THE GENERAL PHARMACOLOGY OF THE HEAVY METALS[1]

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I. INTRODUCTION

The present article on the general pharmacology of heavy metals is devoted to a discussion of predominantly theoretical aspects of metal poisoning. Following Heubner (43), van Leeuwen (60), Zunz (130), and especially Clark (16), the term "general pharmacology" has been chosen to denote that branch of pharmacology in which special emphasis is laid upon attempts to explain in biochemical and physico-chemical terms the mode by which chemical agents alter the functions of living cells.

The term "heavy metals,"[2] although not rigidly defined, is generally held to refer to "those metals with a density greater than five," about forty elements in all. A few of these metals are physiologically important as trace elements. Most of them, including many of the trace elements, exert toxic actions if present in higher concentrations. Since the definition of the term "heavy metals" is based on an arbitrary physical parameter and not on a common chemical property, it is not surprising that the pharmacological actions of each metal exhibit specific features and that the toxicology of each metal poses its own special problems. It may, therefore, be asked what type of useful generalizations can be drawn which bear on the pharmacological actions of most of the members of the group.

Heavy metals are capable of combining with a wide variety of organic molecules. Because of their interactions with ligands present in all proteins, they are particularly potent enzyme inhibitors. Indeed, a listing of the enzyme systems or other biologically important molecules known to be inactivated by heavy metals would include all of the essential chemical components of the cell. This does not imply that all heavy metal ions bind with equal strength to any given ligand. On the contrary, they differ considerably in their chemical reactivity. Nevertheless, as a group, they combine more firmly than do the lighter metals which predominate in living systems. Thus, the heavy metals may be characterized as a group of toxic agents possessing universal reactivity yet individual specificity.

Any further generalizations about the action of heavy metals as toxic substances depend on biological factors, the chemical composition, and the structural as well as the functional organization of cells. In living systems the large numbers of reactive substances compete for traces of the heavy metal. Each chemical constituent has a certain significance in relation to cellular function, but the relative importance of the individual substances in maintaining a specific function varies greatly. Consequently, heavy metal binding will occur simultaneously at "sensitive" and "insensitive" sites, and the toxic action may be produced by only a very small proportion of the total metal fixed; in other words, the diversion of the metals to insensitive sites protects the cell to some

[2] The principles concerning the heavy metals can also be applied to toxicologically important lighter elements, such as Be^{++} and Li^{+}.

extent against the toxic action of the metals, but at the same time tends to obscure the relation between metal binding and pharmacological response. It obviously imposes the most fundamental difficulty on the interpretation of quantitative data concerning heavy metal poisoning and especially on efforts to identify the chemical nature of the interaction with the sensitive ligands.

Cellular structure governs the accessibility of sensitive ligands and decisively influences the time course of heavy metal action. The cell membrane as a diffusion barrier protects the cell interior from the poisonous action of the metal. On the other hand, sensitive ligands located within the membrane structure or on the outer cell surface are separated from the large reservoir of protective complex-forming substances inside the cell. Consequently, functions associated with the cell membrane are particularly susceptible to the action of heavy metals.

On a time scale, the sequence of events must always be from the outside of the cell toward the center. The first reactions of the metal are with ligands of the cell surface, with associated disturbