation analysis

Direct molecular activation analysis combines neutron activation with hot atom (Szilard-Chalmers) chemistry and utilizes the principle that the distribution of the radioactivity of the target element among its possible forms depends on the nature of the target molecule: when neutron activation and the sample matrix are kept constant, a fixed proportion of methylmercury retains its molecular form[88,89]. However, this raciochemical retention is so sensitive to matrix effects that it is different not only for pure methylmercury and methylmercury in fish protein, but also between methylmercury in cod or in swordfish[88]. Moreover, in old samples inorganic mercury can be methylated during activation, although this effect can be prevented by pre-radiation isopropanol extraction[89].

5.3. ANALYTICAL TARGET

Methods for mercury determination after acid digestion or by counting ^{203}Hg do not discriminate between the different forms of mercury. This might be a handicap when the chemical form of mercury is in doubt. However, in many instances non-selective methods, which determine all mercury in the sample irrespective of its chemical form, are adequate because the dominant mercury form can be deducted from the circumstances of exposure. Thus, if one is not exposed to phenylmercury, methoxyethylmercury or ethylmercury, or given a diuretic mercury compound, blood or urine could contain only inorganic or methylmercury. The distribution between these forms depends on the type of exposure.

The background mercury concentration in blood and urine is low: in 74.5% of 609 blood and 80.8% of 778 urine samples the mercury concentration was less than 5 ng/ml[90,91]. In Saskatchewan residents[92] and in hospital employees in Rochester, NY, U.S.A.[57] the blood mercury concentration was less than 10 ng/ml and at this level one third of the mercury was inorganic and two thirds organic. Moderate fish consumption slightly increases the concentration of methylmercury in the blood[57], whereas industrial mercury vapour exposure increases mainly the inorganic mercury concentration[93]. In man, an increase in blood mercury concentration caused by exposure to methylmercury hardly increases the urinary excretion of mercury[94] and thus an abnormally high urinary mercury concentration is indicative of inorganic mercury exposure. The decomposition of the diuretic chlormerodrin[52] or the fungicidal methoxyethyl- or phenylmercury salts[95,96] is rapid, which ensures that 3-4 days after exposure all mercury derived from these com-

pounds is in the inorganic form in both blood and urine. The same rule that restricts mercury to the inorganic and methylmercury forms applies to food and environmental biological samples, e.g., in marine fish mercury is mainly in the methylated form[97,98]. If the possibility of direct contamination with phenyl-, methoxyethyl- or ethylmercury can be excluded, there is no point in considering the presence of these compounds in the analytical programme. Hence, with the exception of their industrial or agricultural use, the analytical work is restricted either to total mercury determination alone or to one of the following combinations: total and inorganic mercury, total and methylmercury or inorganic mercury and methylmercury. The choice depends on the available instrumentation and economics.

5.4. SAMPLING AND SAMPLE STORAGE

Conclusive environmental and epidemiological surveys or clinical diagnosis depend on representative samples. As methylmercury concentrates in red blood cells and not in plasma[99], the determination of total mercury only in the plasma of methylmercury-intoxicated patients is pointless. Thus a knowledge of the distribution and metabolism of different mercurials within a species or in an ecological system is essential for planning a survey. Exploratory tests, when distribution is in doubt, and collection of data on the habits of the target population in question are essential for good economy. Thus, without knowing that artificial hair-waving solutions are popular among Japanese women and that treatment with these solutions decreases the concentration of methylmercury in hair[100], one might use hair as an index media for estimating their past exposure from segmental hair analysis. The same blood mercury concentration has a different meaning when sampling is done before or at the end of the fishing season in a population that consumes fish from mercury polluted lakes or rivers.

Even when the survey is properly planned, a satisfactory number of samples are collected from representative sources and the best analytical technique is used, improperly planned and executed sample handling can make the whole exercise useless. Contamination and losses may occur either before and during the analysis. The use of contaminated glass, plastic or metal surfaces, losses through volatilization (e.g., during thermal neutron activation and decomposition digestion), inadequate absorption (e.g., on gold foil) or extraction (e.g., for gas-liquid chromatography) are the main sources of error. Controlled losses (e.g., when partitioning is used in an extraction procedure) or contamination (high blank) might not affect accuracy, but certainly affect sensitivity. No correction can be introduced when the sample is contaminated with mercury during sampling or mercury is lost during sample storage.

The sampling procedure must be planned and containers prepared. Although there are differences in handling fluid or solid samples, organ tissue or hair, the general rules are the same: clean demercurified containers, which do not absorb or are not permeable to any form of mercury, and conditions that prevent the biological conversion and volatilization of mercury. The quality of containers is important, as inorganic mercury from its acidified solution is lost both by absorption into the wall of the container and by diffusion through the wall. Greenwood and Clarkson[101] found that flint glass, cellulose nitrate, polyethylene and Butyrex were the worst and Pyrex, polycarbonate and Teflon were the best in this respect. The mercury Sampling and Analysis Review Comittee[73] in Canada recommends glass containers with Teflon-lined caps, washed with nitric acid or concentrated chromic acid and rinsed repeatedly with tap water and distilled water. For soft tissues the Committee recommends plastic containers with tight-fitting lids or plastic bags with good closures and with walls that prevent moisture loss. Plastic that becomes brittle when frozen should be avoided. The airspace in the container should be kept to the minimum to prevent changes in the water content of the sample. Cleaning of disposable containers is usually not necessary, but care must be taken to prevent contamination during sampling.

Hair cut at the scalp, while the bunch of hair is held with a pair of haemostatic forceps, is placed in a polyethylene bag and stapled to maintain alignment of the individual strands for storage or transportation[61].

Since it was first reported by Magos et al.[102] that urine or tissue homogenates contaminated with such bacteria as Gram-negative *Diplococcus, Pseudomonas Pyocyanea, Proteus* and *Klebsiella aerogenes,* can volatilize up to 75% of the original mercury concentration within 48 h, the inhibition of bacterial growth in biological samples has become obligatory. Volatilization by bacteria is the result of the reduction of Hg^{2+} to mercury vapour[102]. Other possible bacterial effects are HgS formation and the volatilization of methylmercury in the presence of H_2S[103,104], which can be produced by sulphur-reducing bacteria, methylation of inorganic mercury by intestinal bacteria[103,105] and the decomposition of organomercurials by enterics and pseudomanads[106,107].

Bacterial growth can be prevented by adding 0.1 ml of concentrated HCl to every 10 ml of urine[23]. Alkalinization of the urine or the addition of sulphamic acid with Triton X-100 detergent is also effective, although alkalinity results in the precipitation of inorganic mercury[28]. The general procedure to prevent loss or change in the form of mercury in all samples, with the exception of hair, is to deep-freeze[73]. Hair is best kept at room temperature.

5.5. EXAMPLES OF METHODS

5.5.1. Introduction

Methods in this section are given as examples; their selection does not reflect valued judgement but the author's restricted personal experience. The reagents used are all of analytical reagent grade.

5.5.2. Determination of total mercury in food or other biological samples with the method recommended by the Analytical Methods Committee[22]

5.5.2.1. Principle

Digestion with sulphuric acid, nitric acid and hydrogen peroxide and reduction for atomic-absorption spectrometry with hydroxylammonium chloride and tin(II) chloride.

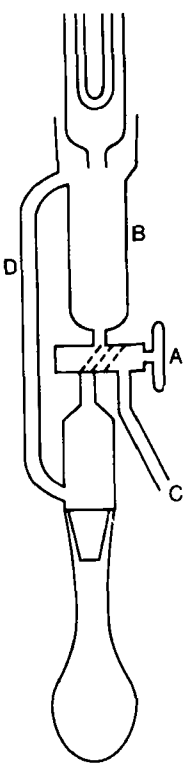

Fig. 5.1. Apparatus for the wet decomposition of organic matter.

5.5.2.2. Apparatus

(1) 200-ml Kjeldahl flask with a B24 socket attached to a standard double-surface reflux condenser as shown in Fig. 5.1.

(2) Heating mantle.

(3) Atomic-absorption spectrophotometer with cold cathode mercury lamp.

(4) Ice-bath.

5.5.2.3. Reagents

(1) Nitric acid, sp. gr. 1.42.

(2) Sulphuric acid, sp. gr. 1.84 (low in lead grade).

(3) Hydrogen peroxide, 50% (w/v).

(4) Potassium permanganate solution, 6% (w/v).

(5) Hydroxylammonium chloride solution: 20 ml of sodium chloride solution (15%, w/v) are mixed with 12 ml of hydroxylammonium solution (21%, w/v) and the volume is made up to 100 ml.

(6) Tin chloride solution: 21 g of granulated tin are heated with 50 ml of water and 50 ml of hydrochloric acid, sp. gr. 1.18.

(7) Standard mercury solution: 0.135 mg of mercury(II) chloride is dissolved in 0.1 *N* nitric acid and the volume is made up to 100 ml with the same acid. A 5.0-ml volume of this solution is diluted to 500 ml with 0.1 *N* nitric acid and from this 5 ml are further diluted with 0.1 *N* nitric acid in the presence of 1 ml of 6% potassium permanganate solution to give a concentration of 100 ng of Hg in 1 ml.

5.5.2.4. Digestion

A 2.5-g amount of homogenate is mixed with 9 ml of sulphuric acid in the Kjeldahl flask. A condenser is attached, the flask is heated on the heating mantle and swirled until a tarry fluid is obtained. The flask is cooled on ice and, while on ice, 2 ml of H_2O_2 are added through the condenser and vertical tap A. The flask is removed from the ice, slowly swirled and when the reaction subsides the contents are heated again and 2 ml of nitric acid are added to the hot sample through tap A. After 2 min the tap is closed, the sample is again heated and, when fumes are evolved, the condensate is run off from tap B into a beaker. The latter procedure is repeated first with 1 ml of nitric acid and 1 ml of hydrogen peroxide and second after collecting the condensate in the same beaker with 0.5-ml portions of hydrogen peroxide and nitric acid. The cool condensate is now returned to the Kjeldahl flask through the reflux system and when the whole contents are cool, potassium permanganate solution is added until a permanent pink colour is produced. The digest is transferred into a 50-ml calibrated flask, the reflux system and Kjeldahl flask are rinsed with water and the sample is diluted with rinsing plus water to the 50-ml mark.

5.5.2.5. Atomic-absorption determination

A portion, usually 10 ml, of the digest is made up to 13 ml with water in the aeration test-tube and mixed with 2 ml of hydroxylammonium solution and 0.2 ml of tin(II) chloride solution. Mercury is flushed out from the digest with air (ca. 740 ml/min) which is passed through magnesium perchlorate and silica-wool, and then through the quartz-windowed gas cell. A standard mercury solution is added to the aerated sample to obtain the peak height caused by a known amount of mercury for calculation of the mercury concentration in the digest. For a blank determination a similar sample of material is selected with a low mercury content (less than 0.03 µg/g).

5.5.3. Selective atomic-absorption determination of inorganic and total mercury by the method of Magos[6] with modifications from Magos and Clarkson[57] and Farant et al.[63]

5.5.3.1. Principle

Inorganic mercury is released from the sample by $SnCl_2$ and total mercury, including methylmercury, by $SnCl_2$-$CdCl_2$ reagent.

5.5.3.2. Apparatus

Reduction vessels for the original method and for the reduced volume of reagents are shown with the whole assembly in Fig. 5.2. The cotton-wool layer filter removes fumes and the ice-cooled midget impinger containing 10 ml of distilled water removes water vapour. The mercury monitor is connected either to a recorder or to a digital voltmeter. It is convenient to use a digital voltmeter which stores and displays the peak output[108].

5.5.3.3. Reagents for wide reaction vessel

(1) L-Cysteine (free base) solution, 1% (w/v).

(2) Sodium hydroxide solution, 45% (w/v).

(3) Tin(II) chloride reagent; 10 g of $SnCl_2$ are added to 20 ml of 16 *N* sulphuric acid and the volume is made up to 100 ml with distilled water.

(4) Tin(II) chloride-cadmium chloride reagent: 25 g of $SnCl_2$ and 5 g of $CdCl_2$ are heated just to boiling with water and the volume is made up to 50 ml.

(5) Antifoam: tributyl phosphate.

(6) Sulphuric acid solution: 16 *N*.

(7) Mercury standard: 0.6767 g of $HgCl_2$ is dissolved in 5% sulphuric acid. A 1-ml volume of this solution is diluted to 1 l with distilled water containing 9.1 g of NaCl, 0.7545 g of sodium ethylenediaminetetraacetate and 100 mg of L-cysteine. This solution contains 500 ng/ml of mercury and is stable at 5°C for at least 6 months.

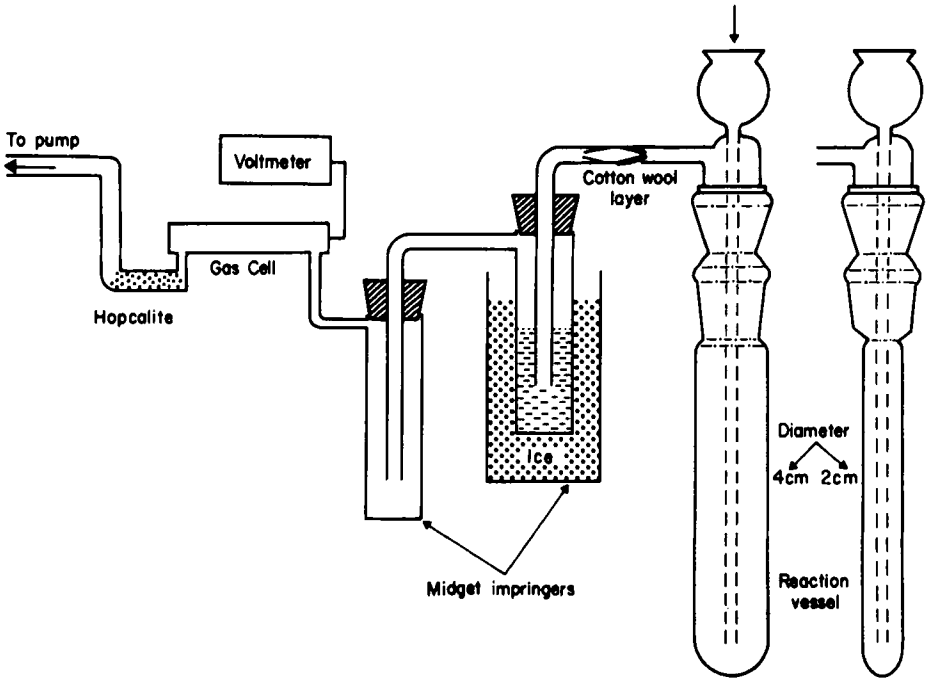

Fig. 5.2. Schematic representation of the assembled apparatus for selective atomic-absorption determination of mercury. The narrow reaction vessel described by Farant et al.[63] is further modified with the intention of automation. It has separate inlets for the two reagents and a valve at the bottom to permit in situ rinsing.

5.5.3.4. Reagents for narrow reaction vessel (Farant et al.[63])

(1) Tin(II) chloride: 10 g of $SnCl_2$ and 1 g of L-cysteine are dissolved in, and made up to volume (500 ml) with, 4.5 *N* sulphuric acid containing 1% (w/v) sodium chloride.

(2) 10 g of tin(II) chloride, 2.5 g of $CdCl_2$ and 1% L-cysteine are dissolved in, and made up to volume (500 ml) with, 4.5 *N* sulphuric acid containing 1% (w/v) sodium chloride.

(3) Sodium hydroxide solution, 35% (w/v).

5.5.3.5. Sample preparation

Tissues are homogenized; hair, fishmeal or grain are dissolved in sodium hydroxide-cysteine solution. When there is a possibility of the formation of mercury colloids, such as HgSe, all other samples are pre-treated with sodium hydroxide-cysteine: blood or homogenates is mixed with equal volumes of 1% L-cysteine and 45% NaOH; 20-80 mg of hair or 500 mg of fishmeal are mixed with

1 ml of 20% NaCl, 1 ml of 45% NaOH and 1 ml of 1% cysteine. Samples are heated on a hot-plate just to boiling. The volume is made up to 10 ml with saline and aliquots are used for atomic-absorption determination.

5.5.3.6. Determination

A 1-ml sample (or more when desired) is pipetted into the reaction vessel followed by the addition of antifoam, 1 ml of cysteine, 20 ml of saline, 10 ml of sulphuric acid and 1 ml of $SnCl_2$ or $SnCl_2$-$CdCl_2$ reagent. The contents are mixed, 20 ml of NaOH solution added and aeration is started. With a narrow reaction vessel, smaller volumes of sample can be analysed. After addition of the sample and antifoam either 5 ml of $SnCl_2$ or 5 ml of $CdCl_2$ reagent are added, followed by 5 ml of NaOH solution and aeration.

The mercury concentration is calculated from the peak height given by the sample minus reagent blank. The difference in peak heights given by the sample with and without a mercury standard divided by the amount of added mercury in nanograms gives the deflection caused by 1 ng of mercury in the presence of the sample. When the blank is high, the difference in deflection caused by a standard added to the reagent and to the sample is used for the proportionate correction of the blank.

When a large number of samples of identical composition (e.g., 1 ml of blood or 1 ml of 10% homogenate) are analysed, the mean deflection caused by internal standards in a representative proportion of samples (e.g., every fifth of 100 samples) can be used to calculate the concentrations in every other sample. In these circumstances the use of the mean deflection actually decreases errors.

5.6. DATA EVALUATION

5.6.1. General considerations

The general aim of the determination of mercury in biological samples is always to assess whether mercury in an ecological system, in a population or in an individual can affect normal function, to assess the role of mercury in existing functional disturbances or to assess the level of exposure. The end-points on the scale used in the assessment of mercury concentrations are the background concentration and the concentration range which is associated with severe, perhaps lethal damage. Between these two points there is the so-called threshold concentration for mild toxic effects. None of these points are absolute.

Tha background concentration is a measure of the range of low-level and practically unavoidable exposure, which can change with time or from location to location. Sensitivity to toxic concentrations may depend on species, age, sex,

nutritional status and physiological conditions, with wide individual variations even within one subgroup. Established threshold limits can change with the discovery of populations with sensitivities higher than those currently known. Moreover, there is always some uncertainty in limits of toxicity: those which are based on animal experiments encounter the problem of extrapolation from one species to another and those based on human observations suffer from other restrictions. Contrary to experiments, the conditions of clinical and epidemiological studies are such that the investigator cannot increase population numbers at will, or select the optimum sampling time, or might not be able to obtain further samples to check suspect determinations.

The determination of mercury in biological samples has an important role in the assessment of exposure. In contrast to the biological limits of the clinical threshold of toxicity, the concepts of threshold limit value (TLV) or permitted concentration levels are administrative, which might or might not correspond to the biological threshold of toxicity. When time is quantitated for inhalation exposure, as is ideally the case in occupational exposure to airborne chemicals, the TLV for a certain chemical is equals to a certain dose and has a more or less well defined relationship to the biological threshold of the chemical. When concentration limits are set in food, as the 0.5-1.0 ppm mercury limits in the edible raw portion of fish, one important parameter, which is required for the conversion of these limits to dose, is missing. This parameter is the amount of fish consumed, which shows enormous individual variation. Limits for mercury in fish aim to protect the most sensitive individuals against the effects of prolonged heavy fish consumption. Unlike concentrations in different food products, the tolerable weekly intake with food is dose equivalent and therefore can be related to threshold toxicity.

5.6.2. Exposure to mercury vapour

The present TLV for occupational mercury vapour exposure in many countries is 0.05 mg/m^3. As the air to urine mercury relationship for time-weighted average (TWA) exposure in µg/m^3 and for urine in µg/l corrected to specific gravity is 1:1[109], the 0.05 mg/m^3 TLV corresponds to 50 µg/l in urine. An upward shift from this excretion level indicates deteriorating environmental conditions. Results above 200 µg/l are considered potentially dangerous[110], as above 300 µg/l excretion in urine the possibility of chronic mercury intoxication with overt clinical manifestations is high[111]. Moreover, Suzuki[112] noted hand tremor with levels as low as 114 µg/l in urine. Nearer to the upper end of the scale a 2400-8300 µg/l urinary mercury excretion was associated with tremor, irritability, skin rash, painful mouth and bleeding gums in a family exposed to mercury vapour in their home after spilling a few hundred millilitres of mercury on to the carpet.

Mercury blood concentrations at the time of the urine collection ranged from 183 to 620 ng/ml[113]. The relationship between urinary and blood mercury concentrations can be shown only by ranking[114], although on a group basis mercury in blood shows a fair correlation with exposure[90]. Smith et al.[115] plotted the weighted average air concentration against blood concentration: every 0.1 ppm increase in the time-weighted occupational mercury exposure caused an approximately 45 ng/ml increase in the blood mercury concentration above the 9 ng/ml background. The background level in occupationally unexposed populations is lower, being less than 5 ng/ml in both blood and urine[90,91].

The WHO Task Group[116] associated 35 ng/ml of mercury in blood and 150 µg/l in urine with an 8-h exposure to 0.05 ppm Hg vapour and 70-140 ng/ml in blood and 300-600 µg/l in urine with 0.1-0.2 ppm exposure. When the period of exposure is 24 h per day, the same urinary and blood concentrations are reached by a third of the above mercury vapour concentrations.

5.6.3. Inorganic mercury salts and methoxyethyl- or phenylmercury

Inorganic mercury salts and methoxyethyl- or phenylmercury compared with mercury vapour or short-chain alkylmercurials have little significance as occupational and environmental toxic agents. They are also less toxic as judged from the recommendation of an International Committee[117] which recommended a mercury TLV of 0.1 mg/m^3 for these compounds, that is, twice the TLV for mercury vapour.

Intentional or accidental ingestion of inorganic mercury salts, mainly $HgCl_2$, results in corrosion of the alimentary tract and renal damage. The determination of inorganic mercury in vomit or stomach contents has a diagnostic value, and in surviving patients the determination of mercury in the urine or blood helps to evaluate the success of the treatment. The probable minimal lethal dose of $HgCl_2$ for children is 120 mg and for adults 180-500 mg, but with chelation therapy recovery was reported after the ingestion of 1.5-20 g of $HgCl_2$[118]. A 7½-year-old child who survived the ingestion of 435 mg of $HgCl_2$ a had blood mercury level of 400 ng/ml with a peak urinary mercury excretion of 9.0 mg/l of $HgCl_2$[119]. Children who suffered from acrodynia excreted 100 to 400 µg Hg/l urine and the excretion of mercury helped to identify calomel as the causative agent[120].

According to post mortem analysis of mercury in victims of $HgCl_2$ intoxication, kidneys have the highest concentration, being approximately 40 times higher than the 0.15-1.2 µg/ml measured in blood[118].

In addition to irritation of skin, caused by all organomercurial fungicides[121], no serious intoxication of occupational origin has been attributed to methoxyethyl- or phenylmercury. Goldwater et al.[122] reported the accidental intoxication of a worker who had been sprayed with phenylmercury acetate. The concentration

of mercury increased to 8.5 mg/l in his urine, but apart from mild proteinuria he showed no signs of intoxication. Data for the evaluation of blood and urinary mercury concentration in relation to toxic thresholds are missing.

Differentiation determination of inorganic and organic mercury within 1-2 days of the last exposure might help to pinpoint organomercury exposure as a cause of elevated levels. After 2 days all of the mercury is expected to be in the inorganic form because of the rapid in vivo decomposition of both methoxyethyl- and phenylmercury[96].

5.6.4. Short-chain alkylmercurials

Methyl- and ethylmercury are the two short-chain alkylmercurials which, after prolonged exposure, are able to cause irreversible damage in the nervous system. Ethylmercury is less stable than methylmercury and probably this factor explains signs of renal damage in ethylmercury-intoxicated patients[123]. In spite of this difference in toxic effects, guidelines for methylmercury may be extended to ethylmercury. Exposure to ethylmercury is restricted to its production or use and to the misuse of ethylmercury-dressed grain. In contrast to ethylmercury, methylmercury is a general environmental hazard, as this form of mercury is synthesized by microorganisms in the aquatic environment and concentrates in the aquatic food chain[4,5].

An International Committee[117] did not set a permitted air concentration level for short-chain alkylmercurials for occupational exposure, but recommended that the maximum permitted blood concentration of mercury should be 100 ng/ml. The WHO Task Group[116] did not recommend any threshold limit for intake of methylmercury with food but proposed that a daily 3-7 µg/kg body weight intake of mercury as methylmercury could lead to 200-500 ng/ml of mercury in blood and 50-125 µg/g in hair and to paraesthesia in 5% of the exposed population with prolonged exposure.

Clarkson and Marsh[124] found ten Korean fishermen with 120-200 ng/ml and one with 265 ng/ml of mercury in their. Although the mercury was organic and the fishermen were probably in a steady state for methylmercury, the neurological findings were negative. The same concentration, whether it represents a steady-state condition or a peak value achieved during a short exposure time, has a different significance from the point of view of neurotoxicity[125]. Skerfving[126] measured 1100 and 1200 ng/ml of mercury in red blood cells in two men who regularly consumed fish with high mercury concentrations in large amounts during the fishing season without any adverse effect.

In the Iraq methylmercury epidemics, paraesthesia became more frequent only in those with 500-1000 ng/ml of mercury in their blood[127]. However, on plotting the frequency of paraesthesia against the blood concentration of mercury measured

65 days after the end of exposure with extrapolation to the end of exposure, the threshold blood concentration could not be less than 350 ng/ml, which is the steady-state blood mercury concentration for long-term methylmercury consumption of about 5 µg/kg/day of mercury[128]. The Research Committee on Minamata Disease[129] also concluded that 5 µg/kg/day is the minimum toxic dose.

The calculations are naturally based on two assumptions: the first if that the population affected by methylmercury is representative and the most sensitive individuals in this population are no less sensitive than the most sensitive ones in other populations; the second is that a threshold for paraesthesia prevents any adverse effect caused by methylmercury. Thus, if one considers the possibility of accelerated cell death without paraesthesia or a biochemical defect in the nervous system without histological abnormality, a threshold for paraesthesia might not be adequate. However, until now efforts to find these types of "silent damage" remained fruitless[130].

The upper end of the scale are the 3.0 and 5.0 µg/ml blood concentrations in patients with severe ataxia and many with hearing and visual defects and/or a lethal outcome[127]. In the hair and blood of methylmercury-intoxicated patients about 90% of the mercury is methylmercury[127]. The mean ratio of the concentration in the hair at the scalp to that in the blood is ca. 250[131] and segmental hair analysis can be used to assess past exposure, limited by the length of hair[60], and to calculate clearance half-time[132] in methylmercury-exposed populations. There appears to be a clinically detectable risk of foetal brain damage when the peak maternal hair concentration is about 100 µg/g, that is 400 ng/ml in blood[133]. The latest follow-up study by Marsh et al.[134] indicates that the foetal brain might be affected at a significantly lower maternal exposure level (67.6 µg/g in hair ≡ 270 ng/ml in blood), and in this case the threshold limit level of 350 ng/ml in blood is not valid for the maternal-foetal unit.

Near to the lower end of the scale are the mercury concentration values of fishing communities around the coastal area of the North-Eastern Irish Sea. Haxton et al.[68] reported a mean mercury concentration of 5.0 ng/ml in blood and 2.0 µg/g in hair with a dietary intake of 1.9 µg/kg/week. In another fishing community in Cornwall with a mercury intake of 1.3 µg/kg/week the corresponding values were 3.5 ng/ml and 1.35 µg/g. About 90% of the blood mercury was organic and a 1 unit increase in blood mercury concentration corresponded to a ca. 250 unit increase in hair mercury concentration. The values reported by Haxton et al.[68] are similar to those found in hospital employees in Rochester, NY, U.S.A.[57] or in Sasketchewan residents[92] and consequently fall into the background category.

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CHAPTER 6

CADMIUM

MARKUS STOEPPLER

CONTENTS

6.1. INTRODUCTION

For years, determinations of cadmium in biological materials were mainly performed by colorimetry, i.e., spectrophotometry[1-5], frequently by the use of dithizone subsequent to a prior separation from interfering substances[1,5,6]. However, emission spectroscopy[2,5], neutron activation analysis (NAA)[2,5,7,8] and

classical polarographic methods[9,10] were also applied. With the exception of radiochemical NAA, which is sensitive but not used as a rapid routine screening technique, these methods typically had absolute detection limits of the order of 1 µg of cadmium. Thus, if lower levels had to be analysed, pre-concentration was always necessary. These methods, however, were sufficiently sensitive for the determination of cadmium in organs and hair with average concentrations at the micrograms per gram level[2,5,11]. The same, in principle, also applies to flame atomic-absorption spectrometry (FAAS), which owing to its specifity and speed has been increasing and successfully used for cadmium analysis from its introduction in 1955[1,2,5,12]. Normal cadmium levels in body fluids, however, are in the low nanograms per gram range[11]. Even in occupational exposure, average levels of cadmium in urine and whole blood not exceeding a maximum of about 100 ng/ml were reported[11].

Some of these data were obtained by FAAS after pre-concentration, but the commercial introduction of more powerful trace analytical methods in the decade from 1970 greatly extended routine analytical potentialities, particularly for cadmium. Graphite furnace AAS (GFAAS)[5,11-13] anodic stripping voltammetry (ASV) and differential pulse anodic stripping voltammetry (DPASV)[5,11,14-17] decreased the absolute detection limits to 1 pg absolute (GFAAS) or less than 10 pg/ml (DPASV), with potential for determinations down to 0.1 ng/g in biological materials. The recently introduced mercury film electrode (MFE) for DPASV again improved detection limits to below 0.1 pg/ml, thus rendering possible in principle precise and accurate determinations at the lowest known natural concentrations[17,18].

6.2. THE ANALYTICAL CHAIN

6.2.1. Sampling, sample preparation and pre-treatment

Cadmium levels in body fluids, without occupational or environmental exposure, are at the low nanograms per millilitre level. Thus, contamination during sampling or sample preparation from laboratory ware, made from different plastics, is highly probable. Proper selection and cleaning of all vessels and instruments used and blank determinations are therefore mandatory for reliable analysis. Only a few examples will be given here, because this is generally treated in the chapter on instrumentation.

The syringes used for whole blood sampling by venepuncture, available with different anticoagulants (heparin, potassium EDTA, ammonium citrate), all contain detectable amounts of cadmium. The cadmium blank level can vary significantly depending on the anticoagulant and the manufacturer.

Urine voidings are extremely prone to cadmium contamination during sampling from the body and from outside, and also from the containers used. As coloured urine sampling bottles can show contamination without the possibility of being satisfactorily cleaned for urine sampling, storage bottles made from colourless high-pressure polyethylene should be used. Cleaning procedures for those bottles have been described recently[19,20].

The determination of cadmium in materials with higher levels such as placentae, liver, kidney and faeces has also to be performed with great care, but precautions against contamination are not so stringent as for body fluids. Autopsy materials, however, should be taken as early as possible after death, because of changes in weight and elemental composition during post mortem storage[21].

Sample pre-treatment for cadmium analysis to obtain an analyte solution generally consists in operations for complete homogenization, if necessary, followed by, for instance, solvent extraction pre-concentration or the application of various decomposition procedures[22,23]. The most important methods for cadmium are mentioned below and a few references given.

Low-temperature ashing is applicable prior to all determination methods and shows, if properly applied, very low blanks[24-26].

Dry ashing owing to the volatility of cadmium and its compounds, requires strict temperature control, e.g., by programmable furnaces which limit the maximal temperature to $\leq 500^{o}C$[27]. For low concentrations ashing in quartz tubes is recommended[28].

Wet ashing using different acids and oxidants and mixtures of acids (nitric, sulphuric and perchloric acids, hydrogen peroxide, etc.) usually achieves complete mineralization at temperatures up to $310^{o}C$, also with mechanized systems[29-31]. Wet ashing is recommended for all modes of DPASV but can introduce elevated blanks if proper selection of reagents and vessels is not performed. Acids with high boiling points and/or a high oxidation potential, if not completely removed, can lead to difficulties in graphite furnace AAS (GFAAS) owing to the low ashing temperature possible for cadmium.

Pressure decomposition up to about $170^{o}C$, mainly with nitric acid, if properly operated, has very low cadmium blanks and in general is very useful for flame AAS and GFAAS[32,33]. However, owing to incomplete mineralization[34] the method is not applicable for subsequent electrochemical analysis without particular programmes and/or a further treatment.

6.2.2. Determination methods

6.2.2.1. Atomic spectroscopy

Analysis using different modes of AAS provides a very sensitive determination

of cadmium at 228.8 nm[12]. Detection limits of <3 ng/ml are attainable for the flame method and of <0.002 ng/ml for the graphite furnace method. These data show potential also for direct analysis. However, light scattering due to Rayleigh's law and molecular absorption deriving from the matrix strongly increase below 300 nm, particularly for cadmium. The low boiling point of cadmium additionally prevents very effective removal of interfering matter at the temperatures applied (300-450°C) during the charring step. This often severely influences compensation efficiency. In such a case improvements are possible by sophisticated temperature programming with oxygen addition, so-called matrix modification and/or solvent extraction. However, more recent compensation principles such as Zeeman compensation seem to be very promising for cadmium[35]. A disadvantage, particularly for GFAAS, is the poor precision obtainable owing to the properties of graphite tubes discussed elsewhere[35].

Atomic-fluorescence spectroscopy (AFS) has also been shown to be a valuable and sensitive approach for determining cadmium, based on relatively inexpensive flame techniques[36]. Owing to the lack of commercially available instruments, however, the routine application of AFS is not possible in numerous laboratories.

The growth of atomic-emission spectrometry (AES) based on (e.g., inductively coupled) plasma sources also offered new potential for cadmium determination in the frame of a multi-element approach[35]. With optimal detection limits of about 5 ng/ml for sequential and about 20 ng/ml for simultaneous multi-element analysis (compromise conditions), the detection power is comparable to that of flame AAS but considerably poorer than that of GFAAS. Thus ICP-AES usually is the method of choice if higher levels have to be analysed and additionally a multi-element approach is required. However, if such expensive instrument is available, solvent extraction/pre-concentration may also be useful. It is well known, however, that all methods with higher pre-concentration factors are particularly prone to contamination.

6.2.2.2. Electroanalytical methods

At present electrochemical methods provide, in addition to wide potential as oligo-element approaches, the highest possible detection power for cadmium in solution without interferences from inorganic constituents[17,18,35]. The most sensitive DPASV system is equipped with a rotating glassy carbon electrode (RGCE) in situ coated with a thin mercury film and achieves detection limits of <0.1 ng/l in analyte solution. This excellent detection power would give absolute superiority if it could be applied directly to, e.g., body fluids as is possible in sea, inland and rain water analysis[17]. Unfortunately, there is always more or less interference from organic matrix constituents, which drastically influences the detection limits attainable in practice. Thus, if accurate and precise determina-

tions at lower concentration levels are required for body fluids and solid materials as well, complete mineralization prior to electrochemical analysis is necessary. This to some extent balances the advantages of DPASV in comparison with AAS. However, low blank digestion procedures in clean laboratories and clean benches[36,37] are feasible without extreme expense. Despite the discussed limitations, electroanalytical methods always constitute, based on very reliable and cheap instrumentation, an invaluable checking potential for other methods used in cadmium analysis such as AAS and ICP-AES. Further, DPASV competes favourably with GFAAS, and even flame AAS if precision analysis is mandatory.

6.2.2.3. Other methods

As cadmium levels in biological materials on average are low, less sensitive methods such as colorimetry and wavelength- or energy-dispersive X-ray fluorescence are of minor importance. This is also true of the sensitive but less precise spark-source mass spectrometry (SSMS).

Several other methods, however, were and still are of benefit for the establishment of "true" cadmium levels in different materials. This is particularly the case for double reflecting X-ray fluorescence[35,38], radiochemical NAA[7,35,39,40] and isotope dilution mass spectrometry (IDMS) with spark source and thermal ionization excitation[35,41]. These techniques are less suitable for routine applications but are excellent tools to confirm independently very low cadmium levels, as was the case for radiochemical NAA and cadmium in whole blood using a very sensitive method for cadmium determination[40,42].

6.2.3. Quality control

Routine analysis is usually performed by a single method, frequently depending on the standard equipment available in a particular laboratory. At present the determination of cadmium in biological materials is predominantly performed with various more or less equally sensitive GFAAS systems. From inter-laboratory comparisons carried out in the last decade a considerable lack of accuracy is obvious[11,43]. This is disappointing because of the importance of cadmium data for medical, occupational and legislative decisions. Thus current quality control, also including other toxic metals such as Pb, Hg, As and Ni, has to be established by different measures, as follows.

To minimize systematic analytical errors the evaluation, production and use of a satisfactory number of appropriate control and standard reference materials for continuous calibration and methodological developments is urgently needed[44]. Fortunately, owing to efforts from national and international organizations and industry, the number of such materials now available or in the final preparatory

stages is increasing. Recently it could therefore be stated that in addition to NBS materials also a first selection of dry (hair, blood) and lyophilized materials that can easily be reconstituted (bovine blood, artificial urine) are in preparation[11]. Cadmium concentrations range from very low to elevated levels typical of occupational and/or environmental exposure.

Additionally, the application of independent methods in either the same or different laboratories with recognized expertise can be of value in establishing normal or elevated cadmium levels and in controlling routine procedures. A physically independent method, e.g., DPASV, NAA or IDMS, compared with GFAAS can thus also serve to detect bias due to an inadequate method or the improper functioning of a particular instrument.

To elucidate the comparability of data reported by different laboratories and to improve analytical skill, the analysis of control samples in numerous laboratories (inter-laboratory comparisons) is also of high value. The routinely performed distribution of control blood samples to numerous laboratories has, e.g. within the EEC, resulted for lead in a considerable improvement of analytical performance which can now also expected for cadmium[45].

6.2.4. Handling and interpretation of results

The discussion of the items of this section is based on the assumption that the reliability of the method(s) considered is confirmed by measures outlined in Section 6.2.3.

If analytical data were reported for further evaluation, e.g., in environmental or medical affairs, the user should be properly informed of the typical error of the given value. Frequently this error is reported as the standard deviation of a few (rarely more than three) independent determinations in subsamples of the specimen to be investigated. In most instances these determinations, owing to routine requirements and costs and in order to obtain the results as early as possible, were carried out consecutively. Thus the value, e.g., 10 ± 0.5 ng/ml of cadmium in whole blood, is reported for within-run precision. If, however, these determinations were carried out on three different days within 1 week, the value might change to, say, 9 ± 1 ng/g. The latter value doubtless represents a more realistic estimation of the true error of the reported value. Thus, the day-to-day precision for typical and routinely analysed levels in given materials with a given method has to be evaluated in order to report typical errors, which is of particular importance if threshold limits have to be considered. Results reported on such a sound basis are meaningful for the user and clearly demonstrate the uncertainty of a given result for a given matrix if, e.g., increasing or decreasing levels have to be considered.

If biopsy and autopsy materials have to be analysed for cadmium it should be further borne in mind that particularly in blood, liver and kidney the cadmium levels of smokers on a group basis are higher than those of nonsmokers. Monitoring of moderately exposed subjects should always be carried out by comparison with a control group, and the control group should have a similar distribution of smokers and nonsmokers as the exposed group.

6.2.5. Cost-benefit relationships

Cost-benefit relationships for cadmium analyses have to consider, in addition to the bare deduced investment costs for the applied instruments and manpower, also the general duty of a laboratory, that is, if it is performing occasional or routine analyses of cadmium and other toxic metals, the instrumentation at hand and the experience of the laboratory staff.

If various or numerous trace metals have to be analysed routinely at different levels it can be assumed that AAS systems, probably also ICP-AES and possibly also DPASV, will be available. This, together with an experienced staff, allows cadmium analyses to be handled without particular difficulties and with easy calculations.

If, however, there is some experience of higher concentrations and the analysis is usually performed by, for example, XRF, ICP-AES and/or FAAS and additionally cadmium analysis at trace levels, possibly together with other toxic metals, is necessary, the first question is the number of analyses to be performed per unit time and the quality (precision, determination limit) required. It is also of interest if there is a possibility of a single analytical campaign for a distinct period (month) or if a rapid analysis after sampling is necessary (day, week). The second question then is whether the instrumentation available can be completed by accessories allowing the desired quality of analysis, for example the supply of a graphite furnace to a basic instrument. If this is the case, and storage of samples for about one analytical campaign (fortnight or month) is possible, this is the simplest and also least expensive approach. The staff, already experienced with AAS, now have only to learn careful contamination control and the particular requirements of GFAAS. If the instrumentation available does not allow simple completion and an additional instrument is needed, another situation is faced. For high sample throughputs, even if the investment is high, at present AAS offers wide element coverage and, owing to automated sample introduction operation control and data evaluation, fairly economic use. If the total number of analyses to be performed, however, is relatively low or a very high frequency with smaller sample numbers is required and additional elements that have to be determined are readily accessible to electrochemical methods,

the introduction of an electroanalytical system with low investment costs but high detection power, precision and accuracy is probably the better choice. This is true even if the laboratory staff initially are not familiar with DPASV, because in the long run this method will be useful as an independent approach without expensive accessories.

If a laboratory has to take over trace metal analysis as a new duty, the same is true as described above. AAS or, e.g., ICP-AES will be superior if numerous elements and samples have to be analysed, and DPASV is superior if the elements to be analysed are well within the exceptional abilities of that technique and the sample throughput is not excessively high.

A description of typical costs and sample throughput for analytical systems can be found elsewhere[35,46].

6.3. PARTICULAR ANALYTICAL PROCEDURES

Owing to continuous progress in methodological and instrumental performance, the given examples should be taken only as a more or less general introduction to practical analysis. For details of the described procedures, the references cited should be consulted.

Table 6.1 summarizes the most important facts, typical cadmium levels and/or ranges of the materials discussed below in about the order of appearance in the text, together with some additional references.

6.3.1. Whole blood and serum (plasma)

6.3.1.1. Atomic spectroscopy

A very rapid, simple and cheap but nevertheless effective approach is based on the Delves cup AAS method[47]. This involves the introduction of 10-20 µl subsamples of whole blood, etc., placed on small nickel cups, initially brought to dryness, into the air-acetylene flame of an AAS instrument. This produces rapidly high signals for relatively volatile elements such as Pb and Cd. Based on some improvements to this method described by Ediger and Coleman[48] and Ulander and Axelsson[49], Elinder et al.[50] were able to analyse numerous whole blood samples with excellent differentiation between smokers and nonsmokers. The detection limit of this method is ≤0.3 µg/l of cadmium and it has been used successfully in many laboratories within a WHO biological monitoring programme[51]. Cernik[52] and Vesterberg and Bergström[53] used the method after a prior dry ashing. Delves[54] later modified the procedure by phosphate addition in order to resolve better the cadmium atomic signal from the molecular absorption signals, and Carter and Yeoman[55] applied rapid low-temperature ashing using carbon tetrafluoride prior

to the cup procedure. The latter method, however, requires skilled staff and proper alignment of deuterium compensation, but still competes successfully with other, more expensive techniques and thus doubtless is the method of choice if only flame AAS is available. More recently automation of the method was proposed[56].

Many methods based on the graphite furnace have been published and several of them routinely applied. Attempts were made to overcome the interference of non-atomic absorption effects by a particular sample preparation or new instrumental concepts. Schumacher and Umland[57] mixed whole blood with acetone and perchloric acid and injected 20 µl of the 1:10 diluted solution into an HGA-72 instrument. This procedure minimized spectral interferences and a detection limit of about 0.1 µg/ml of cadmium in whole blood was reported. A disadvantage is poor precision even at moderately high levels[58]. Perry et al.[59] digested 0.5 ml of blood by addition of nitric acid followed by hydrogen peroxide and Brodie et al.[60] digested with nitric acid at 75°C in a water-bath. Hudnik et al.[61] used pressurized decomposition with nitric acid-perchloric acid (3:1). Hinderberger et al.[62] digested whole blood at 100°C with nitric acid and analysed the solution using the L'vov platform by phosphate addition for matrix modification. Lagesson and Andrasko[63] ashed the blood outside using graphite microboats prior to GFAAS.

Solvent extraction, i.e., complete separation of cadmium from the matrix, has been also advocated. Ulluci and Hwang[64] extracted cadmium directly from blood with APDC-MIBK after addition of saponin and formamide[64]. Boiteau and Metayer[65] wet digested the blood with nitric acid and hydrogen peroxide, extracted cadmium with dithizone-chloroform, back-extracted with dilute HCl and injected into an HGA-72 instrument. Allain and Mauras[66] haemolyzed blood by addition of deionized water, extracted cadmium with APDC-MIBK and injected an aliquot of the MIBK phase directly into an HGA-2100 instrument. Dornemann and Kleist[67] digested whole blood with HNO_3-H_2SO_4 or with HNO_3 under pressure and extracted Cd with hexamehtylene-ammonium hexamethylenedithiocarbamidate (HMA-HMDC) into a diisopropyl ketone-xylene mixture. They injected the organic phase directly into an HGA-72 instrument. Sperling and Bahr[68,69] wet digested whole blood with HNO_3-H_2SO_4, neutralized with $NaHCO_3$ extracted with APDC in CCl_4 and injected 20 µl of the organic phase into an HGA-74 instrument. The detection limit was reported to be about 0.1 µg/l of cadmium.

A relatively simple pre-treatment, proven for lead in whole blood, is deproteinization with, for example, trichloroacetic acid prior to AAS, as formerly advocated by Einarsson and Lindstedt[70]. If, however, dilute nitric acid is used as deproteinizing agent matrix modification with considerable background minimization in GFAAS was also found by Stoeppler et al.[71] for lead. Stoeppler and Brandt[73] applied the same principle to cadmium in whole blood: protein was precipitated with 1 *M* nitric acid, the sample centrifuged and the supernatant

TABLE 6.1

TYPICAL CADMIUM LEVELS IN HUMAN BIOPSY AND AUTOPSY MATERIALS WITH METHODS AND REFERENCES

Values in ng/g (ppb) wet weight if not otherwise stated. For probable averages only those data are included that have been confirmed recently and independently by different methods or were obtained applying rigid quality control measures.

Material	Probable average	Range (approx.)	Remarks	Applied methods	Ref.
Whole blood, non-exposed nonsmokers	<1	0.2-3		GFAAS, DPASV/HMDE, NAA, flame AAS (cup), Zeeman GFAAS	25, 40, 49, 50, 51, 53, 66, 68, 69, 73, 74, 82, 84, 85, 87, 154, 155
Whole blood, non-exposed smokers	<1.5	0.2-6			
Whole blood, occupational exposure		Up to 150 or higher		GFAAS, AFS, flame AAS (cup)	25, 73, 75, 86, 90, 156, 157
Serum	<0.1	0.05-0.4		GFAAS, DPASV	76, 91
Urine, non-exposed	≤0.5	0.1-3.0	Values to some extent age dependent	AAS (cup), GFAAS, Zeeman GFAAS, DPASV	65, 66, 68, 69, 72, 82, 95, 96, 99, 103, 104, 107, 158, 159
Urine, occupational exposure		Up to 100 or higher		Flame AAS, GFAAS, DPASV	35, 75, 104, 129, 156
Parotid saliva	<2		Cd in protein fractions also determined	GFAAS	107

Teeth	<500	50-2.500	Cd content may vary for different parts of a tooth	GFAAS, solid sampling flame AAS (boat), DPASV/MFE	108,109, 110
Hair, non-exposed	<500	100-2000	If only taken close to scalp the value mainly reflects endogenous influence	NAA, GFAAS, GFAAS with solid sampling	112, 113, 115, 117, 118, 120, 121
Hair, exposed	-	Up to several ppm	The distal region seems mainly to reflect exogenous influences	Flame AAS, GFAAS	111, 112, 119, 157
Faeces	-	100-800	Different in different countries: Sweden, U.S.A., Japan	GFAAS, flame AAS	103, 122, 123, 124, 160
Placentae		20-160 (dry weight)	Different values found in different geographical regions (rural-industrial)	GFAAS, NAA	125, 126, 127
Liver, non-exposed non-smokers		1000-3000	Limited study	In vivo NAA	128
Liver, non-exposed smokers		2000-6000			
Liver, exposed		5000-15,000		In vivo NAA	129
Kidney, non-smokers	<3000		Limited study, µg per kidney	In vivo NAA	128
Kidney, smokers	<6000		Limited study, µg per kidney		

(Continued on p. 210)

Table 6.1 (continued)

Material	Probable average	Range (approx.)	Remarks	Applied methods	Ref.
Kidney, occupational exposure		Up to 30,000	µg per kidney	In vivo NAA	129
Kideny cortex, unexposed		10,000-130,000		NAA, flame AAS	2, 122, 134
Kidney cortex, exposed		20,000 500,000			2
Muscle		20-300	Very different in different countries: Sweden, U.S.A., Japan	GFAAS and flame AAS, checked by NAA	122
Pancreas		10-800 (Sweden)	Smokers show higher values different averages: Sweden 500, U.S.A. 700, Japan 2200	Flame AAS or GFAAS	122, 124
Lung		400-2500		Flame AAS (cup)	119, 131
Bone		250	Much lower than hitherto supposed	GFAAS, solid sampling NAA, GFAAS and DPASV	140, 141, 142

used for automated determination of cadmium by GFAAS[72]. The method served successfully for baseline studies on normal levels (smokers and nonsmokers) in medical and occupational investigations[73-75]. With high-performance instruments, a dilution factor of 1:2 (blood + 1 *M* nitric acid) and a 20-µl injection, a detection limit as low as 0.1 µg/l can be achieved. Precision, expressed as day-to-day precision, typically is in the range from about 30 to about 4% depending on concentration, at the 1.3 ppb level, e.g., routine day-to-day precision is <15%. The low detection limit permitted a preliminary study of cadmium in serum with an average below 0.1 µg/l, while whole blood averaged 1 µg/l[76]. This method was also checked by independent methods[37,72,77]. Owing to its adaptability to all existing GFAAS systems with background correction, the method was adopted with minor alterations for routine use as a Deutsche Forschungsgemeinschaft (DFG) reference method[78].

Matrix modification with ammonium phosphate in combination with in-tube ashing in an oxygen atmosphere (HGA-500) was proposed by Delves and Woodward[79]. They reported a detection limit of 0.15 µg/l for a 20-µl injection. The precision of the method at the 1.6 ppb level was 23%.

The simplest and least vulnerable approach, if contamination is considered, is GFAAS determination of cadmium in whole blood with minimal pre-treatment. With commercially available GFAAS instrumentation this seems to be possible without any technical modification at present only by Zeeman compensation[77,80,81] Pleban and Pearson[82] added to whole blood samples the same amount of a 0.1% Triton X-100 solution and manually transferred 7 µl of this solution into the graphite cup cuvette of a Hitachi 170-70 instrument. They reported a detection limit of 0.12 µg/l and a precision of about 11% at the 0.6 µg/l level. The values also given for smokers and nonsmokers agree perfectly with those reported by others[50,73,74,83-85]. Owing to the properties of commercial AAS instrumentation, particularly problems with tubes and thus frequently occuring compensation deficiencies, the direct determination of cadmium in whole blood at normal levels (i.e., ≤2 µg/l) is extremely difficult. With the exception of Cernik and Sayers[86], who described the application of their paper disk technique also to the direct determination of cadmium in whole blood by using an elaborate temperature programme, all other workers proposed instrumental modifications for this purpose. Posma et al.[87] used blood, only diluted with ultrapure water, and a rapid response system to separate the atomic peaks of cadmium from the background without any compensation. The instrumentation consisted of a Varian-Techtron AA5 with carbon cup and carbon tube atomizer. The method was later modified by Castilho and Herber[88], who introduced an AA-6 spectrometer equipped with a Varian-Techtron simultaneous background correction system (Model BC-6). The sensitivity (i.e., the concentration that produces a 1% absorption signal) was 0.1 ppb and at

3 ppb of cadmium in blood a precision of 7% could be achieved. Lundgren[89] diluted the whole blood 1:9 with distilled water and added 20 µl of 2% Triton X-100. He applied a temperature-controlled graphite tube atomizer of his own design. The temperature control permitted the selective vaporization of cadmium and matrix at a high heating rate with an atomizing temperature of 830°C for cadmium. The sensitivity was 0.1 µg/l of cadmium and the detection limit 0.6 µg/l.

Using flame atomic fluorescence, Fell et al.[90] reported the determination of cadmium directly in whole blood diluted with hydrochloric acid and centrifugation prior to analysis. They claimed a detection limit of 0.1 µg/l and a typical run-to-run precision of 8.1%.

6.3.1.2. Voltammetry

Sinko and Gomiscek[91] applied anodic stripping voltammetry after nitric-perchloric acid decomposition under pressure of blood serum and reported a detection limit of 0.01 ng/ml of cadmium.

Valenta et al.[25] first digested the blood sample in a low-temperature asher, then dissolved the ash in dilute HCl and simultaneously determined Cd, Pb and Cu by DPASV-HMDE (pH 2.0-2.4). The determination limit was 0.1 µg/l of cadmium in whole blood with a contamination interference of about 0.1 µg/l of cadmium. The precision, expressed as day-to-day precision at the 0.75 ppb level was 23%. The authors also reported a typical cadmium level of 0.79 µg/l in whole blood for 60 non-exposed persons.

Franke and de Zeeuw[92] used a relatively simple and rapid DPASV procedure for systematic screening for 10 heavy metals, including cadmium. Identification was made from peak potentials in two different electrolyte solutions with the hanging mercury drop electrode (HMDE) as well as the mercury film electrode (MFE). For whole blood and cadmium, however, a prior digestion and the use of the MFE is recommended[92]. Christensen and Angelo[93] described a direct ASV method with an MFE in whole blood using a metal-exchange reagent to determine cadmium. The reagent led to rapid decomplexation of cadmium. If EDTA was present in the material, the inhibition was reported to be overcome by addition of nickel chloride. The detection limit was about 0.4 µg/l of cadmium with a precision of about 9% at the 3 ppb level and about 4% at the 25 ppb level. Intercomparisons with other laboratories were performed and showed satisfactory precision but less acceptable inter-laboratory variations.

6.3.2. Urine

6.3.2.1. Atomic spectroscopy

The determination of cadmium in urine has been performed mainly by two approaches: solvent extraction-flame or graphite furnace AAS and direct GFAAS by

making use of the easy volatilization of cadmium by particular temperature programmes. Owing to severe contamination problems in flame AAS, it can no more be recommended for routine use and thus will not be discussed here.

Kubasik and Volosin[94] extracted cadmium with NaDDC-MIBK and injected the organic phase directly into a carbon rod atomizer. The same procedure was applied by Stoeppler and Brandt[73] with a detection limit of <0.1 µg/l and a day-to-day precision of 26% at the 0.6 µg/l level. Kovats and Böhm[95] and Allain and Mauras[66] used the same procedure but with APDC-MIBK. Elinder et al.[96] initially wet digested the urine samples, ashed in a muffle oven at 450°C and finally extracted with APDC-MIBK at pH 8.3. The cadmium values found ranged for different groups (smokers and nonsmokers) from 0.07 to about 1 µg/24 h with slightly higher averages for smokers. These values were in acceptable agreement with those reported by other workers studying occupationally unexposed groups[66,73,94,97-102]. The method used was compared with other AAS techniques and NAA and good agreement was found.

Sperling and Bahr[68,69] used the same procedure for urine as described above for whole blood[68,69] but stated that direct extraction is not able to recover completely the cadmium present in urine. A precision of about 8% was obtained at a level of 0.2 µg/l.

Another approach was applied by Lund and Larsen[97], viz., electrolysis of cadmium from urine on to a thin platinum wire, followed by electrothermal volatilization.

Direct determination of cadmium in graphite furnaces with or without pretreatment has been described by several authors. Perry et al.[59] wet digested urine as already mentioned for whole blood and analysed the resulting solutions by GFAAS with a detection limit of 0.1 µg/l.

Ross and Gonzalez[103] injected acidified urine samples directly and compared the results with an extraction procedure. The temperature programme of the HGA-2100 instrument gave a good resolution of the cadmium peak from interfering matrix constituents; the atomization temperature was 1300°C.

Vesterberg and Wrangskogh[98] diluted urine samples with an equal volume of 0.3 *M* HNO_3 and analysed the solution using a carbon rod with temperature control by electronic feedback. The detection limit was about 0.1 µg/l with an average precision of 8% in the range 2-10 µg/l. Reliability was tested by a chelation-extraction method. Pleban and Pearson[82] diluted urine 1:1 with 5% nitric acid, injected a 10-µl aliquot into a graphite cup and analysed by Zeeman GFAAS. They reported a detection limit of 0.25 µg/l in urine and a within-run precision of 11% at the 0.62 µg/l level.

Carmack and Evenson[104] injected 10-µl urine samples directly into molybdate-coated tubes of an HGA-2000 instrument. Optical, temperature-controlled heating

of the furnace allowed the selective atomization of cadmium at 900°C. The detection limit was 0.05 µg/l.

Gardiner et al.[99] described the optimization of the working conditions of three commercial GFAAS instruments (CRS-90, SP 9-01 and HGA 72) for the direct determination of cadmium in urine. At atomizing temperatures from 1100 to 1500°C, depending on the instrument, a sufficient separation of the cadmium signal from the background could be achieved. Comparative analyses of a series of urine samples with injection volumes of 5-10 µl showed good agreement and it was stated that all three instruments could be used at cadmium levels down to 0.2 µg/l. The precision at the 1.2 µg/l level was 25%. Individual standard addition was necessary. Comparison with the AFS method in use in the same laboratory[100] showed similarity in terms of detection limit and precision. The AFS method, however, had the advantages of speed owing to the use of a flame and that standard addition is not required. Regrettably, AFS instruments up to now are not commercially available.

Legotte et al.[101] first digested the urine, prepared a concentrated solution and injected into an HGA-2100 instrument with atomization at 2100°C. Standard addition was reported not to be necessary.

Stoeppler and Brandt[72] injected diluted, slightly acidified (HNO_3) urine into an HGA-74 instrument by using a ramp charring up to 350°C and atomization at 1200°C with good resolution of the cadmium peak. A detection limit of 0.2 µg/l and a precision of 8% at the 3.3 µg/l level (moderately exposed persons) is obtainable. For precision analysis individual standard addition is recommended. The accuracy was found to be acceptable by intercomparison with solvent-extraction GFAAS and DPASV[18,37,72]. The same workers also performed storage experiments with ^{109}Cd radiotracer in order to establish whether an initial acidification to about pH 2 suffices to hold cadmium in solution. For normal urines no statistically significant losses could be observed. Even in a series of samples with significant proteine precipitation due to proteinuria[75], which were acidified later to pH 2, losses of cadmium due to adsorption on these precipitates were rather low and in no instance exceeded 5%[102].

6.3.2.2. Voltammetry

The already described direct method of Franke and de Zeeuw[92] seems to be applicable also to the determination of cadmium in urine at least for higher concentrations in cases of occupational exposure with rather high levels[105].

Lund and Eriksen[106] used DPASV with an HMDE for the simultaneous determination of Cd, Pb and Cu in urine. They reported that urine can be analysed directly for Cd and Pb without any pre-treatment. A significant sensitivity enhancement was observed at elevated temperatures. For comparison purposes also a complete

digestion with HNO_3-$HClO_4$-H_2SO_4 mixture was performed and showed good agreement. Higher values obtained after decomposition were explained as an influence of varying blanks. Golimowski et al.[102] described a rapid high-performance trace analytical procedure based on a low blank wet digestion with HNO_3-$HClO_4$ after freeze-drying of the urine samples. Subsequently the analyte solution was adjusted to pH 4.5 and Pb and Cd were determined simultaneously by DPASV at a rotating mercury film electrode (MFE). The quartz digestion vessel was used as the voltammetric cell. Theoretically, the detection limit is far below 0.1 µg/l, but is limited in practical use by the cadmium blank of ca. 0.05 ng/ml. The precision expressed as day-to-day precision was 43% at the 0.32 ppb level (controls) and 5.3% at the 8.3 ppb level (exposed persons). The authors emphasized that the direct determination of cadmium in urine, although feasible, does not always give the whole cadmium content of the sample, because a certain amount of cadmium might be trapped by organic chelators. To avoid this uncertainty, prior wet digestion of the urine is regarded as mandatory.

6.3.3. Other biopsy and also autopsy materials

In addition to the routinely performed determination of cadmium in blood and urine, other biopsy and autopsy materials can also be of value as bioindicators. In environmental, balance, epidemiological and occupational exposure studies biopsy materials such as parotid saliva, teeth, hair, faeces, placentae and autopsy materials such as liver, kidney, pancreas, lung, muscle, bone and foetal tissue have been analysed in numerous studies by different methods. As the normal cadmium concentrations, except for parotid saliva, are higher for these materials than for blood and urine, the analysis in general poses no extreme difficulties provided that experienced laboratories are involved. Thus it can be expected that the precision for GFAAS, DPASV and other methods such as NAA and ICP-AES can usually be maintained at around 5% and for flame AAS at around 2%, which means a total error around or even below 10% for most investigations.

6.3.3.1. Biopsy materials

The cadmium content of parotid saliva is of the same order as in whole blood or urine. Langmyhr et al.[107] applied matrix modification with nitric and sulphuric acid to minimize the influence of sodium chloride prior to GFAAS.

The determination of cadmium in teeth with typical levels of up to about 1 ppm is carried out by different methods. Langmyhr et al.[108] pulverized the material prior to atomization of 0.5-3-mg amounts in a graphite furnace of their own design by the solid sampling technique at 1400°C. Hydroxyapatite was used as a solid standard. Fosse and Berg-Iustesen[109] dissolved the pulverized materials in

2 *M* HCl, extracted cadmium with APDC-saturated xylene, back-extracted into 2 *M* HNO_3 and determined cadmium using a boat technique. Pinchin et al.[110] treated whole teeth with HNO_3 and $HClO_4$ and analysed the analyte solutions with DPASV-MFE at pH 5.

Analysis of hair can give information on trace metals and also on cadmium exposure, as discussed by Creason et al.[111], Valkovic[112] and the IAEA 113. A fair discrimination between endogenous and exogenous influences on trace element levels is possible if hair is analysed along its length, starting close to the scalp[114-117] with typical levels in the ppb range. The levels found were approximately 50-800 ppb in the proximal region and up to ppm levels in the distal region[112,117]. Various procedures have been applied to this task. In addition to the frequent use of NAA[113,118], AAS has been increasingly applied in baseline and in particular studies on occupational or environmental exposure to cadmium[111, 112,115,117,118]. After prior washing with various solutions, as recently comparatively studied by Stoeppler et al.[117], several types of digestion procedures have been applied: Oleru[119] used dry ashing, Bertram and Kemper[120] wet ashing with HNO_3-H_2O_2 and Stoeppler et al.[117] and Bagliano et al.[121] pressurized decomposition with nitric acid, with subsequent analysis by flame or graphite furnace AAS[111,117-121]. Alder et al.[115] atomized directly from the solid state. The availability of a hair control material with a reasonable cadmium content can now further improve reliability[37].

For balance and exposure studies, the determination of cadmium in faeces is a valuable tool for intake estimation, as demonstrated by Kjellström[122]. This material also poses no analytical problems, as shown by inter-laboratory comparisons[122]. For analysis, flame AAS, sometimes after solvent extraction, and also GFAAS have been applied[103,122,123,124].

Cadmium levels in placentae were determined by Thieme and co-workers[125,126] and Copius-Peereboom et al.[127], and significant correlations with environmental factors[125,126] and smoking habits[127] were found. Thieme et al. either wet ashed and determined cadmium by GFAAS[125] or irradiated the dried material in a nuclear reactor and subsequently determined cadmium after digestion and radiochemical separation[126]. Copius-Peereboom et al.[127] used either wet ashing with nitric-perchloric acid or pressurized decomposition in PTFE bombs with nitric acid and rigid quality control. The solutions were subsequently analysed by GFAAS.

Cadmium stores in the liver and kidney can be reliably determined by in vitro NAA, which is performed by irradiating the target organs with a collimated beam of thermal neutrons. The isotope ^{113}Cd (abundance 12.2%) captures these neutrons by forming ^{114}Cd, which promptly decays ($<10^{-14}$ sec) to the ground state by emitting a cascade of γ-rays that can be detected using a Ge(Li) detector external to the body[128]. Ellis et al.[128], using a mobile irradiation unit, were able to discriminate between smokers and nonsmokers with typical values of about

6 mg cadmium per left kidney in smokers versus about 3 mg for nonsmokers. For liver they found 4 ppm for smokers and about 2.3 ppm for nonsmokers. A similar approach was used by Roels et al.[129] to determine the cadmium burden in occupationally exposed workers. Despite its importance for investigating possible correlations between cadmium stores in the liver and kidney and cadmium levels in whole blood and urine, owing to the drawbacks of radiation exposure and the expensive, complex instrumentation used, in vivo NAA, however, does not seem suitable for routine application.

6.3.3.2. Autopsy materials

For autopsy materials the same in principle applies as for biopsy materials if methodology, average precision and accuracy are considered. Thus, when experienced laboratories were involved, no difficulties were observed during intercomparison studies for cadmium in the liver, kidney, muscle and pancreas[122].

Livingston[130] applied NAA after drying and subsequent γ-counting to the determination of cadmium, mercury and zinc in sections of human kidney tissue, particularly the kidney cortex. Ullucci and Hwang[64] digested kidney samples with nitric acid and hydrogen peroxide and used the tantalum ribbon electrothermal AAS method. Jackson and Mitchell[131] homogenized kidney, liver and lung samples, diluted the homogenate and analysed the samples in nickel microsampling cups in an air-acetylene flame by AAS. Evenson and Anderson[132] digested human liver tissue in nitric acid and injected the solution into an HGA-2000 instrument. Oleru[119] dry ashed kidney, liver and lung samples and analysed the residue, dissolved in HNO_3, by flame AAS. Elinder and Kjellström[133], Elinder et al.[134] and Kjellström[122] dry ashed liver, kidney, muscles and pancreas, dissolved the ash in nitric acid and determined cadmium by flame AAS for higher and by GFAAS for lower concentrations. Accuracy checks were performed by NAA. Wet or pressurized decomposition of tissues with subsequent direct GFAAS was applied by Hudnik et al.[61], Stoeppler et al.[37] and Bertram[135]. Dornemann and Kleist[67,136] used wet or pressurized decomposition, followed by HMA-HMDC extraction and GFAAS. Sperling[137] applied a wet digestion with subsequent APDC-CCl_4 extraction and GFAAS[137]. Schramel[138] demonstrated the ability of ICP-AES to determine cadmium in biological materials using the solution after pressurized decomposition with nitric acid[138]. Casey and Robinson[139] dry ashed human foetal tissues, dissolved the ash in hydrochloric acid and determined cadmium by flame AAS.

The determination of cadmium in human bone can be performed by procedures similar to that described above for teeth. Langmyhr and Kjuus[140] applied direct determination by solid sampling using hydroxyapatite as the standard, Lindh and Brune[141] used NAA for the determination of cadmium in human bone and Simon[142] used wet digestion followed by electrothermal AAS and by DPASV[142].

6.4. FUTURE PROSPECTS

The very low levels of cadmium in body fluids and in some environmental samples, e.g., rain, sea and inland waters[17,37], have been confirmed recently by improved sampling techniques, the application of clean workplaces and extremely powerful methods. Precision analysis, however, at trace and ultratrace levels is still difficult if biological materials are considered. Also the time required is excessive at least for the lower levels. Hence methodological improvements are needed.

Prerequisites of paramount importance for methodological development and the introduction of faster, cheaper and more reliable routine procedures is a wide selection of control and certified reference materials[44]. This challenge fortunately has been recognized, so that the appearance of a number of additional materials of this type together with rigid quality control regulations can be expected in the near future[11,37]. This, together with some positive methodological prospects discussed below, should change the present situation, which still is characterized by highly biased trace analysis at least if daily routine is considered.

In AAS it has to be investigated whether flame AAS at moderately low concentrations can be more frequently applied in contrast to the present practice favouring GFAAS whenever possible. This would improve both precision and accuracy because the flame is always less prone to random errors. Thus, new flame procedures, carefully checked by appropriate control and standard materials, could probably compete successfully with GFAAS at levels down to 20-50 ppb, provided that the sample sizes are sufficient or injection techniques can be used[143]. This, of course, would include the improvement of existing sample preparation methods with respect to capacity and efficiency as well as for blank minimization under clean workplace conditions.

GFAAS suffers from some particular disadvantages. For cadmium the severe limitations of existing continuum source compensation and the disappointing properties of graphite tubes frequently prevent precision analysis, even if the signal heights obtainable in the analyte solutions are optimal. For both of these disadvantages at least partly successful approaches are now on the horizon. The Zeeman compensation introduced commercially by a few manufacturers promises to solve the most severe compensation deficiencies encountered in cadmium analysis[77-82].

A particular improvement in GFAAS is the recent introduction of the platform technique with minimization of matrix interferences and a considerable enhancement of the analytically useful lifetime[62,144-146]. It is to be expected that more precise and accurate determinations in complex solutions and even directly from the solid state will now be feasible[147].

The most severe sample throughput and sensitivity limiting factor for voltammetric cadmium determinations within an approved oligo-element approach (at least Pb, Cd and Cu can be determined simultaneously in digests of biological meterials) is still sample pre-treatment. Additionally the currently commercially available automated electrochemical instruments do not have a performance comparable to that of AAS or ICP-AES systems. If this can be improved, a further increase in the application of voltammetry in cadmium analysis could be expected. Automation at moderate cost and with a possibly good overall performance could lead to an expansion of this simple and already very reliable analytical principle[18,148-152]. This is particularly true if direct analysis at higher, i.e., toxic, levels in body fluids will allow rapid and inexpensive screening in occupational exposure, which from present experience seems to be achievable to some extent[92,93,105].

For higher levels and the multi-element approach, particularly for fingerprint studies, in addition to the still continuing application of other approved trace analytical methods such as NAA and XRF, an increasing use of plasma emission spectroscopy can be expected. Technically improved devices, permitting simultaneous multi-element determinations, can now successfully compete, if costs are considered, with computerized AAS systems for automated flame analysis[153].

Thus, future prospects for the determination of cadmium in biological materials are positive. Substantial improvements in sample pre-treatment, sample throughput, precision and accuracy together with fruitful competition of methods can be expected.

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CHAPTER 7

ARSENIC

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CONTENTS

7.1. INTRODUCTION

7.1.1. Bioavailability

Arsenic falls between phosphorus and antimony in Group Vb of the Periodic Table and has an atomic number of 33. Elemental arsenic is obtained by reduction of the oxide. The metal burns in air to form the oxide(s) and reacts readily with some non-metals to yield covalent compounds. Alloys can be formed with metals such as iron, cobalt and copper. As a Group V element, arsenic can assume the formal valence states of -3, +3, and +5. The -3 state is seen as the hydride or halide and the positive formal valences predominate as oxides, mixed oxohalides or organoarsenicals.

Arsenic trioxide (As_2O_3) appears to be the predominant form of naturally occuring arsenic. Other formulations usually contain a metal ion such as iron,

copper, nickel or lead, either as the salt such as lead arsenate or as arsenic-containing ores. The treatment of arsenical ores to obtain the base metals and the use of arsenic trioxide for industrial purposes are the two causes of most of the toxicity problems related to arsenic. Arsenic trioxide is used as an insecticide in vineyards, as a dip for sheep and goats, in combination with mercury(II) chloride as a fungicide for fence-posts and as a decolorizing additive in glass manufacture.

The predominant concern about the effects of human exposure to arsenic centres about industrial procedures that either produce arsenic trioxide as a by-product or use it in the preparation of herbicides or pesticides. In addition, arsenic is present at appreciable levels in coal and oil shales and increased usage of these fuel sources in the present "energy crisis" will cause increased human exposure to arsenic[1]. Studies of workers in the smelting industry exposed routinely to arsenic trioxide have demonstrated a correlation between airborne levels of arsenic and urine concentrations[2,3] Also, urine and hair arsenic levels were elevated in residents of a community close to a smelting plant[4]. Thus, occupational and environmental exposure to arsenic does occur and results in increased body burdens of the element.

The major cause of exposure to arsenic trioxide, however, is by ingestion through either homicidal, suicidal or accidental causes. The use of arsenic trioxide in insecticides provudes a ready source of the material, which apparently is tasteless and hence historically popular for homicidal purposes. The effectiveness of arsenic trioxide as a homicidal poison results from its solubility in gastric fluids and ease of absorption from the alimentary tract[5]; it continues to be used nefariously[6]. In suicidal incidents the ingestion of ant or rat poisons is the most popular mode. Accidental ingestion, usually in children, follows the same modus. There is at least one instance where arsenic trioxide was ingested as a result of residual insecticide being dehydrated from oxoacid as arsenic trioxide, then volatilized and deposited on food during a cooking process[7].

Exposure to the ionic forms of inorganic arsenic in the form of the trivalent arsenites and pentavalent arsenates occurs by the same pathways as the trioxide, i.e., inhalation, direct skin contact and ingestion. Industrial occurrences predominantly involve the first two routes and non-industrial occurrences involve the last route.

Arsine and the arsenic trihalides do not occur naturally and are both unstable and extremely toxic. These compounds usually present an accidental exposure risk in industrial settings. Both are severe haemolytic agents and manifest their toxicity in this manner.

Further evidence of the ubiquity of arsenic was demonstrated by Schroeder and Balassa[8], who analysed commercially available common foodstuffs and vegetables grown on virgin soil and soil fertilized with superphosphate high in arsenic. Examination of their data shows the amount of arsenic present in the "market basket survey", highlighted by the high levels in seafood and the concentrations of arsenic in certain of the vegetables.

The organic arsenicals, e.g., methylarsinic and dimethylarsinic acids, are generally present in low concentrations in the environment relative to the inorganic forms. There are some lakes and ponds that have higher concentrations of arsenic, probably as a result of biomethylation of inorganic forms of arsenic to higher methylated forms and to methylated arsines. The biotransformation reactions and their effects on the food chain are subjects of current investigation. The ability of aquatic organisms to accomplish this transformation and accumulate the organoarsenicals has been demonstrated in marine invertebrates[9,10]. Because of the relative difficulty posed by the analytical problems the elucidation of the actual biochemical mechanisms by which the transformation occurs is difficult and there is little information available concerning this extremely important aspect of arsenic toxicity.

7.1.2. Absorption and excretion

The arsenicals are absorbed slowly through the skin, but absorption via respiratory and digestive routes in relatively rapid. Immediately after absorption 95-99% of the arsenic is found in erythrocytes bound to the globin of haemoglobin. Within 24 h the metal is distributed to the liver, kidney, lung, wall of the gastrointestinal tract and spleen. After about 2 weeks the skin, hair and bone begin to accumulate the metal[11]. The primary route of excretion is urinary[8], with a minor amount being cleared in the faeces[5]. Arsenicals apparently are excreted mainly through the biliary route. The trivalent form of arsenic supposedly is concentrated in the leukocytes[12] and, as such, has been evaluated as an anti-leukaemic agent[13]. Transplacental passage of arsenic has been demonstrated by studying a case of foetal death of a 30-week pregnancy[14] where the mother had taken As_2O_3 about 72 h prior to delivery. Analyses of fetal organs 11 h after delivery showed 0.74 mg/dl As_2O_3 in the liver, 0.15 mg/dl in the kidneys and 0.021 mg/dl in the brain.

The toxic effects of arsenic are dose related. Its effects are manifested by its ability to form covalent bonds with thiol anions, hence the greater toxicity of the trivalent than the pentavalent form, i.e. the trivalent form can accept a nucleophile and increase its number of bonded atoms in a relatively easy reaction. Some of the enzymes known to be inhibited by arsenic are monoamine oxidase, urease, glucose oxidase, choline oxidase, alanine aminotransferase,

aspartate aminotransferase[3], pyruvate oxidase, 2-glutamic acid oxidase[13], fumarase and pyruvate dehydrogenase. Within the pyruvate dehydrogenase enzyme complex, arsenite is presumed to exert its inhibitory effect by interaction with dihydrolipoamide, which is an essential coenzyme. After transfer of the "active acetyl" group to coenzyme A, a reduced lipoamide results. This free dithiol moiety then may react rapidly with arsenite to form a six-membered ring. The nucleophilicity of thiol anion and expandability of the polarizable arsenic molecular orbitals contribute to the stability of the AsO_2 lipoamide species. The usefulness of 2,3-dimercaptopropanol (BAL) in treatment is thought to arise in part from the metathetical replacement of the enzymic or prosthetic group thiol(s) by the vicinal dithiol groups of BAL to yield the theoretically more stable five-membered ring conformation. Arsenic also has an effect on DNA synthesis[15] and repair[16], presumably by binding to thiol groups of DNA polymerase.

Arsenate may follow a different method of exhibiting toxicity, that of an uncoupler of oxidative phosphorylation by formation of unstable arsenate esters which substitute for phosphate esters in ATP formation[17]. In this case arsenate is a structural analogue of phosphate.

7.1.3. Clinical features of exposure to arsenic

Clinical manifestations of chronic arsenic poisoning include peripheral neuritis with ataxia, weakness, numbness and tingling in the limbs. Transverse striate leukonychia (Aldrich-Mees lines)[18] appear 40-60 days following ingestion of a large amount of arsenic. However, this finding is not pathognomonic of arsenic poisoning as it is found also in cases of thallium and isoniazid poisoning. Bone marrow depression frequently is observed in patients with chronic arsenic poisoning. Leukopenia and normochromic anemia predominate and are considered to reflect the bone marrow depression.

When a large amount of a soluble arsenic compound is ingested, especially on an empty stomach, death may occur within a few hours. This fatal result is from acute poisoning of the myocardium, which may or may not be associated with brainstem medullary failure. A less dramatic result occurs with ingestion of smaller amounts of arsenic. Usually swallowing of arsenic is painless but occasionally some epigastric pain is experienced. Shortly after the ingestion, vomiting and diarrhea occur. During the next several days there may be inflammation of the conjunctival and respiratory mucous membranes, epistaxis, transient jaundice, cardiomyopathy, erythematous or vesicular rashes, sweating and then haematological, renal or pancreatic dysfunction may be observed. Symptoms of neuropathy develop 1-2 weeks later and typically consist of numbness with parasthesis in

the extremeties. A comprehensive review of inorganic arsenic and the nervous system has been published[19].

Arsine has been implicated in several hundred poisoning cases, many of which (25-30%) proved to be fatal. Generally a delay of 2-24 h occurs before onset of symptoms. Characteristic symptoms are abdominal pain, nausea, vomiting, haematuria and jaundice. The clinical laboratory abnormalities of greatest significance are anaemia and in vivo haemolysis.

There is strong epidemiological evidence that arsenic is carcinogenic; however, carcinogenicity in experimental animals has not yet been confirmed. The main risks of cancer involving arsenic are skin cancers from iatrogenic poisoning or from some other source such as water. Cancers of the respiratory tract are an occupational hazard in manufacturing plants and mines where arsenic is present at high levels. A review by Sunderman[20] covers the relationship between arsenic exposure and cancer.

7.2. ANALYTICAL PROCEDURES

The determination of arsenic in biological samples has caused considerable problems and a great deal of effort has been expended in the search for good practical methods. Several choices present themselves to the analyst, but all are subject to serious problems of accuracy, precision, sensitivity and technical complexity.

Biological samples usually are oxidized prior to analysis. Most of the procedures described to date involve the use of such oxidizing materials as sulphuric acid, nitric acid, perchloric acid and hydrogen peroxide. Oxidation with these materials requires heat and it is essential to exercise care to avoid losses of arsenic during the heating process. An oxidation step in the analysis must be avoided when methylarsenic compounds are being measured.

The most common first step in arsenic analyses has been the separation of arsenic from interfering sample matrix materials by conversion to arsine. Reduction of arsenic in acidic media by active metals such as zinc or magnesium is the classical approach. Some zinc reduction methods require up to 90 min. Sodium borohydride reduction is a more recent modification and provides a more rapid reduction than zinc. A further advantage of sodium borohydride is that usually it is not subject to the same arsenic contamination as is the zinc reagent. Electrolytic reduction using an apparatus incorporating a mercury cathode also can be used. Methylarsenic compounds and inorganic arsenic acids are all reduced to the corresponding arsine or methyl arsine.

Tissue samples homogenized with 0.05 *M* sodium hydroxide can be analysed directly with arsine generation using sodium borohydride reduction.

This review covers the various instrumental approaches to arsenic analysis. The pre-treatment of a biological specimen has already been summarized and further details are included in some of the more significant techniques.

7.2.1. Methods based on spectrophotometric techniques

As already mentioned, among the earliest techniques for detecting arsenic with reasonable sensitivity were those involving the volatilization of the metal as arsine and comparison of its reaction under certain experimental conditions with standard solutions of arsenic. Various approaches have been proposed. Marsh utilized the formation of the "mirror" of arsenic produced by the action of heat, while the method of Gutzeit compared the colorations formed on discs of dry paper impregnated with mercury(II) chloride. The reduction to arsine can be accomplished electrolytically in an apparatus incorporating a mercury cathode, with the use of arsenic-free zinc and hydrochloric acid or with sodium borohydride.

Several spectrophotometric methods can be used for arsenic analysis. An extremely sensitive modification[21] of the Gutzeit test detected 0.1-0.8 μg of arsenic. This modification used mercury(II) chloride-impregnated paper as an indicator and a special reflectometer to detect the arsine-mercury spots.

The molybdenum blue method has been widely used but possesses serious sensitivity limitations and interference from phosphorus has been observed. The procedure involves oxidation of As(III) to As(V) by nitric acid-sodium sulphate solutions followed by treatment with ammonium molybdate solution. The resulting heteropoly molybdiarsenate (arsenomolybdate) is reduced to the soluble blue complex "molybdenum blue". The most popular spectrophotometric method, because of its simplicity, is based on the complex of arsine with silver diethyldithiocarbamate. In one version of this method[22], arsine was bubbled through a pyridine solution of silver diethyldithiocarbamate and the resulting complex measured spectrophotometrically at 560 nm. Hydrogen sulphide caused interference in this method but could be removed by a glass-fibre filter impregnated with lead acetate. The silver diethyldithiocarbamate method has poor precision at arsenic levels below 1 μg and consequently is of value only in detecting toxic levels. Methylarsenic compounds complicate the procedure. Methylarsinic acid and dimethylarsinic acid are reduced to the methylarsines by nascent hydrogen and both form coloured complexes with silver diethyldithiocarbamate which have different molar absorptivities to the arsine complex. This difference has been used as a means of measuring methylarsenic compounds[23].

The Reinsch test has been used extensively for the detection of arsenic in biological samples, particularly urine. This simple rapid method involves the

deposition of a grey coating of arsenic on a pure copper foil or wire. Antimony and bismuth both deposit on the copper. The Reinsch test will only detect highly toxic levels of arsenic and, therefore, is not reliable enough for a screening test. In the authors' laboratory many cases of acute arsenic poisoning have had negative urine Reinsch tests.

A novel approach has been reported[24] using the effect of arsenic(III) as an auxiliary catalytic agent in the osmium(VIII)-catalysed redox reaction of bromate-iodide. When applied to 1 ng of tissue, digestion was accomplished by mineral acids or by ashing in a radiofrequency low-temperature dry asher. The reaction mixture consisted of the sample containing arsenic(III), acetate buffer, potassium iodide solution, starch solution and osmium(VIII). The absorbance was measured after 40 min at 580 nm. The limit of detection was anproximately 1 μg of arsenic in the sample (40 μg/l), which is not sensitive enough for broad application in instances of occupational exposure or in mild poisoning.

An extremely sensitive enzyme-catalysed reaction rate method has been described for the determination of arsenic in water samples[25]. The enzyme glyceraldehyde-3-phosphate dehydrogenase was used to perform an oxidative arsenolysis of D-glyceraldehyde-3-phosphate. The rate of reaction, as measured by fluorescence due to production of NADH, is first order for arsenic(V). The method had a detection limit of 20 μg/l. Possible application of this approach to the analysis of biological specimens is debatable owing to interference from sodium chloride.

Spectrophotometric procedures for the measurement of arsenic in biological samples should continue to play an important role in many laboratories. Careful analytical technique is essential for satisfactory results. However, the use of conventional instrumentation makes spectrophotometric techniques extremely attractive.

7.2.2. Neutron activation analysis

In certain laboratory situations neutron activation analysis can provide a satisfactory means of detecting arsenic in biological samples. Arsenic can be readily identified and quantitated by the radiation emitted from the radioactive nuclide ^{76}As, which is produced when the biological sample containing the stable isotope is bombarded in a neutron flux. Arsenic has a high cross-section for thermal neutron capture (5.4 barn), and it decays with a half-life of 26.5 h, which is short enough to provide a good activity but long enough to allow chemical separation procedures of some length to be accomplished. Workers using neutron activation analysis to study trace metals in biological samples have used a number of techniques to overcome the problems encountered due to the

presence of several elements in the activated sample. Some of these elements, especially ^{24}Na, greatly confuse and obscure the pulse-height spectra of the trace elements. In purely instrumental analyses of biological material workers using Ge(Li) detectors have allowed their samples to decay from 2 to 8 weeks before measuring the γ-ray spectra, as interferences due to sodium, potassium and bromine decay completely over this period of time. However, with modern counting equipment measurements of ^{76}As can be made on the irradiated sample after 2-3 days. Thus, it is possible to use non-destructive techniques for arsenic provided that the time of analysis is no problem. Smith[26] measured arsenic in hair by irradiation for 24 h in a thermal neutron flux of 10^{12} neutrons/cm^2/sec. He then allowed the sample to decay for 2 days before attempting to measure the arsenic. This type of method is satisfactory if a multichannel gamma-ray spectrometer is available to detect specifically the ^{76}As.

More commonly in the analysis of biological samples the element of interest is separated, in the presence of a carrier, from interfering elements prior to counting.

Mackintosh and Jervis[27] separated the arsenic from the digested biological sample by precipitating metallic arsenic with ammonium hypophosphite. The arsenic was purified further by dissolution in hydrochloric acid. The arsenic triiodide then was extracted with chloroform and reprecipitated as arsenic metal using hypophosphite. The procedure took 90 min per sample. Smith[28] followed irradiation with addition of inactive arsenic as a carrier followed by acid digestion. Arsenic was isolated by a Gutzeit separation using tin(II) chloride reduction and collection of the arsine in a mercury(II) chloride solution, which was counted. Another approach used for the analysis of a variety of biological tissues and fluids employed irradiation of whole samples. Separation of arsenic was accomplished by addition of 20-30 mg of non-radioactive arsenic pentoxide followed by acid digestion and precipitation of arsenic trisulphide by bubbling hydrogen sulphide gas through the digest. The precipitate was isolated by filtration and counted. Krishnan and Erickson[29] followed digestion of biological samples by separation of the arsenate ion from interferences such as sodium and copper using a cation-exchange column. Following elution from the column, the arsenic was counted using a sodium iodide scintillation crystal and a 400-channel pulse-height analyser. Heydorn[30] measured arsenic in plasma and erythrocytes by irradiation at a thermal neutron flux of 7×10^{12} neutrons/cm^2/sec for 30 min. Longer irradiation times led to coagulation of the plasma samples with subsequent handling difficulties. After a 16-20 h decay, a carrier mixture of arsenic, copper and antimony oxides was added, followed by acid digestion and precipitation of arsenic with thioacetamide. The precipitate was redissolved in ammonium sulphide and counted in a NaI(Tl) scintillation detector connected to a multi-

channel. This technique was applied to a study of patients with Blackfoot disease in an area of Taiwan where the artesian well water contains a high concentration of arsenic. Sjöstrand[31] followed the irradiation and acid digestion stage by addition of dilute hydrobromic acid and distillation of the arsenic at 100-110°C. The method was used for the simultaneous measurement of arsenic and mercury and achieved a sensitivity as low as 0.1 µg/l. A separation technique involving acid digestion and collection of arsine on mercury(II) bromide-impregnated paper was found to lower the limit of detection to 0.001 µg/l[32]. A procedure using distillation of arsenic and other volatile elements following activation has been described[33]. The sample after addition of carrier is burned in a combustion tube with a stream of nitrogen to flush the arsenic into a cold trap. This procedure was applied to the analysis of orchard leaves, bovine liver, tuna fish, coal and residual oil.

Neutron activation analysis in general has not proved to be a useful technique for the trace metal analysis of biological samples. Problems of sodium interference together with the extremely limited availability of reactors has hindered greatly any wide application of the technique. Arsenic, however, is one element where neutron activation analysis has contributed considerably as an analytical approach. The sensitivity of this technique, together with the relatively long half-life of ^{76}As plus the ease of separation of arsenic from interferences, has made neutron activation analysis attractive to several workers.

7.2.3. Atomic-absorption spectrometry

Atomic-absorption spectrometric detection of arsenic provides better sensitivity than spectrophotometric methods. The instrumentation is only moderately expensive; it is also available in most service and research toxicology laboratories. The determination of arsenic by atomic-absorption spectrometry using the conventional air-acetylene flame has serious difficulties owing to the intense absorption of the flame itself at wavelengths below 200 nm, which is where the most sensitive resonance lines of arsenic exist. The resonance lines of arsenic are at 189.0, 193.7 and 197.2 nm. The 189.0 nm line is the most sensitive, followed by the 193.7 nm line. Early work involving atomic-absorption methods for arsenic was hindered by the difficulties of designing hollow-cathode tubes because of the high volatility of this element; this technical problem has now been overcome. The argon-hydrogen air-entrained flame has been applied to the measurement of arsenic, resulting in reduced flame interferences[34]. However, the relatively low temeprature of this flame inevitably resulted in interferences from molecular absorption and incomplete salt dissociation. The use of a nitrogen-

hydrogen air-entrained flame also has been reported[35], with a minimization of the interferences and sensitivities in the range 6-20 µg/l of arsenic have been achieved.

Most analytical techniques for arsenic use the conversion to arsine and it is this approach that appears to offer the best means of preparing the sample prior to atomic absorption measurements. Holak[36] was the first to employ this approach, using an argon-hydrogen air-entrained flame, and other workers[37,38] have employed a similar system. These flame methods appear to have a limit of detection of approximately 40 ng in the sample or 5 µg/l on a concentration basis[2-4]. Chu et al.[39] used a flameless system where arsine was swept into a Vycor glass tube (150 x 4.5 mm I.D.) heated to 700°C with asbestos-covered Chromel A wire (arsine decomposes readily to arsenic and hydrogen at temperatures above 230°C). Arsine generated from an acid digest of the sample was first collected in a balloon reservoir and then swept by a stream of argon into the heated tube. This system greatly reduced background absorption over the flame methods; an essential feature of the low background was a high flow-rate of argon through the tube. The sensitivity of the electrothermal method was twice that of a flame system, having detection limits of better than 0.05 µg in the sample. The precision on an in-run basis was 0.36% (relative standard deviation) and recoveries were quantitative.

Fitchett et al.[40] deviated from the arsine generation protocol and developed a graphite tube electrothermal atomic-absorption method for arsenic in urine and water. In their technique urine was heated with hydrochloric acid and treated with iodide ion. Arsenic species, as iodides, were extracted into chloroform and either re-extracted into deionized water for measurement of inorganic arsenic, or re-extracted into dilute dichromate solution for total arsenic determination. The extracts were analysed using electrothermal atomic-absorption spectrometry with a graphite furnace and an arsenic electrodeless discharge lamp. The sensitivity was as low as 10 µg/l and recoveries were essentially quantitative. This method offers the possibility of differentiating between organic and inorganic arsenic. In another report from the same laboratory[41], the procedure was adapted to the measurement of inorganic arsenic, monomethylarsenic and dimethylarsenic in homogenates of liver, kidney, etc.

The recent introduction of sodium borohydride as a reducing agent for the generation of arsine was initially adapted to an atomic-absorption method with an argon-hydrogen air-entrained flame[42]. In the authors' laboratory use of this reduction technique and a modification of the electrothermal system (heated Vycor tube) of Chu et al.[39] provided an extremely convenient system for measuring arsenic in biological specimens[43]. The tube used in this procedure was 150 x 8 mm I.D. heated to 700°C with asbestos-covered Chromel wire. The reaction vessel for the analysis was a modified 50-ml graduated cylinder fitted with a syringe for

adding reagents, an inlet for argon and a connection to the quartz tube. For the analysis, 1 ml of urine plus 0.5 ml of saturated oxalic acid contained in the reaction vessel was treated with 1.5 ml of 5% sodium borohydride solution. The arsine generated in the reaction was swept by a stream of argon through the quartz tube and the absorption recorded at 193.7 nm with background correction at 191.5 nm. The limit of detection was 50 µg/l and the calibration graph was linear up to 200 µg/l. Interferences were not observed and the precision of the method is adequate for detecting toxic levels. This procedure has been used in our laboratory to confirm several cases of arsenic poisoning resulting from homicidal and accidental incidents.

An automated version of this system has been reported by Rose and Renoe[44]. The module could be quickly set up in place of the burner on the atomic-absorption instrument to provide a precision-controlled and timed analytical sequence for analysis. The device incorporated control valves for direction of purge gas flow and for control of the reagents used for sample acidification and borohydride reduction. Timing and logic circuitry provided exact timed intervals for the analysis events (purge gas flow through samples, sample acidification, final metal reduction, triggering for the data acquisition cycle of the atomic-absorption spectrometer and final reset). The module has provided improved analytical integrity for arsenic in comparison with a manually manipulated and timed procedure, as well as providing the flexibility necessary to analyse the other easily volatilized metals.

Some of the difficulties in the determination of arsenic by atomic-absorption spectrometry have been discussed by Robinson et al.[45]. The arsine generation methods were criticized, as arsine can decompose at room temperature, and this suggests that the absorbance obtained in a heated tube might not indicate the total amount of arsine produced by the sample. Also, in flame atomizers, atomic arsenic can be changed quickly to molecular arsenic (As_4 and As_2) with a severe decrease in sensitivity. In non-flame atomizers the problem is further complicated by a possible interaction with carbon. Only under rigidly controlled conditions will reproducible data be obtained. However, in many laboratories, atomic-absorption spectrometry will be the most convenient method for the determination of arsenic in biological specimens as the instrumentation is usually readily available. It is important to recognize that the problems of sensitivity of the assay will limit the applicability of atomic absorption and probably exclude careful studies of arsenic speciation in environmental specimens.

7.2.4. Gas chromatography

For a metal such as arsenic, which readily forms volatile derivatives, gas chromatography offers a useful approach. Most analytical procedures involve total arsenic measurement without regard to the specific chemical form(s) of arsenic present. A number of reports indicate that there is some value is assessing arsenic in its various forms, particularly the methylated derivatives.

Daughtrey et al.[46] described a procedure for the determination of inorganic and methylated arsenicals in urine by gas chromatographic measurement of the diethyldithiocarbamate complexes. Urine was treated with potassium iodide, forming the iodides of inorganic and mono- and dimethyl-arsenicals. These iodides were subjected to reaction with diethyldithiocarbamate and the complexes were extracted and then measured by gas chromatography using electron-capture detection. A later report from the same laboratory[41] adapted a similar procedure to the analysis of tissue homogenates. The tissue samples were handled as homogenates (10%) made up in deionized water and then freeze-dried. Total arsenic was measured by wet ashing the lyophilized samples with nitric and sulphuric acids. To the cooled residues 8 *N* hydrochloric acid and potassium iodide solutions were added, followed by benzene plus diethylammonium diethyldithiocarbamate. After the initial step the benzene extracts were then transferred into vials containing hydriodic acid and solutions of chelating agent and sodium metabisulphite were then introduced. Metabisulphite, a reducing agent, served to prevent the presence of iodine, which reacts with and destroys the complexing efficiency of the chelant. The organic extract now containing the arsenicals as the diethyldithiocarbamate complexes were treated with dilute alkali to remove chromatographic interferences and to minimize an effect on electron-capture detector sensitivity due to the generation of sulphur-containing fragments when the chelant was introduced in acidic media. Subsequent to alkali clean-up the layers were dried over anhydrous sodium sulphate and aliquots injected into a gas chromatograph equipped with an electron-capture detector and a 5% OV-17 glass column. The order of elution for the column was solvent, dimethylarsine complex and monomethylarsine complex.

A gas chromatographic determination of arsenic in biological materials using a nitrogen-phosphorus detector has been reported[47]. The method employed arsine production with sodium borohydride with sample introduction via a headspace technique. The method was reported to possess adequate sensitivity for specimens of toxicologic interest. Gas chromatography-mass spectrometry has been used to detect dimethyl arsinate in the blood, urine and faeces of rats. This powerful technique undoubtedly in the future will contribute a great deal to the understanding of arsenic metabolism and toxicity.

Gas chromatography has not fully realized its potential as a tool for arsenic analysis. The capability of detecting species of arsenic is exciting and should be of considerable importance in future environmental studies.

7.2.5. Electrochemical methods

A coulometric procedure for measuring arsenic in urine has been reported[48] that involved acid digestion and extraction of arsenic(III) as arsenic(III) chloride. Following back-extraction, the arsenic(III) was titrated with electrogenerated iodine. The sensitivity was only 500 µg/l in urine using a 10-ml sample for the analysis. Anodic-stripping voltammetry has been applied to the measurement of arsenic in urine[49]. The sample (1-2 ml) was digested with mineral acids followed by isolation of arsenic(III) chloride from the digest by distillation. The arsenic in the distillate was plated on a gold electrode for 1-2 min using a unique instrumental configuration with a highly efficient stirring system. After deposition of the arsenic, the potential of the electrode was scanned in a positive direction and quantitation made from the area of the curve as the arsenic was stripped from the electrode. The sensitivity was 1 µg/l, recoveries were 80-100% and the precision was approximately 10% (relative standard deviation) at 10 µg/l. This method appears to have considerable potential for the analysis of biological specimens and future applications will undoubtedly be forthcoming.

7.2.6. Emission spectrometric methods

Emission spectrometric methods have been applied in some instances to the measurement of arsenic. Only a few papers in the literature are concerned with flame emission. One of these[50] used aspiration of a methyl isobutyl ketone solution of organic arsenic compounds into an acetylene-oxygen flame with measurement of arsenic emission at 235 nm. The detection limit was 2.2 mg/l and it is unlikely that such a method could have widespread applicability to biological samples. However, some of the best sensitivities have been obtained by the emission detection methods. Lichte and Skoberboe[51] coupled an arsine-generation device to a microwave-stimulated plasma discharge and obtained a limit of detection of 5 ng (2.5 µg/l) using a 2-ml sample. Braman et al.[52] used the sodium borohydride method for generating arsine and stibine, which were swept out of the solution by helium through a d.c. discharge detector. This approach gave a lower limit of detection of 1 ng (0.1 µg/l) using a 10-ml sample. When a liquid nitrogen-cooled U-trap was used to trap out arsine and reduced organic arsenicals, the limit of detection was improved to 0.2 ng (0.002 µg/l) using the

same d.c. discharge method[53]. Separation of the arsines was accomplished in this method of Braman and Foreback[53] in the following manner. After removal of the liquid nitrogen and gentle warming of the U-tube, the arsines evolved from the trap one at a time and detected in the d.c. discharge emission spectrometer. Excellent separation of the arsines was obtained with limits of detection near 0.5 ng (0.05 μg/l) for the methylarsines. Crecelius[54] introduced several modifications to the method of Braman et al. The modifications involved adding three extra traps to the system: a water vapour trap, a second carbon dioxide trap and a water trap.

The microwave-stimulated plasma detector combined with gas chromatographic separation of the arsines in the method of Talmi and Bostick[55] exhibited a limit of sensitivity of 0.02 ng. Arsines were trapped in 5 ml of cold toluene, 10 μl of which were separated on the gas chromatograph. Unfortunately, as only a small fraction of the original sample was analysed, the concentration limit of detection was only of the order of 1 μg/l.

The sensitivity of these emission detection systems appears to be adequate for studies of arsenic in the environment and they offer considerable potential where methods of the highest sensitivity are required.

7.2.7. Miscellaneous techniques

Electron microprobe analysis has been used for the analysis of hair samples from inhabitants of an area close to a smelter emitting arsenic trioxide[56]. The technique is non-destructive, sensitive and has the capability of measuring many elements simultaneously.

The scanning proton microprobe with its intense collimated beam and low background signal has advantages over the electron microprobe. The technique has a high sensitivity for most elements heavier than sodium. One report using this technique described the analysis of single strands of hair from poisoning victims[57].

These highly sophisticated techniques are exciting for certain specific studies but the complexity of the instrumentation limits the approach to only a few centres.

7.3. CONCLUSION

Arsenic is ubiquitous in the environment and probably is an essential trace element in nutrition. Exposure to high concentrations of arsenic causes a debilitating and often fatal illness that affects most organ systems. Chronic exposure appears to cause cancer, particularly of the skin and lungs. Arsenic

is a difficult element to measure in biological materials and the most succesful approaches have involved the separation of arsenic from the sample matrix by means of evolution of the volatile arsine. Detection of arsine by atomic-absorption or-emission techniques shows considerable promise for adaptation to a wide variety of laboratories, although spectrophotometric, electrochemical, gas chromatographic and neutron activation approaches have received considerable attention. Proton microprobe analysis should be of value in certain specific studies requiring non-destructive techniques and high sensitivity.

At the present, no ideal reference method for the measurement of arsenic in biological materials has been described. The availability of instrumentation will determine the analytical approach to be used in any particular situation.

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CHAPTER 8

THALLIUM

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CONTENTS

8.1. INTRODUCTION

Thallium is an important metal in toxicology. It has a relatively high toxic potential, is easy to obtain as a rodenticide and thus has become a well known poison. In the U.S.A. the use of thallium compounds (ca. 5 tons/year at its peak) in rodenticides and insecticides was terminated in 1972 because of its toxic potential[1]. The current usage in the U.S.A. is now estimated at 0.5 ton/year. In various other countries, however, thallium is still in use as a rodenticide.

Thallium forms compounds in both the monovalent and the trivalent states. Tl(I) resembles the alkali metal cations, e.g., K^+, in ionic radius and chemical behaviour, as appears from the replacement of K(I) by Tl(I) in potassium iron(III) hexacyanoferrate(II) (Prussian Blue), giving the latter powerful properties as

an antidote in thallium intoxications[2-4]. However, in contradiction to alkali metals, Tl(I) forms sparingly soluble compounds with sulphides, iodides, chromates, etc., analogous to the heavy metals of Group Ib (Cu^+, Ag^+ and Au^+). The latter properties of thallium have been used in methods of analysis, e.g., in pre-concentration techniques, extraction and precipitation, and also in quantitative assays by gravimetry or iodimetric titration.

Inorganic thallium(III) compounds are more water soluble but hydrolysis occurs, resulting in precipitation of thallium hydroxide or oxide. The organic Tl(III) compounds are more stable than organic Tl(I) compounds.

Electrochemically, the standard potential of the Tl^{3+}/Tl^+ couple in most instances favours Tl^+. Recent investigations, however, indicate that the formation of thallium chloride complexes in media with a high chloride concentration would favour the trivalent state[1].

The clinical aspects of thallium poisoning, such as toxicity, clinical course and treatment, have been reviewed by Moeschlin[5].

8.2. HISTORICAL REVIEW

In the past, the detection of thallium was effected mainly by spectroscopy. As thallium has an emission line at 535.0 nm, thallium salts would give a green colour to the flame. This property is of limited value in detecting thallium in biological materials.

The older quantitative assays were not very sensitive and destruction of the organic matter, followed by a pre-concentration, was required to achieve detection limits low enough to establish intoxications. The pre-concentration steps used were extraction of thallium(III) chloride or bromide ($TlCl_3$ or $TlBr_3$) in diethyl ether, or precipitation of thallium(I) sulphide (Tl_2S) from slightly alkaline solutions. The latter procedure is still used to increase the sensitivity of modern spectrophotometric assays.

In the final step, thallium was quantitated either by a gravimetric procedure in which thallium(I) iodide was precipitated and the precipitate weighed, or by a titrimetric assay in which Tl(III) was formed, then subjected to reaction with iodide, forming iodine and thallium(I) iodide, and the liberated iodine was subsequently titrated with sodium thiosulphate solution[6].

The detection limits obtained with the above assays is in the low milligram range. Hence, even in acute intoxications, large samples of biological material were required, e.g., 50 g[7].

Recently, methods have been introduced that can determine thallium much more sensitively, precisely and accurately owing to the development of instrumental techniques such as flameless atomic-absorption spectrometry and mass spectrometry.

8.3. SAMPLE PREPARATION

In general, little attention has been paid to sampling and sample preparation, yet with most methods reasonably accurate and precise results have been obtained, indicating that thallium assays are not very sensitive to sampling conditions. For instance, interferences from reagents, laboratory utensils, etc., in contrast to many other heavy metals, are not likely to occur as the concentration of thallium in these materials is very low compared with the expected concentrations in body fluids in intoxications. However, if normal levels have to be determined the reagents have to be carefully examined for their thallium content, as the thallium levels in body fluids are in the ppb (ng/g) range[8].

Losses of thallium may occur during sampling as the monovalent form of thallium may precipitate as the sulphate or halide. Hence, for urine analysis centrifugation of the sample should be avoided. On the other hand, the physicochemical properties of thallium(I) indicate that losses during analysis due to complex formation or hydrolysis are not likely to occur.

Destruction of the biological sample prior to analysis is often required. It has been shown that there is no reason to expect difficulties with the recovery of thallium when adequate destruction methods such as wet digestion, oxidative fusion in a closed system (Parr bomb) or low-temperature ashing in an oxygen atmosphere are properly applied. Dry ashing has also been used, but with this technique there is more risk, as nanogram to microgram amounts of thallium may be lost[9]. Low recoveries of thallium have been reported in dry ashings in crucibles owing to silicate formation and to volatilization[10].

8.4. STANDARD METHODS

8.4.1. Spectrophotometry and colorimetry

Spectrophotometric assays are based on the extraction of thallium as a complex in an organic phase, after which the absorbance of the organic phase is measured. For instance, thallium(I) can be extracted by dithizone into chloroform at pH values of 11 or higher. As many other metals may also react with dithizone at this pH, a separation step is required[11].

Thallium(III) may be extracted as a coloured ion-association complex between a thallium(III) halide anion (TlX_4^-) and a large coloured organic cation. An advantage of this method is that this extraction is more selective for thallium. Only large negative ions, e.g., perchlorate, or anionic detergents may interfere. Dyestuffs such as Brilliant Green, Rhodamine B, Malachite Green and Gentian Violet have been used as the organic cation[12-16].

Some colorimetric assays directly in urine without destruction have been described[12,13,16]. Other workers, however, reported interferences from organic compounds such as drugs, proteins, detergents, etc. Thus, although thallium can be detected directly in urine or blood serum by spectrophotometry, we recommend that for quantitative assays a destruction is carried out to minimize the chances of interference.

A reliable quantitative assay that can be recommended is that described by Ariel and Bach[13] and modified by De Wolf and Lenstra[14], based on extraction of the thallium(III) bromide-Brilliant Green complex in toluene. The latter workers incorporated a wet digestion by means of a sulphuric acid-nitric acid mixture. Prior to extraction nitrate was removed as it can interfere at higher concentrations. Perchloric acid should not be used in the digestion because trace amounts of perchlorate can seriously interfere. The sulphuric acid concentrations just before extraction must be in the range 1-3 *N*. Other possible interferences in this method are lead (>250 µg) and anionic detergents. The latter may be removed by thoroughly rinsing the glassware. The method was originally developed for urine, but can also be used for other biological fluids.

Quantitation is effected by means of calibration graphs, which were linear for thallium levels up to 16 µg. The detection limit of the assay is about 0.5 µg (10 ng/ml in urine). The method is accurate as recoveries of more than 98% were found with spiked urine samples in a concentration range from 60 ng/ml to 1.2 µg/ml with a precision of better than 10%.

Advantages of the spectrophotometric assay are that the method is accurate and precise, that the basic equipment is present in most laboratories and that no skilled personnel are needed. Disadvantages are that the total procedure is very lengthy (careful destruction, oxidation of Tl^{+} to Tl^{3+}, removal of oxidizing reagents and extraction), that a large sample is required so that blood analyses cannot be recommended and that the method is metal-directed so that in spite of the long procedure only thallium is determined: no screening system.

Spectrophotometry has been in use for many years in toxicology. Although nowadays colorimetry has been replaced by newer techniques such as atomic-absorption spectrometry, it is still a useful tool in smaller clinical laboratories with a low sample throughput for metal analysis.

8.4.2. Flame atomic-absorption spectrometry (FAAS)

8.4.2.1. Direct aspiration

FAAS may be used in the analysis of thallium without an extraction procedure, i.e., by direct aspiration of the sample or diluted sample in the flame. This makes the method very rapid and simple, especially for biological fluids such as

urine. However, this method is rather insensitive, with a detection limit of about 0.2 ppm and a sensitivity of 0.8 ppm in aqueous solutions. In AAS sensitivity is usually defined as the concentration of element required to give a 1% change in absorption. According to Veenendaal and Polak[17] and Curry et al.[18], various inorganic anions and cations can interfere, resulting in a considerable increase in the thallium signal (1-25%). Therefore, quantitative results obtained by the direct aspiration technique must be regarded carefully, although for urine analysis this interference with the thallium signal has not always been confirmed[19]. In order to minimize these phenomena, the use of salt buffers at concentrations of about 1000 ppm for both the sample and standards is recommended so that the thallium signal is measured against similar backgrounds. Thus, in acute poisoning cases thallium can be detected in urine by the direct aspiration method and an impression can be obtained of the severity of the intoxication. However, for reliable and sensitive quantitative results a selective extraction procedure before the FAAS determination is recommended.

8.4.2.2. Extraction

The extraction of thallium into a suitable organic solvent results in an enhancement of sensitivity and, moreover, such an extraction may introduce a pre-concentration[20]. The determination of thallium after extraction in different kinds of samples has been described[19,21,22] and in general body fluids such as whole blood, blood plasma and urine did not appear to require destruction.

The extraction is influenced by the following parameters:

The pH of the aqueous solution. Although Curry et al.[18] found an optimum in the recovery of thallium using sodium diethyldithiocarbamate and methyl isobutyl ketone at pH 6.0, other workers[21,22] reported that the thallium extraction is efficient in the pH range 5-8.

The complexing agent. Sodium diethyldithiocarbamate (SDDC) and ammonium pyrrolidine dithiocarbonate (APDC) have both been used as complex-forming agents. They both give stable complexes in the organic phase. Although APDC is more stable in the aqueous phase at lower pH values, higher recoveries were found using SDDC[23].

The organic phase. Usually methyl isobutyl ketone (MIBK) has been used with success. Diisopropyl ketone (DIPK) may improve the extraction because of its good properties in the flame, its good phase separation ability and its lower solubility in water[24].

In conclusion, for the determination of thallium in whole blood the method described by Armore[22] can be used, whereas for urine and tissue the procedure described by Berman[21] was found to be adequate[18], with a sample size of 5 g for blood and tissue and 30 ml for urine.

The detection limit obtained for thallium in blood and tissue was about 0.08 µg/g and for urine about 0.01 µg/ml. Recoveries of more than 95% were found with a coefficient of variation of well under 10%. Interference from the masking agent EDTA was not seen.

The FAAS method with extraction is very suitable for the determination of thallium in body fluids in cases of intoxication. The procedure is precise and sensitive enough, and about 30 samples can be analysed per day, including the preparation of calibration graphs, which are obtained by the same procedure as for the samples. A disadvantage is that the sample size required is relatively large. In comparison with other methods, FAAS is relatively cheap and can be readily automated.

8.4.3. Non-flame atomic-absorption spectrophotometry (NFAAS)

Non-flame atomic absorption is more sensitive than flame atomic absorption, but for thallium this increase in sensitivity is accompanied by more interference problems. Only urine, after a 10-fold dilution with 1% sulphuric acid can be injected directly into the furnace of the atomic-absorption spectrophotometer. Other samples require either destruction or extraction, or both. In the direct determination of thallium in urine, quantitation has to be carried out by standard additions, as the aqueous standards give different responses to standards in a urine matrix[25]. Kubasik and Volosin[26] even recommended standard additions for the quantitation of thallium in urine after an extraction procedure.

An important advantage of NFAAS is that only a small sample is required. This is of special interest in cases in which only a small sample is provided, for instance blood or hair[27,28].

The detection limit is about 0.2 ng of thallium injected into the furnace and, depending on the sample size and dilution factor, determinations can be carried out in the lower ng/g range; however, this sensitivity is not high enough for the measurement of normal levels in biological materials.

In conclusion, NFAAS is a good technique when FAAS determinations fail. The latter may happen when the sample size is limited or when a high sensitivity is required, for instance in subclinical intoxications or when the influence of therapy with Prussian Blue has to be followed. Disadvantages must be borne in mind: a slightly lower precision, more interference problems and higher costs, although automation can improve the precision considerably. Automated equipment is now commercially available.

8.4.4. Emission spectrometry

Classical emission spectrography, i.e., with a spark source and photographic plates as the detection system, allows the sensitive detection of thallium. In a 2-ml urine sample about 0.02 µg could be detected[19]. Moreover, in the same run other metals can also be detected. A reliable, quantitative assay, however, is more troublesome.

New excitation sources in emission spectrometry such as the inductively coupled plasma (ICP), laser beam excitation and electrically heated furnaces allow much smaller samples to be used (0.2 ml or less) and, in combination with photomultiplier detection of the emission signal, a more reliable quantitative assay can be obtained[29,30]. At present, however, these newer techniques are still very expensive, so that their use is recommended only for forensic laboratories with a high throughput of samples that have to be screened for unknown metal poisonings.

Although atomic-absorption spectrometers may be used in an emission mode, allowing flame emission measurements, the detection limit for thallium in biological material of about 10 µg/g is often not sensitive enough for the detection of thallium intoxication in blood or urine[18].

8.4.5. Polarography and anodic-stripping voltammetry

Classical polarography has been used to determine thallium in biological materials[31,32]. However, for a sensitive and reliable assay a relatively large sample (5-30 g) and a lengthy procedure was necessary, including destruction of the sample and either an extraction or precipitation step for pre-concentration and clean-up. Therefore, polarography as such, is no longer in use.

The development of anodic-stripping voltammetry (ASV), especially in combination with the differential pulse technique, resulted in a number of applications as thallium proved to be very suitable for ASV determinations[33-43]. ASV allows screening for a series of toxicologically interesting metals[41]. One of the problems encountered with ASV is that overlapping peaks may occur. For thallium, however, this problem can be circumvented as other potentially interfering metals can be masked by the addition of a complexing agent such as EDTA[33]. The thallium peak is not affected by this addition so that thallium can be identified unequivocally by ASV[40].

In ASV the following parameters are of importance:

The electrolyte solution. As the presence of EDTA is recommended, to prevent metal interferences, an electrolyte solution with a pH higher than 4 is to be used to allow stable complexes. However, as the peak potential (identification

parameter) depends on pH, it is advisable to use a buffer. An acetate buffer is often used in the pH range 4.5-6.4[34,36,38,43]. Alternatively, an ascorbate buffer pH 4.5 may be used. The latter has the advantage of removing most, if not all, dissolved oxygen. Hence, the time for deaeration by bubbling an inert gas such as nitrogen or helium through the solution can be substantially reduced[40-42,44]. Perchloric acid (0.1-0.2 *M*) should not be used as it may result in interference from cadmium, indium or lead[37].

Working electrode. The choice of the working electrode is important in relation to sensitivity and reproducibility. With the hanging mercury drop electrode (HMDE) according to Kemula and with the differential pulse technique, thallium levels in urine in the ng/g range could be determined directly with a detection limit of about 5 ng/g and with a precision better than 6%[40]. Other types of electrodes that can be used are the mercury-plated graphite electrode or the mercury-plated glassy carbon electrode (MFE), the latter being preferred because of its reproducibility and stability. This electrode is more sensitive than the HMDE, mainly owing to its larger surface area. The lower limit of detection is in the sub-ng/g range, which may be decreased even further by longer electrodeposition times[38]. The precision, however, is lower, but still better than 10%.

Quantitation by ASV is effected by the standard additions technique. This means that the time required for a total qualitative and quantitative analysis is relatively long (30-60 min). On the other hand, a direct determination, i.e., without destruction of the sample, can be carried out in urine and in blood plasma with 1-ml samples or less[42]. For the determination of thallium in tissue and whole blood, destruction is required. This destruction must be very thorough as the presence of trace amounts of organic material can influence the voltammetric behaviour of thallium or may exhibit electrochemically active behaviour by itself (nitro compounds). Oxidative fusion at increased pressure (Parr bomb) or low-temperature ashing under an oxygen atmosphere are the methods of choice[36,43], although a thorough wet digestion with mineral acids may also be used.

The amount of sample needed is small, e.g., 1 ml or less for body fluids. Even human skin and fingernails can be analysed by this method[35].

In conclusion, ASV in the differential pulse mode can be used for screening purposes and is an appropriate technique to identify thallium unequivocally even at low concentrations or when small amounts of sample are present. A total quantitative determination, however, requires 30 min or more. Hence, when many samples per day have to be analysed, a metal-directed technique such as atomic-absorption spectrometry is faster but not necessarily more sensitive or more precise. Although automation in ASV is still at its infancy, important developments may be expected in the near future.

8.4.6. Neutron activation analysis (NAA)

Naturally occurring thallium is a mixture of 29.5% ^{203}Tl and 70.5% ^{205}Tl. Irradiation of thallium with thermal neutrons results in the formation of ^{204}Tl and ^{206}Tl, respectively. ^{204}Tl decays to the extent of 98% into stable ^{204}Pb under β-radiation with a half-life of 3.5 years and 2% into stable ^{204}Hg. ^{206}Tl has a half-life of 4.2 min and is therefore not of interest. Thus the β-radiation at 0.77 MeV can be measured for qualitative and quantitative purposes. As many interfering activities are to be expected after irradiation of a biological sample, separation of the thallium isotope from the interfering material has to be carried out[45].

The facts that NAA is not readily available in many laboratories owing to lack of access to a neutron source and that after sample irradiation a chemical clean-up or enrichment step is required make NAA unattractive for the routine determination of thallium in forensic and clinical laboratories. However, as the NAA assay is very sensitive, with a detection limit below 0.1 ng, the analysis of very small samples, e.g., parts of one hair, is now possible, so that the technique has specialized applications[46].

8.4.7. Mass spectrometry

Isotope-dilution mass spectrometry (ID-MS) allows the determination of ultra-trace amounts of thallium in various biological materials. Weinig and Zink[8] determined normal thallium concentrations in various human tissues by ID-MS with a detection limit of about 5 pg, a precision of better than 10% and high accuracy.

Although mass spectrometers are now more generally available, their use for routine analysis is still difficult, particularly as a special inlet system is required, the enriched ^{203}Tl is not generally available and a lengthy procedure including wet destruction and extraction is necessary. Hence, ID-MS can be used as a reference or standard technique, and for the determination of very low concentrations of thallium in specialized and experienced laboratories.

8.5. INTERPRETATION OF RESULTS

Smyth and Carson[1] reviewed thallium concentrations found in human organs, tissues and body fluids in normal subjects and in cases of poisoning. They calculated that the average daily human excretion of thallium in urine and faeces is about 1 μg, whereas the daily dietary intake is estimated to be less than 2 μg. Weinig and Zink[8] determined thallium in six human bodies not abnormally exposed to thallium. They concluded that all organs contain thallium and that

the concentrations found (0.15-29.5 ng/g) are more than 100 times lower than those found in poisoning cases. Fatty tissues, cerebrospinal fluid and blood contain lower thallium concentrations, whereas higher concentrations were found in bones, hairs, teeth, nails and kidneys. In the lungs of workers in mines slightly higher thallium concentrations could be detected. According to Armore[22], thallium in blood is divided equally between the blood cells and plasma.

Thallium is accumulated in tissues and thus in cases of poisoning serum levels are much lower than those of tissues. The thallium concentrations in the kidneys and livers of humans killed by thallium were in the range 18-74 μg/g[47].

After an oral intake of thallium, the urine first shows high levels (over 10 μg/ml), but they fall rapidly. However, as thallium continues to be excreted slowly in the urine, it can still be detected in urine after several months[5]. Thallium is also excreted in saliva at concentrations even higher than those in urine[48].

In conclusion, thallium concentrations above 0.01 μg/g in biological materials, except hair, will indicate some form of thallium poisoning.

8.6. CONCLUSIONS AND FUTURE PROSPECTS

In the metal-directed techniques an increase in sensitivity is accompanied by an increase in cost (colorimetry < FAAS < NFAAS < ID-MS). FAAS allows determinations of thallium at concentrations higher than 0.2 μg/g; quantitative assays by NFAAS are reliable at concentrations higher than about 10 ng/g, whereas assays by ID-MS even allow determinations down to the 10 pg/g level.

FAAS and NFAAS allow quantitative determinations of more than 30 samples per day, whereas colorimetry is much more time consuming. ID-MS does not lend itself well to automation, but as this method is the most sensitive, reliable and precise, it can be used as a reference/standard technique and to establish normal levels of thallium. However, the specialized instrumentation restricts this technique to only a few experienced laboratories.

Methods suitable for screening purposes are NAA, emission spectrometry and ASV. NAA is interesting because of its low detection limit, but as the equipment is not generally available it is used only for special studies such as the analysis of parts of a hair.

Emission spectrometry has the advantage that all metals and some non-metals can be detected in one run. Modern equipment that allows the quantitative analysis of small samples with photomultiplier detection is, however, very expensive and rather insensitive for thallium.

ASV is a much less expensive screening technique. The number of metals that can be detected is still limited to about ten but ASV is very sensitive and highly selective for thallium.

In the future it may be expected that flame atomic-absorption spectrometry will become generally available in forensic and larger clinical laboratories because of its simplicity in use and the still decreasing costs. As thallium can be determined by FAAS, although not very sensitive, another metal-directed technique will be required only for studies in which lower detection limits are required.

In cases in which the underlying metal is not known beforehand, ASV in a more automated set-up lends itself to use in clinical laboratories, whereas modern emission spectrometry, in spite of the costs, will find a place in larger forensic laboratories.

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CHAPTER 9

CHROMIUM

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CONTENTS

9.1. HISTORY OF CHROMIUM DETERMINATION IN BIOLOGICAL MATERIALS

In light of collaborative studies and inconsistencies in the analytical data reported by investigators for biological fluids and certified SRMs*, the history of chromium analysis in biological materials is one of the most puzzling among trace elements. Recently, though, there has been better agreement among scientists concenrning valid analytical methods, as well as normal levels of chromium in body fluids.

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*For abbreviations see p. 273.

The development of more accurate analytical methods for chromium has particularly suffered for lack of suitable SRMs at the same concentration levels as those of biological materials. It was not until 1978 that the NBS issued bovine liver (SRM 1577) for chromium. This was the first and so far the only suitable SRM for chromium in biological materials whose chromium content is approximately 100 ng/g. No SRM is yet available for chromium at the concentration level of 1 ng/g, i.l., that which exists in body fluids.

In the past, attempts have been made to employ numerous analytical means for the determination of chromium in biological materials. These include methods based on colorimetry[1-3], electrochemistry[3], XRFS[4], AES[5-9], SSMS[10], GLC[11-13], FAAS[14,15], GFAAS[16,17], INAA[18-20] and DNAA[19,21,22]. MS[23], MED[24], AAS[25] and ECD[26] have been employed as detectors for GLC. Recently, methods based on ICPAES[27-29], CEWM-AA[30,31], IDMS[32] and, at higher concentration levels, PIXE[33] have been used for chromium analysis. Recently the most popular methods have been GFAAS and NAA.

TABLE 9.1

CHROMIUM IN PLASMA OR SERUM AS REPORTED BY DIFFERENT WORKERS

Mean (ng/ml)	No. of subjects	Analytical method	Ref.
185	25	AES	Monacelli et al.[6]
25	39	AES	Paixao and Yoe[5]
28	5	AAS	Glinsmann and Mertz[34]
30	132	AAS	Feldman et al.[14]
23	16	AAS	Levine et al.[35]
10	4	NAA	Behne and Diehl[36]
5.1	7	AAS	Davidson and Secrest[16]
9.3	127	NAA	Kasperek et al.[37]
45	3	NAA	Maxia et al.[38]
14	21	GLC-ECD	Savory et al.[39]
3.1		AES	Hambidge[8]
150	11	Cl	Li and Hercules[40]
0.73	50	AAS	Grafflage et al.[41]
1.62	15	AAS	Pekarek et al.[17]
0.5		AAS	Seeling et al.[42]
7.0	5	GLC-MED	Black and Sievers[24]
0.160	20	NAA	Versieck et al.[43]
0.14	8	AAS	Kayne et al.[44]
4.4	196	AAS	Vir and Love[26]
6.09	32	NAA	Newman et al.[45]
0.45	7	NAA	Kasperek et al.[46]
1.1	20	AAS	Rabinovitz et al.[47]

The values reported for chromium in human serum or plasma by different workers (Table 9.1) clearly reflect the difficulties found in chromium analysis in biological materials. These data indicate serious errors in sample collection and/or analysis. The large downward trend with time in reported concentrations shows, however, that considerable improvements in methods of contamination control and analysis have been made.

The situation at a concentration level two orders of magnitude higher than that found in serum is not much better. Table 9.2 summarizes the concentrations reported for NBS bovine liver (SRM 1577).

TABLE 9.2

REPORTED VALUES FOR CHROMIUM IN NBS BOVINE LIVER (SRM 1577)

NBS certified value: 88±12 ng/g.

Concentration (ng/g)	Analytical method	Ref.
3500-1500	INAA	Nadkarni and Morrison[20]
1000	DNAA	Stella et al[22]
490	INAA	Brill et al.[48]
210	DNAA	McClendon[21]
150	INAA	Grimakis[49]
130	GFAAS	Mertz and Roginski[50]
130	INAA	Plantin[51]
94	GFAA	Chao and Pickett[52]
80.6	DNAA	Versieck et al.[53]
51	INAA	Pierce et al.[54]
50.1	GFAAS	Christensen et al.[55]
5	INAA	Kasperek et al.[56]

Even at concentrations higher than 2 µg/g, the accuracy was not satisfactory in an inter-laboratory comparison study conducted using NBS brewer's yeast (SRM 1569) as an unknown sample[57]. Only two results out of twenty two were within the certified value of 2.12±0.05 µg/g, with a range of 0.351-5.40 µg/g[57]. In this collaborative study, NAA showed better accuracy than GFAAS. However, Tables 9.1 and 9.2 demonstrate that results obtained using NAA can be far from satisfactory. One can therefore conclude that inaccuracies have not been due mainly to the analytical principle or to the instrumental capability used, but rather to the inability of investigators to recognize important sources of error. This has especially been the case with contamination control in sampling, sample handling and analysis.

9.2. SAMPLING, SAMPLE HANDLING AND DIGESTION FOR CHROMIUM DETERMINATION

9.2.1. Control of airborne contamination

Chromium is present at relatively high concentrations in the dust particles of any standard laboratory. A clean room or a Class 100 laminar flow hood is necessary during sample handling and digestion to avoid airborne contamination[58,59]. During transfers between rooms, samples must be kept in covered plastic containers.

For dry digestions, the use of muffle furnaces whose thermocouples contain chromium (Ni-Cr) should be avoided[31]. Additionally, at the ultratrace level, crucibles must be covered during dry ashing[60] as air currents present in the hot oven carry particles high in chromium.

9.2.2. Sampling

9.2.2.1. Selection of tools and container materials for sampling

Knives, homogenizer blades and needles for venepuncture and liver biopsies are usually made of stainless steel, which contains 8-20% of chromium, and therefore cannot be used for sampling in chromium analysis. It has been shown that the use of normal venepuncture or biopsy needles may introduce chromium contamination in blood or liver biopsy samples 100 times higher than that which is naturally present[58]. Tools made of a low-chromium steel, quartz, titanium and plastic are suitable[58]. For the sampling of blood, over-the-needle catheters made of polypropylene are necessary and also commercially available[61].

For storage of samples, high-pressure polyethylene, PVC, PTFE and synthetic quartz of the higher purity have been found suitable[58,59,62].

9.2.2.2. Cleaning of containers

Thorough cleaning of containers before use is necessary, and various combinations of acid treatment have been proposed[58,61,63]. It is very important that acids used for the cleaning of containers should be low in chromium. Sulphuric acid is the most efficient of the mineral acids for extracting chromium[64]. Further, even reagent-grade concentrated sulphuric acid is very low in chromium (0.2 ng/ml). Soaking crucibles overnight in 10% sulphuric acid and rinsing several times with doubly distilled water has been found sufficient for ultratrace chromium analysis[60].

9.2.3. Sample handling and storage

9.2.3.1. Pre-treatment of hair samples

Collection of hair samples for chromium analysis does not cause any special problems. More problematic is the washing procedure necessary to remove the exogenous chromium due to environmental contamination. With the exception of a few published data this area is largely unexplored[65,66]. A recent methodological study showed that 1% sodium lauryl sulphate of hexane-ethanol (1:1) gave the same chromium concentration plateau after a 2 x 20 min wash, removing about 70% of the chromium, whereas acetone was much less effective and did not plateau even after six successive washes[67].

9.2.3.2. Homogenization

Homogenizer blades are usually made of a high-chromium steel. For oven-dried biological materials, therefore, it is of the utmost importance to avoid use of blades of this type. The chromium content of dried bovine liver pieces may increase 10-fold during 3 min of homogenization in an ordinary homogenizer[68]. Blades specially fabricated of a low-chromium steel (0.5% Cr) have been shown not to cause significant contamination of diet samples even after 11 min of homogenization[31]. However, for dried biological materials and especially meat, blades made of a chromium-free alloy should be used.

9.2.3.3. Storage

For storage of samples, high-pressure polyethylene, PVC, PTFE and synthetic quartz of the highest purity are suitable[58,59,62]. Biological materials have to be stored frozen, unless they are dried and sealed in airtight containers[31].

9.2.4. Sample digestion

For most methods, and particularly with samples whose chromium content is at the ultratrace concentration level ($\leq$5 ng/ml), chromium must be in solution before the instrumental analysis. Dry, wet or oxygen plasma ashing can be employed. For all of these digestion methods, however, certain precautions must be exercized.

First, certain biological materials, especially brewer's yeast and some plant materials, may contain large amounts of silica, which is insoluble in most acids. Significant proportions of the chromium in samples have been found to be adsorbed on the silica particles, resulting in erroneously low values[52,69]. Hydrofluoric acid, however, has been used successfully for the dissolution of silica in the solution[57].

9.2.4.1. Wet digestion

In wet digestion, acid mixtures containing perchloric acid must also contain sulphuric acid to prevent losses of chromium. Losses of up to 94% have been reported for chromium if perchloric acid is used alone, and 90% for a mixture of perchloric acid and nitric acid[52]. This is obviously due to the formation of chromium(VI) oxychloride (CrO_2Cl_2, b.p. 118°C). The presence of sulphuric acid in the digestion mixture seems to prevent the synthesis of this compound[52] by forming stable sulphate complexes with chromium.

Another potential source of error in wet digestion is the contamination due to the acids used. Commercial high-purity nitric acid usually contains about 100 ng/ml of chromium and after double distillation may still contain more than 10 ng/ml[59,62,70]. Commercial reagent-grade sulphuric acid and high-purity hydrochloric acid usually contain less than 0.5 ng/ml of chromium[59,70], but can be used for wet digestion in mixtures with oxidizing acids only. Therefore, it is extremely difficult to use wet digestion for samples whose chromium content is at the ultratrace level[52].

9.2.4.2. Oxygen plasma ashing

For samples containing chromium at the ultratrace level, oxygen plasma ashing has been found to be a reliable digestion method[71]. The disadvantage is its inefficiency, which prevents its application to certain samples[72].

9.2.4.3. Dry ashing

A long-standing confusion concerning the validity of dry ashing in chromium analysis was generated by investigators who claimed that some chromium compounds naturally present in biological materials were volatile in dry ashing[20,47,73-76]. The volatile chromium was assumed to be in the form of the glucose tolerance factor. However, numerous experiments conducted using certified SRMs and ^{51}Cr endogenously incorporated into brewer's yeast did not indicate the presence of any significant volatile chromium fraction at temperatures of 500°C or lower[31,64,78-80].

Loss of chromium by retention on the walls of crucibles can be a problem if temperatures of 600°C or higher are used[78,81]. Gorsuch[82] showed that retention of chromium at 600°C or higher was due to large amounts of sodium chloride that he added to the samples for experimental purposes.

At the temperatures of 500°C or lower normally employed for dry ashing, the extent of chromium retention is usually not more than 2%[31,64].

Dry ashing has been found to be suitable for the digestion or biological materials whose chromium content is higher than approximately 8 ng/g[31,68,80]. For samples whose chromium content is lower, however, porcelain or quartz

crucibles should not be used in dry ashing. It has been shown that chromium present in crucible materials leaches at elevated temperatures and can seriously contaminate samples at the ultratrace concentration level[71].

9.2.5. Preparation and storage of standard solutions

Anand and Ducharme[83] have shown that borosilicate glass is superior to plastic materials for the storage of chromium standard solutions. Depending on concentration, the standards remained stable from 1 to 48 weeks in glass containers[83]. Speeke et al.[58] also preferred quartz to plastic as a container material for the storage of chromium standards.

At the ultratrace concentration level, standards must be prepared daily. It is extremely important to prepare standards in dilute acid solutions, as trivalent chromium has a strong tendency to precipitate in aqueous solutions with pH above 3.5 owing to the olation process[84].

9.3. METHODS CAPABLE OF CHROMIUM DETERMINATION AT THE HIGHER CONCENTRATION RANGE PRESENT IN BIOLOGICAL MATERIALS

Hair, bones and most foods and diets are usually higher in chromium that 100 ng/g dry weight. This allows the use of INAA, which is a non-destructive multi-analytical method.

9.3.1. Instrumental neutron activation analysis

The nuclear reaction usually employed in NAA is $^{50}Cr(n,\gamma)^{51}Cr$. The measurement of the 320-keV γ-ray peak of ^{51}Cr($t_{\frac{1}{2}}$ = 27.7 days) follows after an optimal decay time. With biological materials, which are always fairly high in alkali metals and phosphorus, at least 1 week is needed to allow the shorter lived interfering nuclides to decay in order to measure the small chromium γ-ray peak. This means that a long irradiation time, usually more than 1 week, at high neutron flux density is also needed in order to obtain high enough ^{51}Cr activity. A high-resolution detector such as a Ge(Li) semiconductor detector should be used to avoid possible spectral interferences. Another even more important source of error is the chromium present in irradiation ampoules. Even when quartz of the highest purity is used, this "contamination" source may cause serious errors for most biological materials[85]. Separation of the irradiated sample from its irradiation container before recording the activity is therefore necessary. A detection limit of approximately 10 ng/g of chromium can be obtained with INAA.

In summary, the disadvantage of INAA in the determination of chromium in biological materials is the long analysis time. The advantages are the few operations needed and, after irradiation, freedom from contaminations and chemical interferences, as well as the capability of multi-element analysis. In theory, INAA is almost an ideal analytical tool, but as Table 9.2 shows, special care must be exercized in order to obtain valid results for chromium in biological materials. This is especially important in multi-element analyses.

9.3.2. X-ray fluorescence spectrometry

The detection limit of instrumental X-ray fluorescence analysis has traditionally not been sufficiently low for the determination of chromium in biological materials. Lower concentrations can be determined, however, if a sample is digested and chromium is separated from interfering elements by extraction. Using this technique, Beyermann et al.[4] reported a detection limit of 5 ng/ml of chromium in urine. This type of analysis, however, is impractical and increases the risk of losses and contamination.

9.3.3. Flame atomic-absorption spectrometry

When using FAAS for the determination of chromium in biological materials, chromium must be extracted into an organic solvent after a digestion. This is necessary because of the low concentration level, but is also beneficial in order to avoid matrix effects. Feldman et al.[14] used methyl isobutyl ketone for the extraction of chromium after oxidation to the hexavalent state. In more recent studies, chromium has been chelated prior to extraction with chelating agents such as 2,4-pentanedione, APDC and DDDC.

Using the solvent extraction technique, chromium can be measured in biological materials at concentrations higher than ca. 10 ng/ml[15,86,87]. The extraction technique, however, is complicated, time consuming and susceptible to losses and contaminations[52].

9.3.4. Colorimetric and electrochemical methods

Colorimetric determination is based on the formation of a complex when hexavalent chromium reacts with 1,5-diphenyl carbazide. This complex can be measured colorimetrically at 540 nm. Various adaptations of this principle have been developed[1-3]. However, other metals present in biological materials, especially iron and vanadium, seriously interfere[3]. Therefore, the use of this method should be avoided.

For reduction of hexavalent to trivalent chromium can be employed for the electrochemical determination of chromium. Beyermann[3] has examined amperometric chromium analysis to some extent. This method, however, also suffers from a lack of sepcificity and should be used with caution.

9.3.5. Spark-source mass spectrometry

For the determination of chromium in several biological materials by SSMS, the samples need only be ashed. The weak points of this method have traditionally been unstable source behavior, variable response of the photoplate detector, sample inhomogeneity and matrix interferences[88]. Although the use of an electrical detection system coupled with a computer has improved precision and shortened the analysis time of SSMS[89], the determination of chromium in biological materials by SSMS remains a problem[90]. Chromium has been determined in hair, fingernails, aortic tissue[10] and human liver samples[90]. The results of the last study showed serious sensitivity, precision and matrix interference problems. Therefore, with the present state-of-the-art, SSMS is not recommended for analyses that require good accuracy and precision.

9.4. METHODS CAPABLE OF CHROMIUM DETERMINATION AT THE ULTRATRACE CONCENTRATION LEVEL

In this presentation, the ultratrace concentration level is taken to mean chromium concentrations of 5 ng/ml or less. Methods based on several principles are capable of chromium determination at this level: GFAAS, DNAA, ICPAES, IDMS and GLC.

9.4.1. Methods employing gas-liquid chromatography

In GLC, chromium must be converted into a volatile chelate, usually a fluorinated acetylacetonate. After separation in the GLC column, chromium can be detected by ECD[39], MED[24], AA[25] or MS[23]. The absolute detection limits for these systems are 1×10^{-13} g (ECD), 1×10^{-11} g (MED), 1×10^{-9} g (AA) and 5×10^{-11} g (MS), according to different workers[24,25,91,92]. The advantage of MED, AA and MS is the good specificity. As the detection limits indicate, under ideal conditions, using GLC and the most sensitive detectors, chromium can be determined in ultratrace concentrations. However, the low chromium concentrations in biological materials cause several problems.

First, the many chemicals needed in the sample preparation readily render the analysis impossible owing to the high blanks[92]. In addition, stainles-steel

needles, normally employed for the injection of samples in GLC, must be replaced with non-metallic pipettes or pipettes made of a chromium-free alloy. Further, the flame sealing of reaction tubes causes elevated blanks[92]. Finally, the trifluoroacetylacetone commercially available must usually be purified by distillation before use if an ECD is employed.

GLC-MS can also be used for the measurement of chromium by stable isotope dilution (ID). In this method, the samples are spiked with ^{50}Cr and the chromium is extracted after the digestion as the trifluoroacetylacetonate. The isotope ratio is measured by GLC-MS using dual ion monitoring.

This analytical system has been employed successfully for the determination of chromium in urine at normal physiological concentrations. The concentration of 0.32 ng/ml found in a pooled urine sample of healthy U.S. subjects, was verified by independent means[32].

Owing to the complicated procedure, long analysis time and expensive instrumentation, IDMS is not suitable for routine analyses but is an excellent reference method for the verification of other methods at the ultratrace concentration level.

9.4.2. Inductively coupled plasma atomic-emission spectroscopy

Hambidge[7-9] used the "argon-silver arc" technique for the emission spectroscopic determination of chromium in serum. This arc procedure has recently been replaced by the ICP source. For ICPAES, solid samples must be digested, but biological fluids, after dilution, may be analysed directly if the concentration is sufficiently high. At present, the detection limit for chromium is about 2 ng/ml with an ultrasonic nebulizer and about 5 ng/ml when an pneumatic nebulizer is used[29]. This means that neither system is capable of the determination of chromium in normal urine or serum[27,28,30,43,44,93].

The high salt content of most biological fluids requires considerable dilution to prevent clogging of the nebulizer[27,29]. Hence the sample introduction devices are the weak link in this method and need improvement. The advantages of the ICPAES technique are the relatively high freedom from matrix effects, capability of multi-element analysis, small sample size required, few sample pre-treatments needed and a wide dynamic range[27,28].

9.4.3. Destructive neutron activation analysis

In DNAA, samples are usually not digested before the irradiation. However, if a very high neutron flux (10^{14} neutrons/cm^2/sec) is used, the radiation damage develops such a high pressure in the sealed quartz ampoules that there is the

risk of an explosion. In this instance, digestion before the irradiation may be necessary[43]. After the irradiation, the ampoules must be thoroughly cleaned to remove outside contamination. The samples are then digested in order to allow the separation of chromium. After addition of carrier, chromium can be separated from the interfering nuclides using different procedures[21,43]. The most selective of these is distillation of chromium as chromium(VI) oxychloride (chromyl chloride) (CrO_2Cl_2, b.p. 118°C) by boiling the sample in perchloric acid solution and introducing dry hydrogen chloride gas. A quantitative recovery can be obtained[43].

Although DNAA is a contamination-free method after the irradiation, special care must be exercized to avoid chromium contamination before the irradiation. This is very important when samples such as serum and urine are analysed. The sample size is usually 100 mg or less, which means that at the serum chromium concentration level of 0.15 ng/ml, a very small contamination causes an important blank. It has been shown that flame sealing the ampoules may result in a serious contamination of serum samples if the quartz used is not pure enough[85]. Another source that may cause errors in chromium DNAA is the interfering nuclear reaction $^{54}Fe(n,\alpha)^{51}Cr$. As the nuclear reaction shows, the extent of this interference depends on the Fe:Cr concentration ratio in the sample to be analysed. Parr[19] calculated the magnitude of error due to this nuclear reaction for several biological materials. These calculations showed that the extent of error can be up to 650% for whole blood, 159% for liver, 28% for heart and 7% for animal muscle[19].

Versieck et al.[43], using a very high neutron flux (10^{14} neutrons/cm^2/sec) and an irradiation time of 12 days, was able to show that the mean chromium content in the serum of healthy subjects was 0.160 ng/ml. This concentration level was confirmed by independent means[44]. This demonstrates that DNAA is a very powerful analytical tool when proper care is exercized, and the sensitivity is optimized. However, the long analysis time needed, the complicated procedure and the expensive instrumentation restrict the use of DNAA as a routine method.

9.4.4. Graphite furnace-atomic absorption spectrometry

GFAA clearly has been the most popular analytical tool in the determination of chromium in biological materials in the past. However, although the determination of chromium in pure water and in simple inorganic matrixes by GFAA is relatively simple, biological materials pose several problems and potential sources of error.

9.4.4.1. Problems specific to graphite furnace-atomic absorption

9.4.4.1.1. Background correction. The non-atomic absorption caused by molecular species and light scattering leads to seriously over-large values for chromium in biological matrices unless corrected for. In order to solve the problems of back-

ground correction, three principles have been used: (1) measuring the absorption of a continuum emitting lamp, (2) using the Zeeman effect and (3) using a wavelength modulation technique.

The methods based on measurement of continuum emission usually employ a deuterium arc lamp whose effective emission intensity covers the ultraviolet region up to about 350 nm. Thus, 357.9 nm, which is the most sensitive absorption line for chromium that has to be used in order to measure chromium in biological materials, is just on the border of the efficient emission range of the deuterium lamp.

Recently, the adequacy in chromium analysis of the deuterium background correction system used in commercial AA spectrometers has been challenged[30]. The authors found that excessively high values were obtained for chromium in an ashed urine matrix when using a Perkin-Elmer AA spectrometer.

To improve the efficiency of this background correction system in the 350 nm spectral region, Kayne et al.[44] replaced the deuterium arc lamp of the Perkin-Elmer 603 AA spectrometer with a high-intensity tungsten-halogen lamp and reported dramatically improved background correction capability for the analysis of chromium in urine and serum. In addition to the deuterium arc lamp, this type of lamp has now been installed in the Perkin-Elmer 5000 AA spectrometer. The effective spectral emission region of this lamp covers wavelengths from 350 to 800 nm[94].

Theoretically, the use of the Zeeman effect to solve the background correction problem in chromium analysis looks promising, but the successful adaptation of this technique for chromium in biological materials at the ultratrace concentration level has not yet been reported.

Zander et al.[95] combined a high-intensity continuum source and a wavelength-modulated high-resolution échelle monochromator. In this system, termed CEWM-ASS, the modulation process sweeps the absorption profile repeatedly across the exit slit, resulting in a ripple in the photosignal that is proportional to the intensity absorbed and is independent of band interferences. This system like that of the Zeeman effect, has advantages over the conventional dual source background correction system, because a single source is used and only a narrow spectral region is examined. The background correction capability of CEWM-AA is reportedly superior to that of the conventional deuterium arc background correction system: a correction capability of 3.0 absorbance units (A.U.) has been reported[96] compared with 1.0 A.U. with the conventional deuterium arc background correction.

Guthrie et al.[30] were the first to test CEWM-AAS for the determination of chromium in ashed urine. The concentration they reported in a pooled urine sample of healthy subjects (less than 1.0 ng/ml) was later confirmed by independent means[32]. Kumpulainen et al.[31] successfully used CEWM-AA for the determination of chromium in ashed diets. Unfortunately, AA instruments based on CEWM are not commercially available.

9.4.4.1.2. Temperature control in the graphite furnace. Veillon et al.[97], using ^{51}Cr and a graphite furnace (Perkin-Elmer HGA 2100) without the optical temperature control system, found that a significant amount of chromium is retained in graphite tubes after the atomization step. Less ^{51}Cr was retained in pyrolytically coated tubes than in uncoated tubes. Moreover, the use of a high atomization temperature decreased the extent of ^{51}Cr retention. The chromium retention was apparently due to carbide formation. This hypothesis is supported by the results of other investigators[98,99]. Imprecise temperature control, in addition, may result in losses due to volatility (ashing step, temperatures near 1300°C) and mechanical spattering (drying step).

The results of the above studies indicate the need for precise temperature control in the furnace, a rapid heating rate to the final atomization temperature and the use of a high atomization temperature (2700°C) and pyrolytically coated graphite tubes.

The Perkin-Elmer HGA-500 graphite furnace fulfills these criteria owing to the optimal temperature control system. Moreover, it has been shown that chromium can be reliably determined in ashed urine and human milk[71] and in diet samples[68] using a Perkin-Elmer 703 AA spectrometer and an HGA-500 graphite furnace. In these studies, none of the "background correction related problems" such as deformed or double peaks reported by Guthrie et al.[30] were encountered. The sufficiency of the deuterium arc background correction system used was tested by determining chromium in ashed urine samples, one high (sp. gr. 1.023) and the other low (sp. gr. 1.007) in salt using either 10- or 20-µl sample volumes. No significant difference between measured concentrations was found at a urine chromium concentration level of 5.0 ng/ml[71]). Similarly, the adequacy of the background correction was tested for ashed human milk, the chromium concentration of which was 0.6 ng/ml[71].

Therefore, it may be concluded that inadequate temperature control systems of the older graphite furnace models, rather than too low a photon flux of the deuterium arc lamp, was at least partly responsible for the problems encountered by Guthrie et al.[30]. However, it is strongly recommended that the adequacy of the deuterium arc lamp be tested when chromium is determined in biological materials.

Further, although there is a temptation to determine chromium in biological fluids without preliminary ashing by injection directly into the graphite furnace, the use of preliminary digestion is necessary if a deuterium background correction system is employed. Two investigators concluded that preliminary ashing is not needed for the determination of chromium in urine with that instrumentation[100,101]. However, there is still no evidence that, by using this method and a deuterium arc lamp, reliable results can be obtained at concentrations lower than 1 ng/ml. On the contraty, sighificantly lower values for chromium in urine

resulted after direct injection compared with values obtained after preliminary ashing[102]. However, direct measurement is reliable and recommended if instrumentation comparable to the Perkin-Elmer 5000 and HGA-500 is used (see Section 9.5.4).

9.5. STANDARD METHODS PROPOSED

9.5.1. Applicability

The method employing the dry ashing procedure is applicable to the determination of chromium in biological materials whose chromium content is approximately 8 ng/g, or higher, such as most tissues, biopsy materials, diets, most foods and plant materials.

The method employing oxygen plasma ashing is suitable for the determination of chromium in biological materials whose chromium content is approximately 8 ng/g, or less, such as human milk, urine and other body fluids, when AA instruments with background correction capabilities not comparable to the Perkin-Elmer 5000 are used.

For biological fluids, the method of additions using the direct injection of samples into the graphite furnace is recommended if instrumentation comparable to the Perkin-Elmer 5000 AA spectrometer in terms of background correction capability, and the Perkin-Elmer HGA-500 graphite furnace in terms of temperature control capability, is employed.

9.5.2. Sampling

Samples must be collected, handled and stored so as to avoid contamination, or losses, as described in the Sections 9.2.1-9.2.3. If possible, samples of 0.5 g dry weight should be used. With liquids, a sample size of 0.5-2.0 ml is suitable.

9.5.3. Sample preparation

9.5.3.1. Dry ashing procedure

Preferably, platinum crucibles should be used. Quartz crucibles are also suitable. The weighed samples in shielded crucibles are placed in a cold muffle furnace (see Section 9.2.1 for the furnace type). The temperature is gradually increased to 500°C and kept there overnight. After the crucibles have cooled, the white ash is dissolved in 1 ml of 1 *N* hydrochloric acid (sub-boiling distilled or ultrapure). If the ash still contains carbon after the dry ashing, 20 µl of

concentrated sulphuric acid (reagent grade) and 50 µl of 50% hydrogen peroxide (reagent grade) are added to the crucibles and slowly evaporated to dryness on a hot-plate in a Class 100 clean air hood or in a clean room. The crucibles are kept for an additional hour in the furnace at 500°C. The acid treatment is repeated as needed. For most biological materials, only one acid treatment, or no treatments at all, are needed to complete the digestion. After addition of hydrochloric acid to the crucibles, they are allowed to soak for 30 min before injecting samples into the graphite furnace. If the samples contain insoluble particles (usually silicon) they should not be removed by filtration, but the contents should be tranferred into PTFE containers and the particles dissolved using hydrofluoric acid (sub-boiling distilled).

The recovery of chromium after this dry ashing procedure was tested by adding ^{51}Cr to NBS bovine liver (SRM 1577) before the ashing. The mean recovery after three muffle furnace and acid treatments was 95.2±1.3%[31]. The recovery of ^{51}Cr after the dry ashing without the acid treatments was 100.6±0.62% (n = 7) for NBS brewer's yeast, 99.7±1.1% (n = 8) for NBS bovine liver[31] and 98.3±1.1% (n = 10) for brewer's yeast, in which ^{51}Cr was endogenonously incorporated during the growth of the yeast[64].

The ^{51}Cr which was retained on the walls of porcelain crucibles after dissolution of ash in hydrochloric acid varied from 0.4 to 2.0% when tested with NBS bovine liver and brewer's yeast[31,64].

9.5.3.2. Oxygen plasma ashing

This ashing procedure is recommended almost exclusively for body fluids whose chromium content is about 1 ng/ml or less. After thawing and careful mixing, samples of 0.5-1.0 ml are pipitted into 0.5-1.0-ml platinum or quartz crucibles and the liquid is slowly evaporated to dryness on a hot-plate in a Class 100 clean air hood or, for urine samples, in a vacuum oven. Once dried, the samples are treated for 5 h in an oxygen plasma asher at 1 mmO_2 pressure and 400-W power. Then, 100 µl of 50% hydrogen peroxide (reagent grade) are added to the crucibles and slowly evaporated to dryness as described above. The oxygen plasma ashing treatment is repeated as needed. For human milk and urine samples, only one hydrogen peroxide treatment is usually needed. The ash is dissoluted in 1 *N* hydrochloric acid (sub-boiling distilled or ultrapure) and after 30 min samples are injected into a graphite furnace.

The recovery of ^{51}Cr after the complete ashing procedure was 98.6±0.8% (n = 10) for urine and 97.6±2.4 (n = 7) for human milk[71,103]. The retention of ^{51}Cr on the walls of the crucibles was 0.5±0.1% (n = 5) for urine and 0.6±0.1 (n = 7) for breast milk[71,103].

9.5.3.3. Method of additions

For human milk, a 1:2 dilution with water is necessary in order to obtain correct calibration lines, whereas urine can be determined without dilution[104]. However, dilution with water is also recommended for urine whenever concentration of chromium is high enough to allow it.

Four sample cups are used for each sample:

cup 1: 1 ml of sample + 10 µl of 1 *N* hydrochloric acid

cup 2: 1 ml of sample + 10 µl of 25 ng/ml chromium standard

cup 3: 1 ml of sample + 10 µl of 50 ng/ml chromium standard

cup 4: 1 ml of sample + 10 µl of 75 ng/ml chromium standard

All of the above standards have to be prepared in 1 *N* hydrochloric acid. The chromium additions suggested above are suitable for samples from subjects not occupationally exposed to chromium.

9.5.4. Methodology - apparatus

9.5.4.1. Instrumental

The AA spectrometer employed should preferably have a tungsten-halogen lamp for an effective background correction capability at the 357.9-nm chromium line. Instruments based on CEWM-AA are also suitable. With the deuterium arc lamp, sufficiency of the background correction *must* be tested for the particular type o of samples, as described elsewhere[68,71]. The graphite furnace should rather have the optimal temperature sensor. The analytical results should be recorded using a high-quality chart recorder. The shape of the background-corrected analyte peaks should be checked by using the continuous mode, especially if a deuterium arc lamp is used.

9.5.4.2. Analytical conditions

9.5.4.2.1. Analytical conditions for ashed biological materials

AA instrument settings:

Wavelength:	357.9 nm
Spectral band width:	0.7 nm
Background correction:	Tungsten-iodide or deuterium arc lamp
Mode:	Peak height
Integration time:	4 sec (When coupled to the P.E. HGA-500 graphite furnace)

Furnace programme:

Step 1 (drying):	110°C/25 sec (ramp 10 sec)
Step 2 (ashing):	1100°C/11 sec (ramp 10 sec)
Step 3 (atomizing):	2700°C/3 sec (ramp 0 sec) (for the P.E. HGA-500)

Optical temperature sensor: Yes
Automatic remote baseline correction: 2 sec prior to atomization
Start of integration time: 1 sec prior to atomization
Internal gas flow-rate: 50 ml/min

Other analytical conditions:
Sample matrix: 1 *N* HCl
Injection volume: 20 µl
Purge gas: Argon (purest grade available)
Type of graphite tubes: Pyrolytically coated

9.5.4.2.2. Analytical conditions for biological fluids without preliminary ashing

AA instrumental conditions:
Instrument recommended: Perkin-Elmer Model 5000 or one with comparable background correction capability at the visual spectral region
Wavelength: 357.9 nm
Spectral band width: 0.7 nm
Background correction: Tungsten-iodide lamp (350-800 nm spectral region)
Mode: Peak height
Integration time: 4 sec (when coupled with the Perkin-Elmer HGA-500 furnace)
Expansion: 10x (for normal urine and serum)

Furnace programme:
Furnace recommended: Perkin-Elmer HGA-500

Step	Temperature (°C)	Ramp time (sec)	Hold time (sec)	Read*	B.O.C.**	Rec.*
1 (drying)	100	15	20	-	-	-
2 (drying)	130	10	20	-	-	-
3 (ashing)	1100	10	60	-	-	-
4 (atomization)	2700	0	3	-1	-2	-5

*The moment when the function in question is activated, indicated in seconds from the beginning of the step (Rec. = recorder).
**B.O.C. = automatic baseline offset compensation.

Internal gas flow: 50 ml/min
Optical temperature sensor: Yes

Other analytical conditions:

Method of additions:	For normal urine, serum and human milk add 0, 0.25, 0.5 and 0.75 ng of Cr per ml of sample (see Section 9.2.3.3)
Injection volume:	20 µl
Pure gas:	Argon (purest grade available)
Type of graphite tubes:	Pyrolytically coated

The sensitivity and detection limit of this method are illustrated in the recorder chart tracing of the determination of chromium in a pooled urine sample of nine healthy subjects using a Perkin-Elmer 5000 AA spectrometer and an HGA-500 graphite furnace (Fig. 9.1)[104].

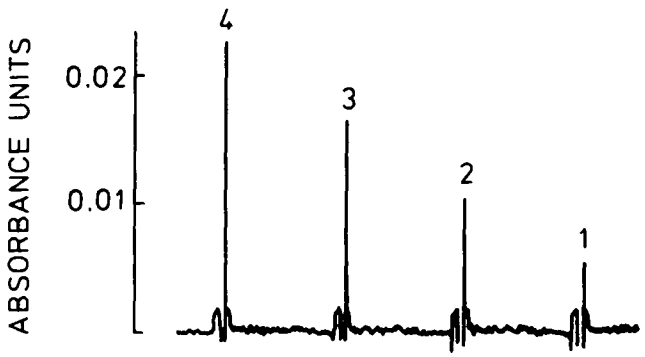

Fig. 9.1. Determination of chromium in a pooled urine sample of nine healthy Finnish subjects using the method of additions. Recorder chart tracing. Expansion 10x. Injection volume, 20 µl. Peak 1, sample; peak 2, sample + 0.25 ng/ml of Cr; peak 3, sample + 0.50 ng/ml of Cr; peak 4, sample + 0.75 ng/ml of Cr. Instrument used: Perkin Elmer Model 5000 AA spectrometer and HGA-500 graphite furnace[104].

9.5.4.3. Standards

The sample response is compared with that of inorganic chromium as potassium chromate or chromium chloride, except when using the method of additions. Working standards in the range 0.5-5.0 ng/ml should be prepared daily and in the range 5.0-50.0 ng/ml at least twice a week from a 1 mg/l stock standard solution. The standards should be kept in glass containers.

9.5.4.4. Blanks

Several blank crucibles should be used for each set to facilite statistical treatment of the blank signals. The calculation of the detection limit must be based on the magnitude and variation of blank signals.

9.5.5. Reliability criteria of the methods

9.5.5.1. Method employing the dry ashing procedure

The accuracy of the method was tested for biological materials whose level of chromium content was 20 ng/g dry weight or higher, using NBS bovine liver. The mean values obtained in three series of analyses during a 3-month period were 88, 85 and 89 ng/g (NBS certified value 88±12 ng/g)[68].

The precision of this method was tested with NBS bovine liver and diet samples. The precision in the triplicate series of analyses of 30 diets expressed as the relative standard error of the mean (RSEM = 100 x SEM/mean) ranged from 0.2 to 12.1% with a mean of 4.1%[68]. The coefficient of vatiation in series as tested with NBS bovine liver was 5.6% (n = 5), 7.1% (n = 8) and 9.1% (n = 6)[68]. The precision between the three series expressed as the coefficient of variation was 2.4%[68]. These three series were analysed at 1-month intervals.

9.5.5.2. Method employing the oxygen plasma ashing procedure

The accuracy of this method for biological samples whose chromium content was at the ultratrace concentration level could not be verified as no certified SRMs for biological materials are available at that concentration level. However, the level of chromium concentration found in a pooled human urine sample (0.55 ng/ml)[103] agrees well with the value of 0.50 ng/ml obtained for this sample using the method of additions and a Perkin-Elmer 5000 AA spectrometer with the improved background correction capability at the visual wavelength region due to the tungsten-iodide lamp[104] (see Section 9.5.5.3).

The precision of this method was also tested using the pooled breast milk sample presented above. Although the coefficient of variation in series was 27% (n = 5) and 23% (n = 7) tested on different days, the day-to-day coefficient of variation between series was only 7.6% (3 days)[103].

9.5.5.3. Method of additions using direct injection

The accuracy and precision of this method were tested using a Perkin-Elmer Model 5000 AA spectrometer coupled with a Perkin-Elmer HGA-500 graphite furnace.

The accuracy was tested using the same pooled human milk sample indicated above. The mean value of 0.50 ± 0.07 ng/ml of chromium was obtained[104,108] compared with 0.55 ng/ml obtained after oxygen plasma ashing[103]. For urine, the accuracy was tested with two pooled round-robin urine samples containing 0.11 and 0.51 ng/ml of chromium as ascertained by another laboratory using IDMS and CEWM-AAS[104,105]. Using the present method values of 0.13 ± 0.01 and 0.54 ± 0.01 ng/ml of chromium were obtained for the same samples, respectively[104,105,108].

The precision of this method was tested using the above-mentioned pooled human milk and urine samples. The day-to-day coefficient of variation for the urine sample was 8.7% (3 days)[104]. For the human milk sample the day-to-day coefficient of variation was 13.5% (3 days)[104].

9.6. FUTURE PROSPECTS

Although the graphite furnace is used principally in AA spectrometry, it may also be employed for atomic-emission spectroscopy. Recently, a successful adaptation of this principle has been described using an instrument constructed from an HGA-72 furnace atomizer with platform atomization, an échelle monochromator and a square-wave wavelength modulation system[106]. A detection limit of 0.023 ng/ml was reported for chromium in a water matrix[106]. As this type of instrument operates in both the UV and visible regions, and has the potential for application to simultaneous milti-element analysis, the future looks promising for instruments based on this principle.

As has already been mentioned in the section describing ICPAES, this method will probably be a good technique for the determination of chromium in biological materials, once the sample introduction system and the detection limit are improved. Owing to its wide dynamic range, multi-element capability and, particularly, its unusual freedom from matrix interferences, ICPAES would be an ideal technique for the direct determination of chromium in biological fluids.

At the higher concentration range, PIXE is a promising new method. PIXE has a considerably better detection limit than that of traditional XRFS[39]. At present, the detection limit should be sufficient for the determination of chromium in hair samples. This method is still under rapid development[107] and may become an important tool for cases in which a non-destructive multi-element analysis is required using very small tissue samples.

LIST OF ABBREVIATIONS

AA	Atomic absorption
AAS	Atomic-absorption spectrometry
FAAS	Flame atomic-absorption spectrometry
GFAAS	Graphite furnace atomic-absorption spectrometry
CEWM-AA	Continuous emission wavelength modulation atomic absorption
AES	Atomic-emission spectroscopy
ICPAES	Inductively coupled plasma atomic-emission spectroscopy
MS	Mass spectrometry
SSMS	Spark-source mass spectrometry
ID	Stable isotope dilution

IDMS	Stable isotope dilution-mass spectrometry
GLC	Gas-liquid chromatography
GLC-MS	Gas-liquid chromatography-mass spectrometry
NAA	Neutron activation analysis
INAA	Instrumental neutron activation analysis
DNAA	Destructive neutron activation analysis
PIXE	Proton-induced X-ray emission spectrometry
MED	Microwave-excited emission detector
ECD	Electron-capture detector
XRFS	X-ray fluorescence spectrometry
SRM	Standard reference material
NBS	National Bureau of Standards, Washington, DC, U.S.A.
PVC	Poly(vinyl chloride)
APDC	Ammonium pyrrolidinedine dithiocarbamate
DDDC	Diethylammonium diethyldithiocarbamate
SEM	Standard error of the mean
RSEM	Relative standard error of the mean = 100 x SEM/mean
Cl	Chemiluminescence

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CHAPTER 10

NICKEL*

F. WILLIAM SUNDERMAN Jr.

CONTENTS

10.1. INTRODUCTION

Bertrand and Macheboeuf[1] in 1925 were the first investigators to measure nickel in tissues from man and animals; they discovered the relative abundance of nickel in marine molluscs. The first analyses of nickel in human urine and faeces were reported in 1950 by Tompsett and Fitzpatrick[2], and the first analyses of nickel in human blood and serum were performed in 1957 by Cluett and

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Yoe[3]. The nickel concentrations obtained in these early studies were artefactually high, as the spectrophotometric methods were insufficiently sensitive and specific. These pioneering studies, nonetheless, initiated the gradual evolution of accurate, reliable technqiues for analysis of nickel in biological materials.

This chapter is an extension of earlier publications that were prepared under the auspices of the National Academy of Sciences (U.S.A.)[4] and the International Union of Pure and Applied Chemistry[5]. The aim of this chapter is to summarize methods for analysis of nickel in biological materials. Attention is focused particularly on clinical applications of such analyses to monitor human exposures to nickel compounds.

10.2. COLLECTION AND CONVERSION OF SAMPLES

Nickel contamination during the collection, storage and processing of samples is a serious problem for measurements of nickel concentrations in body fluids, excreta and tissues. Persons who handle the samples or perform the analyses should wear plastic gloves, as sweat from the hands is rich in nickel[6] and is a source of nickel contamination of containers and utensils. Sample handling and analysis should be performed in a laminar-flow hood, to minimize nickel contamination from dust. Containers, vessels and Pasteur pipettes constructed of polyethylene, polystyrene, polypropylene or PTFE should be used for the collection, transfer and conservation of samples; the plastic-ware should be cleaned before use by soaking in ultrapure nitric acid (3 mol/l), followed by multiple rinses with water that has been demineralized by ion exchange and distilled in an all-glass or quartz still[5]. Plastic syringes and tubes for blood collection and cone-tips for micropipettors are often contaminated with trace metals[7-11]; they should also be acid-washed.

To collect blood for nickel analysis, the antecubital fossa is washed with soap and water and wiped with an ethanol-soaked pledget of gauze or cotton. After the ethanol has evaporated, a sterile polyethylene canula is inserted into an antecubital vein, and the stylus of the canula is removed. The canula is flushed with at least 3 ml of blood (which is either discarded or used for other analyses) before the blood specimen for nickel analysis is aspirated into a polystyrene syringe. The blood sample is allowed to clot in a stoppered plastic tube for 1 h at room temperature. Serum is removed with an acid-washed Pasteur pipette, placed in a plastic tube and frozed at -14°C until the day of analysis.

To collect urine for nickel analysis, stringent precautions are necessary to prevent contamination of urine specimens by dust from the subject's body or clothing. The urine should be voided directly into a wide-mouthed polyethylene bottle and acidified by addition of concentrated, ultrapure nitric acid (5 ml of

acid per litre of urine). A 10-ml aliquot of acidified urine is placed in a plastic tube and frozen at -14°C until the day of analysis. Because of fluctuations of urine nickel concentrations which occur during the work-shift, analyses of nickel in 8-hour urine specimens should be used routinely to monitor occupational exposures to nickel[12]. In situations where timed urine collections are impractical, analyses of nickel in end-shift urine specimens are the best alternative.

Versieck et al.[13] showed that steel scalpel blades or Menghini needles cause many-fold increases in nickel concentrations of small biopsies of human liver. Plastic knives and segments of rigid plastic tubing with sharpened, bevelled ends are satisfactory tools for the collection of post-mortem tissue specimens for nickel analysis. For advice on contamination control in analysis of nickel and other trace metals, readers should consult the monographs by Zief and Mitchell[14] and LaFleur[15].

10.3. PRE-TREATMENT OF SAMPLES

Most procedures for the determination of nickel in biological materials require preliminary steps for oxidation or removal of organic constituents. The merits of various techniques for sample pre-treatment have been discussed by several investigators[5,16-20]. Zachariasen et al.[16] evaluated wet digestion using a combination of oxidizing acids versus dry ashing in a muffle furnace as preliminary steps for the determination of nickel in whole blood, plasma and urine. Dry ashing was judged to be superior, because it required less attention and minimized the use of chemical reagents, which are sources of nickel contamination. Torjussen et al.[17] reached the opposite conclusion in a study of nickel in tissues. Acid digestion of tissues was judged to be superior to dry ashing, because it saved time and the sample could remain in the same vessel throughout the analysis. Mikac-Devič et al.[18] compared four techniques for the destruction of organic constituents of serum and urine, including dry ashing in a muffle furnace and three different procedures for acid digestion. The best results were obtained by digesting the samples with mixed ultrapure acids (nitric, sulphuric, and perchloric acids) in a Pyrex tube inside an electric block heater. This procedure had the following advantages: (a) digestion, chelation and extraction steps were performed in a single tube without the need for quantitative transfer; (b) constant volumes of acids were used for the blank, standard and unknown samples; (c) the samples did not require continuous attention, and (d) the samples did not evaporate to dryness, as sulphuric acid refluxed in the tubes when the digestion was completed. Ader and Stoeppler[19] used ^{63}Ni as a tracer to evaluate losses of nickel during acid digestion versus dry ashing of urine samples. Acid diges-

tion in quartz tubes yielded quantitative recovery of ^{63}Ni. On the other hand, variable amounts of ^{63}Ni were lost during dry ashing in quartz crucibles, owing to the formation of insoluble nickel silicates. Watling and Wardale[20] evaluated five techniques for the oxidation of tissue samples, including two dry ashing procedures, two acid digestion procedures and a low-temperature ashing technique that used an oxygen plasma. One of the dry ashing procedures was unsatisfactory owing to nickel contamination of manganese nitrate, which was added as an ashing aid. The other four procedures yielded equivalent results for nickel analyses. After considering the pros and cons, Watling and Wardale[20] concluded that they preferred digestion with nitric and perchloric acids.

Sunderman and Wacinski[21] tried unsuccessfully to determine nickel concentrations in tissue samples that were subjected to pressure digestion with nitric acid in PTFE vessels inside Parr combustion bombs. Leakage of nitric acid fumes into the steel bomb casings resulted in sporadic nickel contamination. Gaffin[22] noted variable recoveries of nickel when human liver and muscle samples were subjected to pressure digestion in PTFE vessels by a similar procedure. Kaplan et al.[23] determined nickel and other trace metals in rat lung samples (0.3 g wet weight) which were dissolved and digested by incubation at 60°C for 24 h in 6 ml of a toluene solution of tetramethylammonium hydroxide. Although time consuming, this procedure was labour-saving and did not require special equipment.

Schaller et al.[24], Nomoto and Sunderman[25], Gonzalez et al.[26] and Andersen et al.[27] reported that preliminary digestion of organic matter in serum or plasma can be circumvented by precipitation of proteins with trichloroacetic acid (TCA) and hydrochloric acid. At low pH, Ni(II) is liberated from binding to serum albumin and amino acids; Ni(II) can be chelated and extracted directly from the protein-free supernatant fluid. Simplicity and convenience of TCA-hydrochloric acid precipitation make this procedure advantageous for routine measurements of nickel concentrations in serum or plasma of nickel-exposed workers. Sunderman[5] identified a possible pitfall of this procedure, as he found that TCA-hydrochloric acid precipitation did not release nickel quantitatively from jackbean urease, a nickel metalloprotein. Artefactually low values for serum nickel concentrations might be obtained if similar nickel metalloproteins exist in mammalian tissues and are released into serum under pathological conditions. Addition of TCA and hydrochloric acid has been suggested for the determination of nickel concentrations in urine[27], but the validity of this approach has not been thoroughly documented. The present author recommends that the acid digestion technique of Mikac-Dević et al.[18] be used for the determination of nickel in serum or urine. This method can also be used for the determination of nickel in tissue samples (<5 g wet weight) if increased volumes of mixed acids are used. The acid digestion procedures of Nomoto and Sunderman[25] and Elakhovskaya et al.[28] are suitable when larger samples of tissue (1-5 g wet weight) are analysed. Acid digestion of

tissues is conveniently performed in Erlenmeyer flasks on an electric hot-plate that provides precisely regulated and uniform heating[5].

Nickel concentrations in human body fluids, tissues and excreta are low in comparison with the sensitivities of most analytical techniques. Therefore, methods for nickel determinations usually include chelation, extraction, precipitation or adsorption steps to concentrate the nickel prior to quantitation. These steps also help to separate nickel from substances that may interfere with quantitation. In Table 10.1 are listed the separation techniques that have been employed for the determination of nickel in biological materials. Other separation techniques, which have only been used for the determination of nickel in aqueous solutions, include the following: (a) precipitation with benzildioxime[38], polyvinylhydroxyquinoline[39] or tris(pyrrolidinedithiocarbamato)cobalt[40]; (b) adsorption on polyamine-polymer resin[41], dimethylglyoxime-impregnated polyurethane foam[42] or ion-exchange membrane filters[43]; and (c) solvent extraction following complexation with thiothenoyl trifluoroacetate[44], with a mixture of dithizone, quinolinol and acetylacetone[45] or with a mixture of dithizone and dimethylglyoxime[46].

TABLE 10.1

SEPARATION TECHNIQUES THAT HAVE BEEN USED FOR THE DETERMINATION OF NICKEL IN BIOLOGICAL MATERIALS

Modified from Sunderman[5].

Chelation reagent	Extraction reagent	Ref.
Ion-exchange resin (MK-2)	Dilute HCl	Janik and Jankowski[29]
Polydithiocarbamate resin	HNO_3, H_2SO_4	Barnes and Genna[30]
Diethyldithiocarbamate (DDC)	Isoamyl alcohol	Sunderman[31]
Dimethylglyoxime (DMG)	MIBK or CCl_4	Kincaid et al.[32]; Morgan[33]; Zachariasen et al.[16,34]
Pyrrolidinedithiocarbamate (PDC)	MIBK	Nomoto and Sunderman[25,35,36]; Andersen et al.[27]
Furildioxime (FD)	MIBK	Mikac-Devič et al.[18]
Dithizone (DTZ)	$HCCl_3$	Armannsson[37]

Resin adsorption of nickel from urine has been reported by two groups[29,30]. Following acid digestion of urine, Janik and Jankowski[29] neutralized the diges-

tion mixture and filtered it through a column of MK-2 ion-exchange resin at pH 9.5. The adsorbed nickel was quantitatively eluted with dilute hydrochloric acid. Barnes and Genna[30] passed filtered samples of acidified urine through a column of polydithiocarbamate resin. The sequestered nickel was quantitatively recovered by acid digestion of the resin. The studies of Janik and Jankowski[29] and Barnes and Genna[30] were preliminary investigations; neither report contained comparisons with nickel analyses by reference procedures or data for nickel concentrations in urine samples from non-exposed, healthy subjects.

Mikac-Devič et al.[18] tested four chelating agents [ammonium pyrrolidine dithiocarbamate (APDC), dimethylglyoxime (DMG), furildioxime (FD) and benzildioxime (BD)], for the extraction of Ni(II) from digests of urine or serum into methyl isobutyl ketone (MIBK) or *n*-butyl acetate. Maximum analytical sensitivity by electrothermal atomic-absorption spectrophotometry was achieved with FD or APDC as the chelating agent and MIBK as the extraction solvent. Ader and Stoeppler[19] used ^{63}Ni as a tracer to compare the efficiencies of nickel extraction by three chelating agents (APDC, DMG and FD). Under the same conditions that were employed Mikac-Devič et al.[18], Ader and Stoeppler[19] found that the recovery of ^{63}Ni from urine digests into MIBK averaged 99% for APDC, 89% for FD and 88% for DMG. Zachariasen, Andersen and their co-workers[16,17,27,34] evaluated DMG and APDC as chelating reagents for the determination of nickel in biological materials. They concluded[27] that APDC is the best of these chelating agents.

Extraction of Ni-APDC from digested or deproteinated serum and urine into MIBK has been performed at pH values ranging from 2.5[25] to 9.0[27]. Blanton et al.[47] reported that the efficiency of extraction of ^{63}Ni-APDC from aqueous solution into MIBK was constant from pH 1 to 8 and was independent of the nickel concentration up to 100 µg/l. Jenne and Ball[48] found that Ni-APDC extracted from water at pH >4 was stable in MIBK for at least 15 h at room temperature and up to 1 week at 4°C. Ader and Stoeppler[19] observed time-dependent decomposition of Ni-APDC in MIBK at pH 2.6; they advised that quantitation of nickel by atomic-absorption spectrophotometry be performed within 1 h after extraction of Ni-APDC into MIBK. Sunderman[5] noted that decomposition of Ni-APDC in MIBK is more pronounced in extracts of urine or serum than in extracts of aqueous standard solutions, and is greater at acidic than at neutral pH. Quantitative recovery of nickel added to urine was achieved when extraction of Ni-APDC into MIBK was performed at pH 7.0-7.5; low recoveries were obtained when the extraction was performed at pH 2.5-3.0 or 5.0-5.5[5]. Based on these findings, the present author recommends extraction of nickel as the Ni-APDC complex into MIBK at pH 7.0-7.5.

10.4 ANALYTICAL METHODS

10.4.1 Spectrophotometry and high-performance liquid chromatography

In Table 10.2 are listed the molar absorptivities of color reagents that have been employed for spectrophotometric measurements of nickel. Furildioxime was selected by the U.S. National Bureau of Standards (NBS) as the preferred reagent for spectrophotometric determinations of nickel in biological materials[60]. Extraction of nickel furildioximate into chloroform is selective, as interferences by iron and aluminium can be prevented by addition of citrate or tartrate to the reaction mixture, and as interference by cobalt and copper can be minimized by backwashing the chloroform extract with ammonia. The NBS method for the determination of nickel in reference samples of biological origin (e.g., orchard leaves and bovine liver) involves the following steps: (a) wet ashing with nitric and perchloric acids; (b) addition of ammonium citrate and furildioxime; (c) extraction of nickel furildioximate into chloroform and backwashing with dilute ammonia solution; and (d) spectrophotometry of the chloroform extract at 435 nm. The NBS spectrophotometric method is insufficiently sensitive for clinical applications, as the samples must contain 0.5-4 µg of nickel[60].

Sunderman[31] employed dimethylglyoxime as the extraction reagent and diethyldithiocarbamate as the spectrophotometric reagent in a nickel assay that involved the following steps: (a) wet ashing of biological materials with nitric acid, sulphuric acid and hydrogen peroxide; (b) separation of nickel from interfering metals by chloroform extraction of nickel dimethylglyoximate in citrate buffer at pH 8.5; (c) back-extraction of nickel with hydrochloric acid; (d) chelation of nickel as the diethyldithiocarbamate complex; and (e) extraction into isoamyl alcohol at pH 8.5. The absorbance of nickel bisdiethyldithiocarbamate was measured at 325 nm. This method was suitable when concentrations of nickel in serum or urine exceeded 10 µg/l; it was insufficiently sensitive to measure accurately the concentrations of nickel that exist in the serum or urine of healthy, non-exposed subjects. Uden and Walthers[58], Liska and co-workers[61-63] and Saitoh and Suzuki[64] used high-performance liquid chromatography (HPLC) with spectrophotometric detectors to separate and quantitate nickel complexes in aqueous solutions. Uden and Walthers[58] separated nickel and copper complexes of N,N'-ethylenebis(salicylaldiimine) by HPLC on microparticulate silica with a solvent system consisting of 20% acetonitrile in methylene chloride. By means of an ultraviolet detector and a flow cell with a volume of 8 µl, they achieved a detection limit of approximately 5 ng of nickel per sample. Liska et al.[63] separated nickel bisdiethyldithiocarbamate from the corresponding complexes of Zn, Cu, Mn, Pb, Co, Cd, and Fe by HPLC on microparticulate silica with a solvent system consisting of 10%

TABLE 10.2

SPECTROPHOTOMETRIC REAGENTS FOR NICKEL DETERMINATION

Modified from Sunderman[5].

Reagent	Solvent	Absorption max. (nm)	Molar absorptivity, ε ($l\ mol^{-1}\ cm^{-1} \times 10^4$)	Ref.
Dimethylglyoxime	Chloroform	335	0.5	Kuse et al.[49]
Benzildioxime	Chloroform	406	1.1	Banks and Barnum[50]
KCN and NH_3	Water	267	1.2	Scoggins[51]
Thiothenoyltrifluoroacetone	Carbon tetrachloride	480	1.2	Muyle and Khopkar[52]
Cyclohexane-1,2-dione dioxime	Water	460	1.4	Perez et al.[53]
Furil dioxime	Chloroform	435	1.6	Bodart[54]
6-Nitroquinoxaline-2,3-dithiol	MIBK	710	2.1	Bhaskare and Jagadale[55]
3-Nitroso-4-hydroxy-5,6-benzocoumarin	Acetone	395	2.5	Kohli and Singh[56]
Thiotrifluoroacetylacetone	Chloroform	256	3.4	Barratt et al.[57]
Diethyldithiocarbamate	Isoamyl alcohol	325	3.7	Sunderman[31]
N,N-Ethylenebis(salicylaldiimine)	Methylene chloride	254	5.0	Uden and Walthers[58]
Zephiramine	Chloroform	307	5.1	Kuse et al.[49]
Crystal violet	Toluene	611	8.2	Toei et al.[59]

chloroform in cyclohexane. Saitoh and Suzuki[64] separated nickel bistheonyltrifluoroacetone [$Ni(TTA)_2$] from the corresponding complexes of Cu, Zn, Cr, Co, Al, Fe, Be, and Pd by HPLC on poly(vinyl acetate) gel with *p*-dioxane as the solvent. The absorption maximum of $Ni(TTA)_2$ was approximately 365 nm; the molar absorptivity of $Ni(TTA)_2$ was not specified. Saitoh and Suzuki[64] employed an HPLC apparatus in which all components that contacted the solvent were constructed of PTFE or Pyrex. HPLC procedures with spectrophotometric detectors have not yet achieved analytical sensitivities sufficient for the determination of nickel in body fluids; these methods, nonetheless, hold promise for future development. Lower detection limits for nickel could be achieved by use of an electrothermal atomic-absorption detector in place of the spectrophotometric detector.

10.4.2 Gas chromatography

Separation and quantitation of nickel complexes by gas chromatography of volatile chelates has been a topic of numerous investigations during the past decade (Table 10.3). The β-diketone complexes possess thermal stability and volatility which are favourable for gas chromatography; their solubility in polar organic solvents is advantageous for preliminary solvent extraction. Substitution with fluoro or thiol groups increases the sensitivity of β-diketone compounds for electron-capture detection. Substituted thiocarbamates, such as dipropyldithiocarbamate and diethyldithiocarbamate, are also attractive ligands for gas chromatographic analysis. Excellent separations of nickel from zinc and copper can be achieved; the complexes can be quantitated by use of flame-ionization or electron-capture detectors. For the determination of nickel in liver, lung and kidney, Uden et al.[67] ashed the samples in a muffle furnace and dissolved the residue in acid (0.3 g of tissue/ml). Aliquots (100 µl) were made alkaline with gaseous ammonia and 1 ml of an ethanolic solution of $H_2(enTFA_2)$ was added. After addition of 20 ml of water, $Ni(enTFA_2)$ was extracted into 1 ml of benzene. When a 1-µl sample of the benzene extract was injected on to the gas chromatographic column, the detection limit with a ^{63}Ni electron capture detector was 20 pg and that with a scandium tritide electron-capture detector was 4 pg per sample. The detection limit with the scandium tritide detector was equivalent to approximately 15 µg of nickel/kg (wet weight) of tissue. Nickel concentrations in the samples of lung, liver and kidney were not specified, but the authors noted that the results agreed with values obtained by atomic-absorption spectrophotometry. Barratt et al.[66] used a similar procedure to measure nickel in samples of instant tea and hydrogenated triglycerides, in which the nickel concentrations ranged from 4 to 13 µg/g. Gas chromatographic analyses of nickel in body fluids or excreta have not yet been reported.

TABLE 10. 3

CHELATION REAGENTS FOR DETERMINATION OF NICKEL BY GAS CHROMATOGRAPHY

Modified from Sunderman[5].

Reagent	Abbreviation	Extraction solvent	Column packing	Column temperature (°C)	Ref.
Trifluoroacetylacetone	TFA	Benzene	2% Silicone SE-30 on Chromosorb W HP	165-230	Tamura et al.[65]
Monothiotrifluoroacetyl-acetone	T-TFA	*n*-Hexane	5% Silicone E-350 on Universal B	140-170	Barratt et al.[66]
Bis(trifluoroacetylacet-one)ethylenediimine	$H_2(enTFA_2)$	Benzene	1.5% OV-101 on Chromosorb W	225	Uden et al.[67]
Bis(trifluoroacetylacet-one)ethylenediimine	$H_2(enTFA)_2$	*n*-Hexane	3% Silicone QF-1 on Varaport 30	150	Belcher et al.[68]
N,N'-Propylenebistri-fluoroacetylacetoneimine	$H_2(pnTFA_2)$	Benzene	1.5% Dexsil 200 on Chromosorb W	260	Uden et al.[69]
Bis(acetylpivalylmethane)-ethylenediimine	$H_2(enAPM)_2$	Cyclohexane	5% Silicone E-350 on Universal B	285	Belcher et al.[70]
Dipropyldithiocarbamate	DPDTC	Chloroform	1% Dexsil 300 on Chromosorb W HP	245	Gemmer-Colos and Neeb[71]
Diethyldithiocarbamate	DDC	Chloroform	OV-101 in a capillary column	190-246	Riekkola et al.[72]
Diethyldithiocarbamate	DDC	Chloroform	Mixture (1:1) of 5% OV-101 and 5% QF-1 on Gas-Chrom Q	240	Radecki and Halkiewicz[73]

10.4.3. X-Ray fluorescence and particle-induced X-ray emission

Standard techniques of X-ray fluorescence spectrophotometry are insufficiently sensitive for direct determinations of nickel in body fluids, tissues and excreta. Forssen[74] ashed human tissues in a muffle furnace and compressed 30-mg aliquots of the ash into wafers (17 mm diameter) by means of a hydraulic press. The wafers served as X-ray targets. Spectral emission lines of thirteen elements, including nickel, were scanned with a lithium fluoride detector. Forssen[74] reported that the detection limit for nickel was approximately 10 µg/g of ash; she detected nickel in only 20 of 665 tissue samples. Kessler and Mitchell[75] increased the sensitivity of X-ray fluorescence spectrophotometry by preliminary co-precipitation of trace metals with titanium in the presence of diethyldithiocarbamate. The precipitate was confined to a microdot (1.3 mm diameter) on filter disc which served as the X-ray target. Nickel was detected in amounts as small as 0.6 ng/disc, which was equivalent to a nickel concentration of approximately 2 µg/l in aqueous solution. Kessler and Mitchell[75] did not employ this procedure for measurements of nickel in biological materials.

Particle-induced X-ray emission (PIXE) spectrophotometry, which is more sensitive than X-ray fluorescence spectrophotometry, has been used for the detection and quantitation of trace metals in tissues and body fluids[76-78]. A proton beam (2-4 MeV) from a Van der Graff generator is focused magnetically on a dried sample inside a vacuum chamber. The sample is deposited as a spot (10-20 mm diameter) on a target composed of a thin organic film. The proton beam dislodges inner shell electrons from atoms in the sample: the inner shell vacancies are immediately filled by outer shell electrons. This process releases X-rays with energies characteristic of the elements from which they were derived. The intensity of X-ray emission at each specific energy level is detected by a silicon detector and is quantified with a multi-channel analyser. Quantitation of PIXE analyses is achieved by reference to internal standards, such as strontium, or by spiking the sample with known amounts of the element to be analysed[76-79]. Several investigators[80-83] have attempted direct measurements of nickel concentrations in human serum by the PIXE technique; the analytical sensitivities were insufficient to detect nickel. For example, Barrette et al.[82] found that the detection limit for nickel in serum by PIXE analysis was 19 µg/l. Barrette et al.[82] and Campbell[79] concluded that ashing and pre-concentration were required for quantitation of serum nickel by the PIXE technique. Chen et al.[84] used PIXE for measurements of nickel concentrations in tissue specimens obtained at autopsy from patients who had died of Legionnaires's disease and from control patients. Samples (0.1-0.5 g wet weight) were digested in nitric acid; 10 µl aliquots were evaporated to dryness and analysed by PIXE with a 2-MeV proton beam. The X-ray intensity at

7.472 keV was used to calculate nickel concentrations by the method of standard additions. The correlation coefficient was 0.936 between nickel concentrations obtained by Chen et al.[84] using PIXE analysis and those obtained by the present author using electrothermal atomic-absorption spectrophotometry, based on paired measurements of five samples of lung. The concentrations of nickel in lung samples from control subjects and patients with Legionnaires's disease are given in Table 10.4 to show that comparable results were obtained by the PIXE and atomic-absorption techniques.

TABLE 10.4

COMPARISON OF NICKEL DETERMINATIONS IN LUNG BY PARTICLE-INDUCED X-RAY EMISSION (PIXE) AND ELECTROTHERMAL ATOMIC-ABSORPTION SPECTROPHOTOMETRY (EAAS)

Method	Lung Ni concentration (μg/kg dry wt.)*	
	Control subjects	Legionnaires's disease
PIXE**	132 ± 99 (9)	1170 ± 1110 (9)
EAAS***	81 ± 51 (7)	790 ± 470 (5)

*Each value is mean ± S.D. The number of subjects are listed in parentheses.
**Analyses performed by Chen et al.[84].
***Analyses performed by the present author.

10.4.4 Neutron and charged particle activation analysis

Neutron activation analysis of nickel in biological materials is limited by the relative insensitivity of this technique. Lux and Zeisler[85] employed activation analysis using reactor irradiation and γ-spectrometry with a Ge(Li) well-type detector for measurements of trace metals in human connective tissue samples. The detection limit for nickel was 0.5 μg/g (wet weight). Lux and Zeisler[85] did not detect nickel in normal connective tissue, but they did demonstrate nickel in connective tissue samples near nickel-containing metal implants. Swanson and Truesdale[86] used neutron activation for the determination of nickel and other metals in human lenses that had been lyophilized after quenching in liquid nitrogen. Swanson and Truesdale[86] speculated that nickel accumulation might be involved in the pathogenesis of senile cataracts, as nickel was inconstantly detected in lenses from young patients, but was present in readily measured concentrations in cataractous lenses from senile patients. Swindle and Schweikert[87]

described a procedure for the determination of nickel by charged particle activation analysis using an 88-in. cyclotron, based on the reaction $^{58}Ni(p,pn)^{57}Ni$ ($t_{\frac{1}{2}}$ = 36 h). Post-irradiation chemical separation of ^{57}Ni resulted in a detection limit for nickel of approximately 1 μg/g in inorganic reference materials. Versieck et al.[13] measured ^{58}Co produced by the reaction $^{58}Ni(p,n)^{58}Co$ ($t_{\frac{1}{2}}$ = 71 days) to study the influence of contamination from needles and scalpels on nickel concentration in human liver. Ward and Ryan[88] used the same neutron activation reaction to measure concentrations of nickel and other trace metals in NBS reference bovine liver and in human whole blood and serum. Prior to irradiation in a reactor, the samples were subjected to nitric acid digestion in PTFE bomb. Neutron activation analysis of NBS reference bovine liver yielded a nickel concentration of 0.20±0.03 μg/g (dry weight), which agreed with the reference value of 0.18±0.03 μg/g (dry weight). The nickel concentration in a pooled sample of human whole blood averaged 140±20 μg/l; the nickel concentration in a pooled sample of human serum was less than the detection limit of 30 μg/l.

10.4.5 Isotope-dilution mass spectrometry

Paulsen et al.[89], Poore et al.[90] and Kopenaal et al.[91] described techniques for the determination of nickel by isotope-dilution spark-source mass spectrophotometry. In the procedure of Moore et al.[90], the sample was spiked with stable ^{62}Ni; nickel was extracted as the dimethylglyoxime complex from an ammoniacal solution into chloroform and back-extracted from the chloroform with dilute nitric acid. Nickel was separated by cation-exchange chromatography, and the ^{58}Ni:^{62}Ni and ^{60}Ni:^{62}Ni ratios were determined by mass spectrometry with thermal ionization at 2060°C. A rhenium ribbon filament was used to reduce the nickel background. The concentration of nickel was calculated from the relative abundances of ^{58}Ni, ^{60}Ni in spiked and natural samples. The procedure of Koppenaal et al.[91] involved (a) acid pressure decomposition of the sample in a PTFE bomb, (b) electrochemical concentration of the analyte on a gold cathode and (c) isotope-dilution spark-source mass spectrometry. This method minimized sample manipulation and was relatively immune to contamination. Moore et al.[90] and Koppenaal et al.[91] applied their procedures to measurements of nickel and other trace metals in coal, coal products and fuel oil. In the method of Koppenall et al.[91], the detection limit for nickel was approximately 10 ng/g (dry weight). Isotope-dilution mass spectrometry has not yet been used for the determination of nickel concentrations in biological materials. The present author envisions that combination of isotope-dilution mass spectrometry with gas chromatographic separation of volatile nickel chelates will eventually become a definitive method for the determination of nickel in body fluids, tissues and excreta.

10.4.6 Differential pulse polarography

Conventional polarographic techniques for nickel analysis have been described by several workers[45,92-94]; these techniques have lacked sufficient sensitivity to be employed for nickel determinations in biological materials. Flora and Nieboer[95] found that the addition of dimethylglyoxime to ammoniacal tartrate or citrate buffers enhanced by a factor of 15 the sensitivity of derivative polarography of nickel at a dropping mercury electrode. This enhancement phenomenon was also noted by Vinogradova and Prokhorova[96] and Astafeva et al.[97]. By means of dimethylglyoxime-sensitized differential pulse polarography, Flora and Nieboer[95] detected nickel concentrations as low as 2-3 µg/l in buffered reaction mixtures. In a preliminary study, Nieboer et al.[98] applied the pulse polarographic procedure to measurements of nickel in human urine and blood following oxidation of organic constituents by dry ashing. Satisfactory agreement was observed between nickel analyses in body fluids by pulse polarography and by electrothermal atomic-absorption spectrophotometry.

10.4.7 Radiodisplacement and catalytic methods

German et al.[99] described a radiotracer displacement technique for the determination of nickel in aqueous solution. Nickel was isolated by diemthylglyoxime extraction and determined by the displacement reaction between Ni(II) and ^{65}ZnEDTA. ^{65}Zn, displaced from ^{65}ZnEDTA, was extracted into a dithizone-carbon tetrachloride solution and measured by γ-spectrometry. German et al.[99] demonstrated that samples containing 0.5 µg of nickel could be analysed by this technique. The radiodisplacement method has not been used for analyses of nickel in biological materials. Mealor and Townshend[100] developed a kinetic method for the determination of nickel, based on its catalytic effect on the decomposition of permanganate in alkaline solution in the presence of acetodiphosphoric acid. This reaction was employed by Hadjiioannou et al.[101] for an automated spectrophotometric reaction-rate system. Amounts of nickel in the range 0.3-2.1 µg per sample were determined, with a coefficient of variation of 2.5% and measurement times of 10-50 sec. Kurzawa and Kubaszewski[102] described a kinetic method for nickel determination based on the reaction of sodium azide with iodine. Sodium diethyldithiocarbamate catalyses this reaction; nickel diethyldithiocarbamate does not. In the presence of a stoichiometric excess of sodium diethyldithiocarbamate, the nickel concentration in the reaction mixture is inversely related to the velocity of the iodine-azide reaction. Kurzawa and Kubaszewski[102] applied the reaction system to analyses of samples containing 0.14-14 µg of nickel, including measurements of nickel concentrations in margarine and drugs.

10.4.8 Atomic-emission spectrophotometry with inductively coupled plasma excitation

From 1955 to 1964, several investigators surveyed the concentrations of trace metals in human blood and autopsy tissues by emission spectrography; measurements of nickel were frequently included in the tabulated results of these studies[103-109]. Little reliance can be placed on these measurements of nickel, as the nickel concentrations were either below or barely above the detection limits. When more sensitive atomic-absorption procedures were developed during the mid-1960s, measurements of nickel in biological materials by emission spectrography were generally abandoned. Renewed interest in atomic-emission techniques for nickel analysis has been evoked by the recent development of inductively coupled plasma atomic-emission spectrophotometry (ICP-AES)[30,110,111]. Abercrombie et al.[110] prepared biological samples by acid digestion and aspirated the dissolved residues into an inductively coupled argon plasma. Spectral emissions of 32 elements were measured with a 0.5-m Ebert polychromatro. Abercrombie et al.[110] reported determinations of nickel concentrations in orchard leaves, bovine liver, bacterial culture media and bird feathers. The approximate detection limit for nickel was 0.4 µg/g (dry weight). Haas et al.[111] described an instrument for the direct multi-element analysis of urine based on (a) ultrasonic nebulization of the sample, (b) aspiration of the sample vapour by an argon stream into a luminous plasma produced by an induction coil and (c) simultaneous detection of photoemissions at twenty wavelengths by a polychromator. Emission intensities of added amounts of reference elements compensated for variations in the efficiency of nebulization. The ICP-AES instrument lacked sufficient sensitivity to detect nickel in normal urine. Haas et al.[111] stated that the detection limit for nickel was approximately 4-9 µg/l of urine. Barnes and Genna[30] concentrated metals from urine by a factor of 125 by use of a poly(dithiocarbamate) resin. Samples of urine (250 ml) were passed through a resin column and the sequestered metals were recovered by digestion of the resin to achieve a final sample volumes of 2 ml. The sample was then aspirated into an inductively coupled plasma for determination of ten trace metals, including nickel. Barnes and Genna[30] reported that the detection limit for nickel in urine was 0.06 µg/l. This ICP-AES technique offers advantages for routine analyses of trace metal concentrations in urine specimens, as many metals can be measured simultaneously.

10.4.9 Flame atomic-absorption and atomic-fluorescence spectrophotometry

In 1960, Allan[112] employed flame atomic-absorption spectrophotometry (FAAS) for the determination of nickel in aqueous solutions. Applications of FAAS to

measurements of nickel concentrations in human body fluids, tissues and excreta were soon described by several investigators[24,25,35,36,113,114]. In the procedure of Nomoto and Sunderman[25], samples of urine (50 ml) were digested with nitric, sulphuric and perchloric acids, and samples of serum (10 ml) were deproteinized with trichloroacetic acid. Nickel was extracted as nickel bisdiethyldithiocarbamate into MIBK (3 ml); the concentration of nickel in the MIBK extract was determined by atomic-absorption spectrophotometry with an air-acetylene flame at 232 nm. This procedure achieved a detection limit for nickel of 0.1 µg/l of urine or 0.5 µg/l of serum[25]. The coefficients of variation of replicate nickel analyses in urine and serum samples were 10% and 9%, respectively[25]. The large sample requirement for serum and the protracted digestion required for the analysis of urine made this FAAS method cumbersome for routine use.

Emara et al.[115] found that nitric, sulphuric and perchloric acids caused suppression of nickel measurements at 232 nm by flame atomic-absorption spectrophotometry. Nomoto[35] observed interference by Cu (10 mg/l) and Au, Pt and Cd (2.5 mg/l) in the atomic-absorption spectrophotometry of nickel (50 µg/l) at 232 nm in an air-acetylene flame. Sunderberg[116] found that Fe, Mn, Cu and Co (2 g/l) suppressed the atomic absorption of nickel (20 mg/l) in air-acetylene flames. Sunderberg[116] reported that the interferences could be eliminated by careful adjustment of the height of the optical beam above the burner head. A reference method for the FAAS determination of nickel in foods and other organic matter was described by the Analytical Methods Committee[117], involving (a) destruction of organic substances by acid digestion, (b) extraction of nickel as the complex with ammonium pyrrolidinedithiocarbamate (APDC) into MIBK and (c) atomic-absorption spectrophotometry of nickel by aspirating the MIBK extract into an air-acetylene flame and measuring the absorbance at 232 nm. The method of the Analytical Methods Committee[117] closely resembles that described by Nomoto and Sunderman[25]. In the present author's opinion, these FAAS procedures are both suitable for the determination of nickel concentrations in foods, faeces and tissues.

The sensitivity of nickel determinations by flame atomic-fluorescence spectrophotometry (FAFS) is greater than that by flame atomic-absorption spectrophotometry (FAAS)[118-120]. For example, Matousek and Sychra[119] found that the detection limit for nickel at 232 nm by FAFS was approximately 3 µg/l, compared with 20 µg/l by FAAS with the same spectral source and spectrophotometer. By use of a tunable dye laser as the excitation source, Weeks et al.[120] obtained a detection limit for nickel of 2 µg/l by FAAS at 352.4 mm. Because even lower detection limits for nickel can be achieved by electrothermal atomic absorption, there has been little recent interest in FAAS of nickel in biological materials.

10.4.10 Electrothermal atomic-absorption spectrophotometry

When graphite electrothermal atomizers became commercially available in the early 1970s, most laboratories that were engaged in nickel analyses for clinical purposes shifted from FAAS techniques to electrothermal atomic-absorption spectrophotometry (EAAS)[16-18,26,27,34,121,122]. The EAAS technique of Mikac-Devič et al.[18] was typical of these procedures. Samples of serum or urine (1 ml) were digested with nitric, sulphuric and perchloric acids, and nickel was extracted as the furildioxime complex into MIBK (0.7 ml). Aliquots (50 µl) of the MIBK extract were pipetted into the graphite tube furnace, and the temperature programme for drying (up to 120°C), ashing (up to 950°C) and atomization (2600°C) was performed. This procedure achieved a detection limit for nickel in serum or urine of 0.4 µg/l; the coefficients of variation for analyses of nickel in serum and urine were 10% and 7%[18].

Dudas[123] studied the effects of drying parameters on the sensitivity of EAAS analysis of Ni-APDC in MIBK extracts. He noted that MIBK slowly spread laterally within the graphite tube, provided that the during cycle was delayed for 1 min after sample injection. Otherwise, boiling of MIBK caused sputtering of the sample and the analytical sensitivity and reproducibility became impaired. Fuller[124] and Findlay et al.[125] investigated losses of nickel during the pre-atomization heating period in EAAS. Fuller[124] found that heating at 750°C for 60 sec was attended by minimal loss of nickel, whereas heating at 1100°C for 30 sec caused a 10-35% loss of nickel, depending on the sample matrix. Findlay et al.[125] reported that heating at 900°C for 30 sec caused a loss of less than 5% of nickel by volatilization. Kantor et al.[126] measured the vaporization of nickel at temperatures from 1330 to 2100°C. Minimal vaporization of nickel occurred at 1330°C; the plateau of maximum atomic absorption was reached at 2000°C. Under different experimental conditions, Czobik and Matousek[127] found that the plateau of maximum atomic absorption of nickel was reached at 1710°C. Kantor et al.[126] and Czobik and Matousek[127] both reported that >50-fold excess of copper suppressed the atomic absorption of nickel at electrothermal atomization temperatures below 2000°C. Kantor et al.[126] did not observe any significant interference by copper in the EAAS of nickel at 2100°C. Mikac-Devič et al.[18] found that iron (30 mg/l) suppressed the EAAS of nickel (10 µg/l) in the furildioxime-MIBK extraction procedure. No interference was noted when iron was tested at a concentration of 10 mg/l[18]. Sutter and LeRoy[128] found that the effects of iron on the EAAS of nickel were influenced by the concentration of nitric acid in the sample. At a low concentration of nitric acid (1.5 mmol/l), addition of iron (50 mg/l) slightly increased the atomic absorption of nickel (40 µg/l), whereas at a high concentration of nitric acid (1.5 mol/l) a similar addition of iron strongly suppressed

TABLE 10.5

OUTLINE OF THE IUPAC PROVISIONAL REFERENCE METHOD FOR EAAS DETERMINATION OF NICKEL IN SERUM AND URINE

Digestion and extraction procedure (these steps are performed in a single tube)

(1) Serum or urine (2 ml) and 2 ml of HNO_3-H_2SO_4-$HClO_4$ (3:1:1) are digested in a heating block for 4.5 h.
(2) Water (3 ml) and bromophenol blue indicator (50 µl) are added; the mixture is titrated to pH 7.2 with dilute ammonia solution.
(3) 2% APDC solution (0.5 ml) and MIBK (0.7 ml) are added and mixed; the MIBK extract is removed for EAAS analysis.

Parameters for EAAS determination of Ni (in 20-µl samples of MIBK extract)

(1) *Spectrophotometer and accessories.* Perkin-Elmer Model 5000 spectrophotometer; HGA-500 graphite furnace; D_2-background corrector; pyrolytic graphite tube; optical sensing temperature regulator; automatic sampler; peak integrator. The wavelength is 232 nm; the purge gas is argon (300 ml/min).
(2) *Temperature programme.* Drying cycle: 25 sec ramp from 25 to 120°C and 10 sec plateau at 120°C. Ashing cycle: 45 sec ramp from 120 to 1040°C and 10 sec plateau at 1040°C. Atomizing cycle: 7 sec plateau to 2700°C with argon flow reduced to 10 ml/min.
(3) *Detection limit for nickel.* 5 pg injected into the graphite furnace (equivalent to 0.18 µg/l of serum or urine).

Precision data (day-to-day, derived from 21 analyses of a single urine specimen)

(1) Urine nickel concentration (mean ± S.D.) = 4.22 ± 0.33 µg/l.
(2) Range of observed values = 3.6 - 4.8 µg/l.
(3) Coefficient of variation = 7.8%.

Recovery data (derived from addition of Ni to 12 specimens of urine from healthy persons)

(1) Added nickel = 5 µg/l.
(2) Recovered nickel (mean ± S.D.) = 4.91 ± 0.17 µg/l.
(3) Nickel recovery = 98.1 ± 3.4% (range = 94 - 107%).

the atomic absorption of nickel[150]. Julshamn[129] reported that perchloric acid (1 mol/l) caused 18% suppression of the atomic absorption of nickel (1 mg/l) at 232.0 nm, as determined by EAAS at 2500°C. This inhibitory effect could be prevented by preliminary evaporation of the perchloric acid solution. Jackson and West[130] observed >15% suppression of EAAS of nickel by Cr, Be, Sn, Fe, Mg, Mn, Co, Cu, Al and Ca when these metals were present in concentrations 100 times that of nickel, based on EAAS analyses with carbon filament atomization. Interferences by these metals were reduced to an acceptable level by collimating the optical path with a small rectangular slit, so that the light beam passed immediately above the carbon filament. Grove and Sunderman[131] showed that tris(hydroxymethylamino)methane (Tris) buffer suppressed electrothermal atomic absorption of nickel in aqueous standard solutions but did not interfere in analyses of nickel in jackbean urease. Addition of Tris (2 mmol/l) to an aqueous solution of $NiNO_3$ (0.9 µmol/l) caused 57% suppression of atomic absorption of nickel at 232 nm under the instrumental conditions described by Mikac-Dević et al.[18]. Additions of Tris (2-50 mmol/l) to an aqueous solution of jackbean urease (that contained 0.3 µmol/l of protein-bound nickel) did not affect the atomic absorption of nickel under the same conditions. Grove and Sunderman[131] suggested that Tris inhibition of EAAS of free nickel ions might serve as a rapid and sensitive method to distinguish nickel that is free in solution from nickel that is tightly bound to proteins.

Inter-laboratory comparisons of analyses of nickel concentrations in body fluids have been conducted since 1976 by the Subcommittee on Environmental and Occupational Toxicology of Nickel of the International Union of Pure and Applied Chemistry (IUPAC). This ongoing programme is an international endeavour to improve the proficiency of nickel determinations in body fluids. In 1978, Adams et al.[132] published the results of the two initial inter-laboratory surveys which were undertaken by the IUPAC Nickel Subcommittee. In each survey, eight or nine urine samples were distributed to laboratories in seven nations for measurements of nickel concentrations by atomic-absorption spectrophotometry. The surveys disclosed wide discrepancies in nickel concentrations that were reported by participating laboratories[132]. Atomic-absorption procedures with preliminary oxidation and extraction steps were generally superior to direct electrothermal atomization techniques in (a) analytical sensitivity, (b) recovery of added nickel, (c) inter-laboratory precision and (d) concordance of ranking of urine samples in order of increasing nickel concentrations. Adams et al.[132] suggested that a reference procedure for the determination of nickel in biological materials would help to harmonize the discordant results of nickel determinations. The IUPAC Nickel Subcommittee sponsored an International Conference on Nickel Toxicology which was held in Kristiansand, Norway, in May 1978[133]. The working Group on

Nickel Analysis at the Kristiansand Conference agreed on a provisional reference method for the EAAS determination of nickel in serum and urine. The Working Party selected APDC as the chelating agent and MIBK as the extraction solvent. This method is currently being evaluated and modified in laboratories in the U.S.A., Canada, Great Britain, G.F.R., France, Finland, Japan and Yugoslavia, and was published in 1981[146]. The IUPAC provisional reference method for nickel determination by EAAS is outlined in Table 10.5. Illustrative data for day-to-day analytical precision and recovery of nickel are included in Table 10.5. The author considers that this EAAS procedure is the most sensitive, accurate and reliable method currently available for the determination of nickel in urine and serum.

INTERPRETATION OF ANALYTICAL RESULTS

Nickel concentrations in serum or urine specimens from healthy adult inhabitants of several regions of the world are listed in Table 10.6. The analyses were performed by atomic-absorption spectrophotometry; the subjects did not have occupational exposures to nickel compounds. Excellent agreement was observed between the nickel concentrations in serum or urine from subjects in G.F.R.,

TABLE 10.6

AAS DETERMINATIONS OF NICKEL CONCENTRATIONS IN SERUM AND URINE OF HEALTHY ADULTS WITHOUT OCCUPATIONAL EXPOSURE TO NICKEL COMPOUNDS

Modified from Sunderman[5].

Location	Serum nickel concentration (µg/l)*	Urine nickel concentration (µg/l)*	Ref.
Jülich, G.F.R.		2.6±1.2 (21)	Ader and Stoeppler[19]
Matsumoto, Japan	2.1±1.1 (24)	2.7±1.1 (73)	Nomoto[35,134]
Santiago, Spain	2.5±0.5 (5)		Gonzalez et al.[26]
Hartford, U.S.A.	2.6±1.0 (26)	2.0±0.9 (20)	McNeely et al.[135]
Sudbury, Canada	4.6±1.4 (25)	7.2±3.9 (19)	McNeely et al.[135]
Kristiansand, Norway	1.9±1.4 (57)**	4.9±4.2 (57)	Torjussen and Andersen[136]

* Each value is the mean ± S.D. The number of subjects is listed in parentheses.
**Plasma specimens.

TABLE 10.7

AAS ANALYSES OF NICKEL CONCENTRATIONS IN SPECIMENS FROM HEALTHY ADULTS WITHOUT OCCUPATIONAL EXPOSURES TO NICKEL COMPOUNDS

Modified from Bernacki et al.[12].

Specimen	No. of subjects	Nickel concentration		Units	Ref.
		Mean±S.D.	Range		
Whole blood	17	4.8±1.3	2.9-7.0	µg/l	Nomoto and Sunderman[25]
Serum	80	2.6±0.9	0.8-5.2	µg/l	Sunderman[137]
Urine	50	2.2±1.2 2.6±1.4	0.7-5.2 0.5-6.4	µg/l µg/day	Sunderman[137]
Faeces	10	14.2±2.7 258±126	10.8-18.7 80-540	µg/g (dry) µg/day	Horak and Sunderman[138]
Scalp hair	20	220±80	130-510	µg/kg	Nechay and Sunderman[139]
Arm sweat	33	52±36	7-180	µg/l	Hohnadel et al.[6]
Parotid saliva	20	2.2±1.2	0.8-4.5	µg/l	Catalanatto and Sunderman[140]
Palatine tonsils	15	140±70	30-280	µg/kg (wet)	Torjussen et al.[17]
Nasal mucosa	57	130±200		µg/kg (wet)	Tornjussen and Andersen[136]

Japan, Spain and the U.S.A.[19,26,35,134-136]. Increased nickel concentrations were found in urine and serum specimens from inhabitants of Sudbury, Canada, which is a site of large nickel deposits and nickel refineries[135]. Urine nickel concentrations in inhabitants of Kristiansand, Norway[136] were slightly higher than those in inhabitants of G.F.R., Japan and the U.S.A. This finding may possibly be related to the location of a nickel refinery in Kristiansand. Reference values for nickel concentrations in body fluids, excreta and biopsies from healthy adult persons are listed in Table 10.7, and reference values for nickel concentrations in human post-mortem tissues in Table 10.8. Nickel concentrations in urine specimens from workers in twelve occupational groups are listed in Table 10.9. The advantages and disadvantages of various specimens that can be analysed to monitor occupational exposures to nickel compounds are summarized in Table 10.10. Readers are referred to other articles and monographs for information about the metabolism[137], radiochemistry[143], toxicology[144] and carcinogenicity[145] of nickel and nickel compounds.

TABLE 10.8

AAS ANALYSES OF NICKEL CONCENTRATIONS IN POST-MORTEM TISSUES FROM ADULT SUBJECTS, RANKED ACCORDING TO AGE AT DEATH

Modified from Sunderman[5]. The results were reported by Nomoto[36] and Sunderman et al.[141]. All analyses were performed by the Nomoto and Sunderman technique[25].

Subject		Cause of death	Nickel concentration (μg/kg wet weight)				
Sex	Age (years)		Bone	Lung*	Kidney	Liver	Heart
M	18	Hanging		8		7.6	4.3
F	22	CO poisoning		10		13.2	8.3
F	40	Barbiturate poisoning		22		8.6	5.7
M	40	Amyotrophic lateral sclerosis	360	132	9.2	5.8	6.7
M	44	Stab wounds		24		5.2	6.2
F	46	Uterine cancer	270	48	14.8	7.3	5.8
M	48	Oesophageal cancer	190	81	6.9	7.1	4.4
F	49	Hepatoma	290	109	9.6	8.3	5.7
M	55	Hepatoma and cirrhosis	640	134	18.2	10.9	8.6
F	58	Oesophageal cancer		121	6.8	6.1	4.9
F	60	Tuberculous meningitis	340	104	11.0	8.8	7.2
F	72	Cholangiocarcinoma	240	221	7.7	9.6	9.3
Mean nickel concentrations:			333±147	85±65	10.5±4.1	8.2±2.3	6.4±1.6

*The correlation coefficient between the subjects's ages and lung nickel concentrations is 0.82.

10.6 SUMMARY AND CONCLUSIONS

The determination of nickel in biological materials has been reviewed, with emphasis on the following topics: (a) precautions for preventing nickel contamination during specimen collection and processing; (b) preliminary steps for oxidation or removal of organic matter; (c) concentration and separation procedures; (d) instrumental methods for quantitation of nickel; (e) reference values for nickel concentrations in human body fluids, tissues and excreta; and (f) nickel concentrations in urine specimens from workers in various occupational groups.

Electrothermal atomic-absorption spectrophotometry (EAAS) is currently the most sensitive, convenient and reliable technique for the determination of nickel in biological materials. EAAS is rivalled in sensitivity by three other tech-

TABLE 10.9

NICKEL CONCENTRATIONS IN URINE SPECIMENS FROM WORKERS IN TWELVE OCCUPATIONAL GROUPS*

Modified from Bernacki et al.[142].

Occupational	Number of subjects and sex	Description	Urine Ni concentration (μg/l)	
			Mean±S.D.	Range
Hospital workers	19(15M,4F)	Physicians, technologists and clerks	2.7±1.6	0.4-5.1
Non-exposed industrial workers	23(20M,3F)	Managers, office workers and storekeepers	3.2±2.6	0.3-8.5
Coal gasification workers	9M	Hydrogenation process workers	4.2±2.4	0.4-7.9
Buffers/polishers	7(6M,1F)	Buffing, polishing and deburring parts made of Ni alloys	4.1±3.2	0.5-9.5
External grinders	9(7M,2F)	Abrasive grinding of parts made of Ni alloys	5.4±2.4	2.1-8.8
Arc welders	10(7M,3F)	Welding of parts made of Ni alloys	6.3±4.1*	1.6-14
Bench mechanics	8(4M,4F)	Assembling and finishing parts made of Ni alloys	12.2±13.6*	1.4-41
Nickel battery workers	6(5M,1F)	Fabricating Ni-Cd or Ni-Zn storage batteries	11.7±7.7**	3.4-25
Metal sprayers	5(4M,1F)	Flame spraying Ni-containing powders on to aircraft parts	17.2±9.8**	1.4-26
Electroplaters	11M	Intermittent exposure to Ni in combined plating operations involving Ag, Cd, Cr and Ni	10.5±8.1**	1.3-30
Nickel platers	21M	Full-time work in Ni plating operations	27.5±21.2***	3.6-65
Nickel refinery workers	15M	Workers in an electrolytic nickel refinery	222±226***	8.6-813

*$p < 0.05$ vs. hospital workers, calculated by t-test.
**$p < 0.01$ vs. hospital workers, calculated by t-test.
***$p < 0.001$ vs. hospital workers, calculated by t-test.

TABLE 10.10

ADVANTAGES AND DISADVANTAGES OF VARIOUS SPECIMENS FOR NICKEL ANALYSES IN ORDER TO MONITOR OCCUPATIONAL EXPOSURE TO NICKEL COMPOUNDS

Modified from Bernacki et al.[12]

Specimen	Advantages	Disadvantages
Whole blood	Collection is convenient; centrifugation is unneccessary; Ni concentration in whole blood is higher than in serum	Venepuncture is slightly uncomfortable; heparinized blood may clot during storage; acid digestion of whole blood is time consuming; Fe may interfere in EAAS analysis
Serum or plasma	Collection, transportation and storage are convenient; analytical methods are reliable	Venepuncture is slightly uncomfortable; Ni concentration may fluctuate during workshift; Ni concentration is much lower than in urine of exposed workers
Urine	No discomfort; analytical methods are reliable; good discrimination between exposed and non-exposed workers	Dust causes contamination; 8-h collection is inconvenient; precipitate may form during storage
Parotid saliva	Minimal discomfort; transportation and storage are convenient; analytical method are reliable	Special collection equipment is required; validity as exposure index is not established
Scalp hair	No discomfort; transportation and storage are convenient; Ni concentration probably does not fluctuate rapidly	Sampling is variable; dust, soaps, hair dyes, lotions and sweat cause contamination; analytical methods are not standardized; validity as exposure index is not established
Nasal mucosa	Good correlation with chronic exposure to insoluble Ni dust	Biopsy entails discomfort and some risk of bleeding and infection; platinum biopsy utensils are advisable to avoid nickel contamination; analysis is more difficult than serum or urine

niques: differential pulse polarography, particle-induced X-ray emission spectrometry and gas chromatography with electron-capture detection. With further refinements, each of these techniques should be suitable for clinical applications. Combination of isotope-dilution mass spectrometry with the gas chromatographic separation of volatile nickel chelates may eventually become the definitive method for the determination of nickel in biological materials.

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CHAPTER 11

SELENIUM AND TELLURIUM

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CONTENTS

11.1. INTRODUCTION

There has been concern for several years over selenium as a naturally occurring toxicant, as an essential element deficient in foods and feeds of some areas, as a potential industrial hazard in certain manufacturing processes and as an environmental pollutant[1]. Because of these concerns, an extensive literature on methods of analysis for this element has accumulated. On the other hand, tellurium has not been found to be present in toxic amounts in foods and feeds or to be of biological significance, so it has received limited attention. For selenium there are very satisfactory analytical procedures applicable to a wide

variety of materials and covering a wide range of concentrations. For tellurium, methods of analysis are available, but the analytical chemistry of this element is still not well developed. Methods for these two elements will be discussed separately.

11.2. SELENIUM

11.2.1. Introduction

It is not the intent here to review in detail the extensive literature on selenium analysis; this has been done elsewhere[2-7]. Instead, literature relating to historical aspects, the methods recommended here and possible alternatives are cited.

11.2.2. History

Early methods for selenium analysis depended on its separation from other elements by distillation as the tetrabromide[8]. The separation was completed by precipitation of the selenium in its insoluble elemental form with a variety of reducing agents[9,10]. After wet digestion or ashing of the sample, the amount of the element was measured either gravimetrically or by colorimetry based on the pink colour of the finely dispersed elemental selenium[11]. As little as 10 μg of selenium could be detected. Methods such as this were used extensively during the early studies on selenium poisoning in farm animals. The principles involved are the basis for the chemical separation used with some methods of neutron activation analysis or for the gravimetric analysis of selenium compounds for the element.

In 1941, Klein[12] improved these early methods by using titration with thiosulphate and iodine based on the procedure of Norris and Fay[13] to measure down to about 1 μg of the element with reasonable accuracy. This method was fairly widely used until the late 1950s, when interest in selenium as a biologically essential element demanded more sensitivity. Iodimetric methods other than those based on the Norris and Fay procedure have been used with very limited success.

Another early method involved the development of a green-blue complex of selenium with codeine in concentrated sulphuric acid[14]. This did not give reliable quantitative results, but could be used as a qualitative test for the isolated element.

The most rapid strides in selenium analysis have been made during the past two decades as a result of improved instrumentation and the stimulus of research on the role of the element as a nutrient. The procedures now in use are based largely on the developments of that period.

11.2.3. Sample preparation

Sample collection, processing and storage methods are extremely important to the validity of the results of a selenium analysis. This is because of the uneven distribution of the element in nature and of its many chemical forms, some of which are volatile. Methods for destroying organic matter, dissolving the selenium, separating it from interfering substances and otherwise preparing for measurement of the element are as important as the measurement itself. Space will not allow for the discussion in detail of all phases of preparation, so much must be left to the good judgment of the analyst.

11.2.3.1. Sample collection, processing, and preservation

11.2.3.1.1. Animal specimens. Animals biosynthesize volatile selenium compounds and excrete them via the lungs[15]. Methods for collecting these compounds from expired air are discussed later (Section 11.2.3.1.8). Little is known about the concentrations of these volatile compounds in body fluids, tissues or excreta, so it may be best to analyse these specimens without drying. With methods of analysis that require drying the sample, this has usually been accomplished by lyophilization. However, either thermal (60°C) or microwave drying has been reported to be satisfactory for liver, fish or lucerne tissues[16].

Different animal tissues contain widely differing concentrations of selenium[15], and there may be variation within an organ. In addition, blood cells usually contain a higher concentration of the element than does the plasma. These variations make it necessary to take special care in sampling these materials.

Storage methods that preclude significant decomposition of samples are required to prevent loss of selenium. For a period of a few days storage at 0-4°C is adequate, but for longer periods samples should be frozen. Dried samples can be stored at room temperature for long periods without measurable loss of selenium.

11.2.3.1.2. Plant specimens. The species, stage of growth, part of the plant and a variety of soil conditions are all factors in determining the selenium content of plants[15]. Proper sampling techniques require concern for all of these. In most instances, plants may be dried at 70°C without measurable selenium loss, but some plants, notably certain of the *Astragalus* species, contain appreciable amounts of volatile selenium compounds. For the best results, these plants require analysis without drying.

During handling and grinding dry plant material, there is a tendency for the leafy parts to segregate from the fibrous parts[17]. This may affect the analytical results. It can be avoided to some extent by pulverizing in a ball mill.

Undried plant material stores for long periods in a frozen state without measurable selenium loss. Most dried samples store well at room temperature.

11.2.3.1.3. Microorganisms. Some microorganisms are known to produce volatile forms of selenium[18]. It is best, therefore, to analyse microbial samples without drying.

11.2.3.1.4. Feeds. Most of what has been said here about plant tissues applies to feeds. There are additional matters to be considered, however. For instance, when selenium is added as a supplement to a feed, it is often not evenly distributed and great care must be used to obtain a representative sample. Ball-milling to a fine powder helps to distribute the selenium. When the method of analysis used allows pre-digestion with subsampling (see Section 11.2.3.2.3), it is good practice. Liquid feeds require special sampling care because of the tendency for some insolubles containing selenium to settle out. Bentonites are often added to feeds and, as they can cause analytical errors, their presence may require modifications in some methods[19].

11.2.3.1.5. Foods. Foods may be processed and stored much like plant and animal tissues or fluids. Desiccation of eggs with acetone[20] also removes fat without significantly affecting selenium content. The tendency for selenium to distribute unequally between yolk and white in eggs needs some consideration[21]. Most foods are probably best analysed without drying.

11.2.3.1.6. Soils and rocks. Soils may vary widely in their selenium contents over short distances, and there may also be large differences due to depth of sampling. Because of the latter, sampling to plough depth only is a questionable practice[7]. Indeed, the analysis of soil for total selenium may be of doubtful value, as the results do not reflect the capacity of the soil to provide selenium to plants. Measuring the water-soluble or isotopically exchangeable selenium may offer a better measure of this capacity[22].

Soils may contain some volatile selenium[23], but the amount is apparently very small and its loss on drying should have no measurable effect on selenium content. Both soils and rock can be stored air-dried for many years without loss of the element.

11.2.3.1.7. Waters. The precautions normally observed in the sampling of water for other analyses should also be observed when sampling for selenium determination. In addition, if total selenium (soluble plus suspended) is to be measured, the sample should be stored at 0-4°C and analysed within a few weeks. If soluble selenium only is to be measured, waters with sediments should be filtered immediately after collection, preferably through a 0.45-μm membrane. Adding 1% concentrated nitric acid to the filtered water precludes loss of selenium by microbial action[24] or by coprecipitation with iron(III) hydroxide that

may form in waters of high iron content[25]. Treated thus, waters can be stored at room temperature over long periods without selenium loss.

11.2.3.1.8. Air. Both volatile and particulate selenium may be present in air, which introduces some sampling problems[26]. Dry filters have been used successfully in studies on particulate selenium. A variety of liquid trapping agents have been used for collecting volatile selenium from plants, animals, microorganisms or the atmosphere. These agents include water, soda-lime or bromine-hydrobromic acid mixtures[26]; mercury(II) chloride, or mercury(II) oxide in nitric acid[27]; concentrated or 8 *N* nitric acid[28]; and a variety of other materials[29].

Solid materials have also been used for trapping volatile selenium compounds[30-34]. In some instances, gas chromatographic columns are used, so that the adsorbed selenium compounds can later be identified as well as measured by volatilizing them from the column in an appropriate instrument.

11.2.3.2. Preparation for measurement of selenium

Methods for measuring selenium may be divided into two general classes. One requires the destruction of the sample, while the other does not. For non-destructive methods, samples need only be dried and ground before measurement of the selenium. Most methods, however, require destruction of the organic matter prior to its measurement. A variety of techniques have been used to accomplish this, as discussed below.

11.2.3.2.1. Combustion. The open combustion of samples has been used for selenium analysis, usually with the addition of some fixative to reduce the loss of the element. This has not been generally found to be a suitable technique. Low-temperature ashing with excited oxygen has also been tried without success[3]. On the other hand, combustion in the Schöniger flask has given excellent results[17], although it is somewhat inconvenient and therefore not widely used.

11.2.3.2.2. Fusion. Sodium carbonate, sodium peroxide and Parr bomb fusions have all been used for selenium analysis with some success, especially with rocks and soils. However, these techniques are cumbersome and do not work well with wet samples, so their use has been very limited[3].

11.2.3.2.3. Wet digestion. The most widely used technique for destroying organic matter and freeing the selenium in samples has been some type of wet digestion. A number of acid mixtures have been used for this, most including nitric and perchloric acids. Several additives for speeding the digestion have also been used, including hydrogen peroxide, molybdenum, vanadium and persulphate[7]. Wet digestion gives excellent recoveries when properly handled, can be used for a wide variety of materials with or without drying and is adaptable

to a number of procedures for measuring the selenium[3]. It has been adapted to the routine analysis of large numbers of blood samples[35].

When the method of analysis uses wet digestion, some materials that are difficult to sample may be treated by a simple dissolution in acid to overcome this problem. This consists of weighing a larger than usual sample (5-25 g of dry matter) into a flask and adding 10 volumes of concentrated nitric acid. The mixture is heated to dissolution (usually 20-30 min), made up to volume and subsampled for analysis. Fats and oils rise to the top. These contain essentially no selenium, so they can be avoided in subsampling, making the remainder of the digestion faster. The nitric acid solutions can be stored for long periods at room temperature without loss of selenium[7]. This technique has been found especially useful in analysing certain foods, materials of high selenium content and feed supplements or premixes where salts of the element have been added in dry form.

11.2.3.3. Concentration of selenium and separation from interfering substances

When samples contain very small amounts of the element, most methods for selenium measurement require its concentration. In addition, some methods require its separation from substances that might interfere in the measurement process. There are a variety of techniques that can be used, some of which serve both to concentrate and to separate.

11.2.3.3.1. Evaporation. As a rule, waters contain very small amounts of selenium, so most methods of measurement require some kind of concentration. Except in unusual situations, the element seems to occur almost entirely as the selenite or the selenate. Thus, by adjusting the pH to above 8.0 with sodium hydroxide and evaporating the solution to a small volume or to dryness, the selenium is retained[7]. The same cannot be said for urine or other body fluids, as they contain organic forms of the element which might decompose to volatile compounds.

11.2.3.3.2. Distillation. For many years, distillation of the selenium from digests as the tetrabromide and its subsequent reduction to the insoluble element was widely used as a means of isolating and concentrating it for analysis. This method has the disadvantages of requiring working with bromine, of being time consuming and of lacking sensitivity. It is useful, however, in neutron activation analysis where chemical separation is employed[7].

Selenium may also be distilled as hydrogen selenide. This technique is used in atomic-absorption spectrometry. Dry distillation of the element has been used in conjunction with neutron activation analysis[36].

11.2.3.3.3. Precipitation and coprecipitation. In addition to its precipitation as the element, selenium can be precipitated in chemical combination with

several heavy metals or as insoluble organic compounds[5]. It can be coprecipitated with arsenic, tellurium, iron(III) hydroxide or other heavy metals[6]. None of these methods are now in wide use.

11.2.3.3.4. Ion-exchange treatment. Cation-exchange resins have been used to remove metals when they are present at concentrations above which chelating agents are effective[37]. Anion-exchange resins have been used to remove selenite and selenate from solution, the amounts of each being determined by their elution with graded concentrations of hydrochloric acid[38]. Other uses of ion-exchange resins in selenium analysis have been reviewed by Nazarenko and Ermakov[6].

11.2.3.3.5. Solvent extraction. The extraction of selenium by tributyl phosphate, several organic solvents or amines, or as chelates or ternary complexes has been reviewed[6]. The only widely used procedure of this type is the reaction of selenite with an *o*-diamine to give the piazselenol, which is extracted with decahydronaphthalene or cyclohexane. Heavy metal interferences are avoided by the addition of ethylenediaminetetraacetic acid, fluoride, oxalate or some other sequestering agent[3].

11.2.3.3.6. Other methods. Paper chromatography, the ring oven technique, thin-layer chromatography and gas chromatography have also been used to separate selenium from interfering substances[3,4].

11.2.4. Measurement of selenium

Selenium is measured quantitatively by a wide range of methods. The method actually used will depend on the type of samples, the equipment available, the number of analyses to be made, the sensitivity and accuracy required, the need to preserve the sample, the cost, other analyses to be made and other factors. No one method can be considered that of choice for all situations. A few seem most widely used, most readily adaptable to a wide variety of samples and most thoroughly studied, and these will be discussed in some detail. Others are merely mentioned with references in Table 11.1. Among them are some that might well be preferred in certain laboratories.

11.2.4.1. Neutron activation analysis

A number of types of activation analysis have been used for measuring selenium[3]. Of these, thermal neutron activation is the most common. This produces several radionuclides that have been used in subsequent measurement, as follows: ^{81}Se, half-life 18.6 min; ^{81m}Se, half-life 57 min; ^{77m}Se, half-life 17.5 sec; and ^{75}Se, half-life 128 days.

TABLE 11.1

SOME LESS WIDELY USED METHODS FOR SELENIUM DETERMINATION

Method	Refs.	Method	Refs.
Gravimetric	9,11,37,39	Anodic-stripping voltammetry	62
Volumetric (macro)	40,41	Cathodic-stripping voltammetry	63,64
Volumetric (semi-micro and micro)	12,42,43	Spark-source mass spectrometric	65
		X-ray fluorescence	66,67
Colorimetric	9,44-46	Proton-induced X-ray emission	68,69
Spectrophotometric	47-54	Direct injection enthalpimetry	70
Catalytic	55,56	Gas chromatographic	71-74
Isotope dilution	57,58	Inductively coupled plasma emission	75
Coulometric	59		
Polarographic	60,61	High-performance liquid chromatography	76

Neutron activation analysis can be used either with or without destruction of the sample. More recently, the non-destructive techniques have usually been based on counting the ^{77m}Se nuclide[77-80]. Destructive methods are most commonly based on counting the ^{75}Se nuclide[81-86]. While the non-destructive methods lend themselves more readily to multi-element analysis[87-89], destructive methods can also be used for this, and they also permit chemical separation of the selenium to provide for greater reliability[2-5,7].

The most serious drawback to neutron activation analysis is the cost of the equipment required. Thus, relatively few laboratories are in a position to use the technique. Those which are usually develop their own specific methodology based on what is being analysed, the number of samples, the precision required, the equipment available and other considerations.

11.2.4.2. Fluorimetric analysis

The discovery that 3,3'-diaminobenzidine formed a coloured complex with selenium[90] led to a spectrophotometric method based on its use[91]. Extraction of the complex with an organic solvent improved the technique[51], and investigation of its fluorescent properties[92] led to a number of fluorimetric methods based on the used of this reagent[93-96]. When 2,3-diaminonaphthalene was found to give more sensitivity and convenience[97], this reagent was soon incorporated into fluorimetric methods for measuring selenium[17,37,98]. Following this early work, a number of fluorimetric methods based on the use of 2,3-diaminonaphthalene have been published, and the reliability of the technique has been thoroughly studied[99-107]. These methods have been found to be sensitive, reliable and accurate. They do not require highly sophisticated equipment and the cost of the analysis

is reasonable. The methods adapt readily to a wide variety of samples, wet or dry. Therefore, a procedure based on fluorimetry is presented in detail later (see Section 11.2.5). A number of procedures essentially the same and equally as good as that described appear in the literature.

11.2.4.3. Atomic-absorption analysis

Atomic-absorption analysis offers three separate techniques for the determination of selenium: direct flame atomization, heated graphite furnace atomization and hydrogen selenide generation with flame atomization[108-115]. Atomic absorption has frequently been hailed as a comparatively interference-free technique. However, this is justified only for direct flame atomization and even then interferences do exist. When sufficiently high concentrations of selenium are being measured, direct flame atomization can give satisfactory precision, accuracy and freedom from interferences. The limitation to this technique is its lack of sufficient sensitivity to determine selenium accurately in most biological materials. The most sensitive atomic line for selenium is at 196 nm, where a sensitivity of 0.5 μg/ml can be achieved with a hydrogen-argon air-entrained flame or 0.7 μg/ml with an air-acetylene flame[116]. This short wavelength introduces additional problems due to absorption by the atmosphere. The hydrogen-argon air-entrained flame is preferable owing to its greater transparency. Heated graphite furnace atomization and hydrogen selenide generation offer improved sensitivity. Ihnat[108] reported a detection limit of 0.13 ng/ml for hydrogen selenide generation and 10 ng/ml for the graphite furnace. The hydrogen selenide generation method is recommended for the analysis of most biological samples. Automated systems of analysis for submicrogram amounts of selenium by hydrogen selenide generation have been reported[112,117-119]. Hilderbrand and Dillon[120] have developed a scanning method for the analysis of blood for selenium using a multi-step furnace process. The results compare well with those obtained by fluorimetric analysis. The method is described in detail later (see Section 11.2.6).

11.2.5. Method for fluorimetric analysis

11.2.5.1. Reagents

NH_2OH-EDTA solution. Dissolve 9 g of ethylenediaminetetraacetic acid dihydrate and 25 g of hydroxylammonium chloride in water and dilute to 1 l.

Cresol red indicator. Dissolve 0.05 g of *o*-cresolsulphonphthalein in 5 ml of water plus 1 drop of concentrated NH_4OH and dilute to 250 ml with water.

Standard selenium solution. Dissolve 40 mg of elemental selenium in 2 ml of concentrated nitric acid by warming gently. Dilute to 1 l with 0.1 *N* hydrochloric acid.

This solution is stable for many months at room temperature. At about biweekly intervals, dilute this solution 1:100 with 0.1 *N* hydrochloric acid and use it as a working standard. 1 ml = 0.4 µg of Se.

DAN solution. Stir 200 mg of 2,3-diaminonaphthalene with a few drops of 0.1 *N* hydrochloric acid to wet the reagent thoroughly. Add 200 ml of 0.1 *N* hydrochloric acid and heat for 15 min in a water-bath at 50°C. Transfer into a 500-ml separatoring funnel, extract twice with 15 ml of cyclohexane and discard the extract. Filter the hydrochloric acid solution through a wetted filter-paper and use immediately or store in a cold, dark place under a 2 mm layer of cyclohexane. Note: the blank determination should not exceed the equivalent of 0.025 µg of selenium. If it does, the 2,3-diaminonaphthalene should be recrystallized.

11.2.5.2. Apparatus

Micro-Kjeldahl digestion unit. The digestion unit should be provided with an all-glass fume duct attached to a glass aspirator and should be used in a perchloric acid fume hood. Rubber connections should be avoided.

Fluorimeter. The fluorimeter should irradiate the sample at 369 nm and measure the fluoresced light at 525 nm.

Water-baths. A 50°C and a boiling water-bath are needed.

11.2.5.3. Procedure

Weigh not more than 1.5 g (dry basis) of sample prepared as described earlier (Section 11.2.3.1) and containing not more than 0.5 µg of selenium into a 30-ml micro-Kjeldahl flask. (For samples with higher selenium contents, digestion, reduction, dilution to a suitable volume and subsampling, or the pre-digestion procedure described in Section 11.2.3.2.3 should be used.) Add a glass bead and 10 ml of concentrated nitric acid and place the flask on the digester. Heat at low heat, watching for excessive foaming. After the initial vigorous reaction, remove and add 2 ml of 70% perchloric acid. Continue to heat until the appearance of white perchloric acid fumes or until the solution darkens. In the latter case, immediately remove the flask and cool it for a few seconds. Add about 1 ml of nitric acid and continue heating to perchloric acid fumes, repeating the nitric acid addition if necessary. Continue to heat for at least 15 min (30 min for urine) beyond perchloric acid fumes and then remove and cool to room temperature. Add 2.0 ml of 10% (v/v) hydrochloric acid and heat for 30 min in a vigorously boiling water-bath.

After cooling, add 5.ml of the NH_2OH-EDTA solution, 2 drops of the cresol red indicator and 1:1 ammonia solution to a yellow colour of the indicator. Immediately add 10% hydrochloric acid to a pink colour. Treat a blank containing 2 ml of perchloric acid similarly.

Add 5 ml of the DAN solution to each flask and water to a few millilitres below the base of the flask neck. Swirl to mix and place in the 50°C water-bath for 30 min. Remove and cool to about room temperature.

Transfer the solution into a separating funnel and shake vigorously for at least 1 min with 10.0 ml of cyclohexane. Discard the water layer and wash the cyclohexane layer by shaking for 1 min with 25 ml of 0.1 *N* hydrochloric acid. Discard the hydrochloric acid layer and centrifuge the cyclohexane layer briefly to remove water droplets. Read blank, standard and samples in the fluorimeter, correct the readings for the blank, and calculate the selenium content as follows:

$$\text{Se } (\mu g/g) = \frac{0.4 \times \text{corrected sample reading}}{\text{corrected standard reading} \times \text{sample weight (g)}}$$

If the sample contains more than about 5 µg of selenium repeat the analysis with a smaller sample. Simply diluting the cyclohexane extract gives false results.

11.2.5.4. Comments

The results obtained with this method compare very favourably with those obtained by other procedures. It gives good recoveries of added selenium[103,105,106] and the digestion procedure causes no appreciable loss of the element under a variety of conditions[3,105]. The method is sensitive to about 0.02 µg of selenium. Analysis of standard samples gave the results in Table 11.2[106].

TABLE 11.2

RESULTS FOR ANALYSIS OF STANDARD SAMPLES

Sample	Se content (µg/g)	
	Claimed	Found
Bowen's kale	0.148 ± 0.014	0.136 ± 0.002
NBS bovine liver (SRM 1577)	1.1 ± 0.05	1.17 ± 0.06
NBS Orchard leaves (SRM 1571)	0.08 ± 0.005	0.089 ± 0.003

The results in Table 11.2 and other[105] data attest to the repeatability of the method. A single determination can be made in about 3.5 h, but with adequate equipment a single analyst can perform 18 analyses per day.

Automated[121-124] and simplified[35,125] fluorimetric methods have been developed in laboratories where large numbers of samples are routinely handled.

Perhaps the most common error made in this type of selenium determination is the failure to digest the sample adequately. This is often the result of the

feeling that too much digestion will cause a loss of selenium. Actually, the loss of selenium during wet digestion has been over-emphasized.

A qualitative test capable of detecting as little as 10-20 µg of selenium can easily be made on the perchloric acid digest following reduction with hydrochloric acid in the boiling water-bath. Addition of 5 ml of water, 5 ml of 48% hydrobromic acid and about 50 mg of sodium sulphite and boiling will precipitate pink elemental selenium, easily seen on filtering through a pad of asbestos.

A simplified spectrophotometric method based on this fluorimetric method has been developed for use where less sensitivity is required or even desirable, or where a fluorimeter is not available[53]. This procedure is the same as that described above, except that 10 ml of DAN reagent is used, 20 µg of selenium standard are used, the piazselenol is extracted by shaking the solution after removal from the 50°C water-bath and cooling to room temperature with the cyclohexane directly in the digestion flask. Enough of the cyclohexane extract is removed to read it in a spectrophotometer at 378 nm. This method is sensitive to about 1 µg of selenium.

11.2.6. Atomic-absorption method for selenium in blood

11.2.6.1. Reagents

Triton X-100. Prepare a 0.1% solution in distilled water.

Selenium standard solution. Oissolve 0.100 g elemental selenium in 5 ml of nitric acid with warming. Dilute to 1 l with water. Prepare dilutions containing 0.10 and 0.20 µg/ml of selenium.

11.2.6.2. Apparatus

Atomic-absorption spectrophotometer. This should be equipped with background corrector, recorder readout and selenium electrodeless discharge lamp.

Heated graphite furnace. This should be capable of multiple drying, charring and atomization step programming.

11.2.6.3. Procedure

Unclotted blood samples are diluted 1:1 with 0.1% Triton X-100. An atomic-absorption instrument with heated graphite furnace and deuterium background compensation is used for the analysis. The instrument is adjusted to the 196-nm line of the selenium electrodeless discharge lamp. A 0.7-nm slitwidth and background correction are used.

A 20-µl volume of sample, 10 µl of standard and 20 µl of 1000 ppm nickel solution are introduced into the graphite tube. The standards used contain 0.0, 0.1 and 0.2 µg/ml of selenium. The graphite furnace is programmed to follow the heating procedure sequence listed in Table 11.3.

TABLE 11.3

PROGRAMME FOR GRAPHITE FURNACE

Step	Temperature (°C)	Ramp time (sec)	Hold time (sec)
Dry-1	100	30	15
Dry-2	110	20	10
Dry-3	120	10	5
Char-1	300	20	10
Char-2	500	10	20
Char-3	1200	20	10
Atomize	2400	1	7

11.2.6.4. Comments

The maximum sample absorbance during the atomization step is determined and used to plot the standard additions curve. Sample are analysed in duplicate. Samples analysed by this method and the fluorimetric technique reported above gave an average variation of ±6.8%.

Methods using hydrogen selenide generation have also been described[108,109,126] and improved equipment for this generation is available[127].

11.2.7. Interpretation of results

Considerable judgment must be used in drawing conclusions from selenium analyses. It should be recognized that there will be differences due to the tissue or specimen examined and to the species. In toxicosis, both acute and chronic forms must be taken into account. Finally, very low values may reflect a selenium deficiency.

It is not possible to state definite guidelines for use in the interpretation of results. The following is offered as a suggestion only, and it must be applied with caution after consideration of clinical signs or other evidence.

It appears that values falling within the limits shown in Table 11.4 suggest no problem from either an excess or a deficiency of selenium. Values below or above these ranges do not necessarily reflect a deficiency or an excess of the element. However, the greater the deviation of results from these values the greater the possibility of a deficiency or a toxicosis.

TABLE 11.4

LIMITS OF SELENIUM CONCENTRATIONS FOR NO EXCESS OR DEFICIENCY

Sample analysed	Range of selenium concentrations (g/g or g/ml on an undried basis)
Feeds or foods	0.1 - 2.0
Blood	0.1 - 0.6
Plasma (or serum)	0.05 - 0.4
Liver	0.1 - 3.0
Kidney	0.1 - 3.0
Muscle	0.03 - 0.70
Hair	0.1 - 3.0
Urine	0.01 - 0.20 (10-300 g per 24 h)

11.3. TELLURIUM

11.3.1. Introduction

The state of development of the analytical chemistry of tellurium presents a marked contrast to that of selenium. Rather than being able to select the most appropriate method for an individual laboratory from a series of adequate methods, as one can do for selenium, a method which satisfactorily estimates the concentration of tellurium in biological materials is yet to be developed. Several methods have been used for determining tellurium concentrations in spiked biological samples and in tissues from animals exposed to very high dietary or respiratory levels of the element. However, data on normal or baseline concentrations in biological samples are very scanty because of the difficulty in measuring the low levels encountered. Blood levels of 0.25 ng/ml of tellurium were reported by Van Montfort et al.[128]. A study on trace metals in the hair of Tokyo residents yielded a mean tellurium concentration of nearly 1 µg/g with a range of 0.79-47.4 µg/g[129].

11.3.2. History

The general analytical chemistry of tellurium has been reviewed by Green and Turley[130] and more recently by Nazarenko and Ermakov[6]. The analysis of organic compounds and organic materials for tellurium was reviewed by Masson[131]. Low concentrations of the element have been determined by atomic-absorption spectrophotometry of the sol formed on reduction of tellurite with tin(II) chloride[132] or thioacetamide[133]. These procedures are useful for tellurium concentrations of a few parts per million. A tellurium-bismuthiol II complex in chloroform was

used by Cheng and Gaydish[134] for the analysis of non-biological materials in the 0.1-0.5 ppm range. In general, however, these methods do not have the sensitivity required for biological samples unless they are used in conjunction with extensive pre-concentration. Most of the recent research on tellurium analysis has used atomic absorption as the mechanism for quantitation with some emphasis on neutron activation, polarography, spark-source mass spectrometry and emission spectroscopy. In each instance, preliminary sample digestion and concentration are necessary for meaningful data.

11.3.3. Sampling

The same procedures and precautions used for selenium (see Section 11.2.3) must be observed in collecting, processing and preserving samples for tellurium assay. The size of the sample used for analysis is normally much larger than for most trace element work because of the low concentrations encountered. Hanson[132] used 200 ml of urine in his analysis. Cheng and Agnew[135] dissolved the ash of 2.0 g of liver tissue in a final volume of 3 ml of solution for their analysis. The limiting factors for the size of sample are the amount available and the maximum degree of enrichment consistent with quantitative recovery.

11.3.3.1. Sample preparation

The preparation of biological samples for tellurium analysis has three objectives: destruction of organic material, concentration enrichment and removal of interfering species. The last two steps can frequently be achieved simultaneously.

11.3.3.1.1. Destruction of organic matter. Destruction of organic material is satisfactorily achieved by wet digestion of the sample. Fiorino et al.[136] obtained 100 ± 7% recovery of added tellurium using a ternary acid digestion (nitric, perchloric and sulphuric acids) where the solution was heated until the evolution of strong fumes of sulphurtrioxide. Cheng and Agnew[135] reported recoveries of 80-133% using a nitric acid-perchloric acid digestion of liver samples with added tellurium. Hanson[132] achieved a 100 ± 7% recovery of the added element from urine subjected to wet digestion. The above recoveries are for the entire analytical process from digestion to measurement. Thus, most of the deviation in the recoveries might well be attributed to aspects of the procedure other than the digestion step.

Electronically excited oxygen has been used to ash animal tissues prior to analysis for tellurium[128,137]. Samples were freeze-dried prior to their introduction into the low-temperature ashing apparatus. Destruction of up to 6 g of animal tissue organic matter was achieved in 20-30 h at temperatures not exceed-

ing 190°C. Recoveries of 99-107% were obtained. The ashed samples were dissolved in 1 ml of aqua regia. The primary advantages of this ashing process are its low temperature, reduced susceptibility to reagent contamination, safety and absence of the need for monitoring during the ashing process.

Either wet digestion or low-temperature ashing provides an acceptable mechanism for the destruction of the organic material in biological samples. Dry ashing at high temperatures should be avoided owing to the volatility of many tellurium salts and resultant loss expected during the ashing process.

11.3.3.1.2. Concentration enrichment and separation from interferences. The sensitivities reported with atomic-absorption[138], neutron activation[129,139], emission spectrographic[140], polarographic[141,142] and spark-source mass spectrometric[143] techniques all indicate that sample enrichment will be necessary for normal tissue samples.

Tellurium can be concentrated by use of a variety of precipitation and extraction techniques. Examples of both processes will be discussed.

Bedrossion[144] described an extraction procedure for separating tellurium from other elements in steel. A 0.1-2.0-g sample was dissolved in 6 *N* hydrochloric acid and hydrogen peroxide was added to oxidize the tellurium(II) to tellurium(IV). The tellurium was extracted quantitatively with 5% trioctylphosphine in methyl isobutyl ketone. The method may be applicable to biological samples after some minor modifications.

Hanson[132] extracted tellurium from wet-digested urine samples as the iodide complex. As much as 200 ml of urine was digested. The acidity of the digest was adjusted to 1.0 *N*, iodide was added to 0.6 *N* and the tellurium was extracted into an *n*-pentanol-diethyl ether organic phase. The diethyl ether served to decrease emulsion problems without affecting the efficiency of extraction. After evaporating the organic solvent to dryness, the residue was dissolved in 20% hydrochloric acid. Few other elements are extracted by this procedure, providing an efficient separation and also a 50-100-fold enrichment. The process is quantitative. Work in the authors' laboratory evaluated the application of this procedure to other types of samples[138].

Coprecipitation on an arsenic carrier has been used to separate tellurium from interferences prior to atomic-absorption analysis and also to enrich the concentration[145]. Added arsenic was reduced by the addition of hypophosphorous acid to a flocculent precipitate which carried both selenium and tellurium with it. The tellurium was quantitatively removed in the 1-100-μg range by 1800 μg of arsenic and an 8-h settling period. The precipitate was dissolved in a small amount of nitric acid.

Aeremae and Assarsson[146] precipitated tellurium, following reduction by hydrazinium chloride, as a coprecipitate with aluminium hydroxide. A 20-fold excess of aluminium hydroxide was required to achieve >90% precipitation of the tellurium.

11.3.4. Measurement of tellurium

The method of choice for tellurium determination depends on the purpose of the analysis. A number of different procedures can be used to detect concentrations in tissues and biological fluids from species exposed to very high levels of the element. However, none of these methods has the sensitivity to detect it in tissues or fluids from species exposed only to normal levels. The methods that can be used for toxicity and screening studies will be discussed individually and possible extensions to normal concentration analysis will be evaluated.

11.3.4.1. Atomic-absorption analysis

Most of the recent research on methods development has used atomic absorption for the quantitation steps. Lockwood and Limtiaco[137] used flame atomic absorption to determine the tellurium levels in fourteen different tissues from rats administered oral or intraperitoneal doses of the element. They found concentrations ranging from less than 4 to 190 ppm. The procedure was sensitive to 0.5 µg/ml in the test solution and had an average relative error of 2.22%. The method seems relatively rapid, in terms of instrument time, but useful only when very high exposure levels are of interest.

Cheng and Agnew[135] achieved improved sensitivity by using a microsampling boat attachment with the atomic-absorption measurement. They reported a detection limit of approximately 0.03 µg/ml in the sample solutions for liver homogenates. However, the precision of the boat technique is inherently poor owing to severe matrix effects[147], so this procedure is useful for screen purposes only.

Flameless atomic absorption spectrophotometry offers the best sensitivity of the atomic-absorption techniques. Chao et al.[148] used it to determine tellurium at 4-200 ppb levels in geological samples. There is, however, an absence of literature on the technique for tellurium in biological samples. Work in the authors' laboratory[138] indicates a sensitivity of 0.2 ng/ml in the test solution. The method is subject to numerous interference effects and requires a separation step prior to the actual measurement. Corn samples have been analysed for tellurium by an adaptation of this method, briefly described as follows. A 10-g sample is digested with ternary acid in a 100-ml micro-Kjeldahl flask. The digest is neutralized with 1:1 ammonia solution and extracted by the method of Hanson[132],

described in Section 11.3.3.1.2. The organic solvent extract is evaporated nearly to dryness and is subsequently digested with hydrogen peroxide and then with nitric acid to the disappearance of the last traces of oily residue. The solution is diluted to 10.0 ml for atomic-absorption measurement. The graphite furnace is operated at a drying temperature of 125°C a charring temperature of 500°C, and an atomizing temperature of 2700°C. The flow of argon is interrupted during the atomizing step in order to enhance the sensitivity of the method. The 214.3-nm atomic line is used for the analysis. Preliminary data suggest a detection limit of less than 1 ng/g and a recovery of 99% with a coefficient of variation of 16%.

Tellurium has been determined by generation of the hydride with sodium borohydride-sodium hydroxide solution. The hydride was carried directly into the atomic-absorption spectrophotometer flame. A nitrogen-diluted hydrogen flame was used, the hydrogen sweeping the hydride into the system. Selenium, arsenic and antimony can be determined by this procedure. This method has been applied to foods and to biological samples digested with ternary acid. Sample sizes of 1-5 g were used. Standards containing 0.1-0.7 μg of tellurium gave a linear Beer's low plot up to 0.4 μg. The instrumental detection limit was 11 ng, corresponding to 5-10 ppb in the original tissue. None of the element was detected in any of the untreated samples, while a recovery of 100 ± 7% of added tellurium was obtained. This procedure can be partially automated for routine analyses.

11.3.4.2. Other methods

Hanson[132] determined the recovery of tellurium from urine samples that had been spiked with tellurium. His analysis was based on the absorbance of the sol formed following reduction of the element with tin(II) chloride. The sensitivity of the method was about 0.1 ppm for 1% absorbance, which corresponds to 5 ppb in the original urine. He reported poor reproducibility, however, at concentrations below 50 ng/ml in the urine.

Volaire et al.[141] reported a polarographic method for the determination of tellurium. The supporting electrolyte was 1.0 *M* perchloric acid for the reduction $Te^{0} \rightarrow Te^{-2}$. The technique is capable of detecting the element in the ppb range. The standard additions technique was used to overcome interferences by selenium, arsenic and copper.

A sensitivity of about 10 ppb has been reported for neutron activation analysis of geological samples for tellurium[139]. The procedure used, however, would need considerable alteration for use with biological samples. Pre-concentration and separation steps might be most helpful here. Neutron activation analysis has also been used in the determination of tellurium in hair[129].

Paulsen[143] reported that spark-source mass spectrometry has a sensitivity of a few ppb for tellurium. However, the report included no details of the method used.

Kinser and Keenan[140] determined microgram amounts of tellurium in biological tissues with an emission spectrographic method. Thallium was used as the internal standard. The element was quantitated by arc emission based on the intensity of the image of the tellurium doublet at 238.33 and 238.58 nm on a photographic plate. No pre-concentration step was used. The relative standard deviation for 0.1-10 μg amounts was 10.6%, and the mean recoveries of the element added to liver or blood were 94 and 88%, respectively. The method lacks sensitivity, so it is useful only for survey studies where high levels of the element are of interest. However, the use of inductively coupled plasmas in atomic emission appears promising. Barnes and Genna[149] measured the tellurium content of urine by this method after pre-concentration on poly(thiocarbamate) resin and claimed a detection limit of about 0.4 ng/ml.

11.3.5. Method for tellurium determination

A procedure for the determination of tellurium in biological samples containing normal concentrations of the element is not available. Atomic-absorption analysis can be used, however, for tissues that contain abnormally large amounts. Known procedures for tellurium can be enhanced with respect to sensitivity by using an enrichment procedure. The method described below uses the enrichment procedure of Hanson[132] and measures the element by a procedure similar to that of Lockwood and Limtiaco[137].

11.3.5.1. Reagents

Perchloric acid, 70%.

Ammonia solution. Dilute concentrated ammonia solution with an equal volume of water.

Sodium iodide crystals.

Hydriodic acid, 1 *N* solution in water.

Tin(II) chloride. Dissolve 10 g of $SnCl_2 \cdot 2H_2O$ in 20% (v/v) hydrochloric acid.

Tellurium standard solution. Dissolve 0.0625 g of tellurium dioxide and 2 g of sodium hydroxide in water and dilute to 500 ml (100 μg/ml of tellurium). Make appropriate dilutions for use in preparing the calibration graph.

11.2.5.2. Apparatus

Micro-Kjeldahl digestion apparatus; as for selenium determination.

Atomic-absorption spectrometer. This should be equipped with a background corrector and a tellurium hollow-cathode lamp.

11.3.5.3. Procedure

Urine samples should be enriched as follows. Transfer 200 ml into a 400-ml beaker, add 50 ml of concentrated nitric acid, and evaporate with gentle boiling to 15 ml. Transfer into a 100-ml micro-Kjeldahl flask, add 5 ml of perchloric acid and digest as described for selenium determination by the fluorimetric method. Transfer the digest into a 125-ml separating funnel with water and neutralize to phenophthalein indicator with the ammonia solution. Add concentrated hydrochloric acid dropwise until any white precipitate that is present redissolves and then rinse the flask with 6 ml of the acid, adding it to the separating funnel and washing with water to a volume of about 70 ml. Add 6.6 g of solid sodium iodide and shake with 20 ml of *n*-amyl alcohol for 30 sec. Extract the aqueous phase twice more with *n*-amyl alcohol. Extract the individual alcohol fractions with 15 ml of the hydriodic acid solution, combine them and extract with 20 ml of *n*-amyl alcohol. Evaporate the combined alcohol extracts to 15-20 ml and transfer them into a 100-ml micro-Kjeldahl flask with 40 ml of water. Add 5 ml of 30% hydrogen peroxide. Evaporate until the alcohol is removed (the samples should not be allowed to go to dryness). Cool, add 10 ml of concentrated nitric acid and 2 ml of perchloric acid, and heat on the digestion rack to the appearance of perchloric acid fumes. Cool and dilute to 25 ml with water.

For other materials, the digestion procedure described for selenium is used, diluting the digest to 25 ml with water.

The samples are ready for atomic-absorption analysis with either flame or flameless atomization. For flame analysis, standard solutions containing 0, 2, 4, 6 and 8 μg/ml of tellurium and a blank solution should be analysed with the samples. The standards are used to construct a calibration graph. The wavelength used is the 214.3-nm tellurium line. For flameless atomization, standard solutions containing 0, 0.02, 0.04, 0.06 and 0.08 μg/ml of tellurium are used. A 50-μl volume of the standard, sample or blank is introduced into the furnace for analysis. The tellurium content of the original sample is calculated by the following equation for either method of analysis:

$$\text{Te (g/g)} = \frac{\text{g/ml of Te from calibration graph x final volume of solution}}{\text{g or ml of sample used}}$$

11.3.5.4. Comments

Flameless atomic-absorption spectrophotometry is sensitive to about 1 ng of tellurium injected. For 200 ml of urine, this corresponds to 0.25 ng/ml of tellurium. For tissues, 5 ng of tellurium per gram of tissue can be detected if 10 g of the tissues are used. Flame analysis is 100 times less sensitive but has better precision and should be used if higher concentrations are of interest. Flameless analysis for tellurium should be considered as a qualitative or rough quantitative method only.

11.3.6. Interpretation of results

At present, no procedure has been developed that is sensitive enough to measure normal or baseline concentrations of tellurium in biological samples with reasonable reliability. Most of the analyses reported have been made on samples with added tellurium or from sources exposed to abnormally high levels of the element. Hence the interpretation of the results of tellurium analyses is difficult.

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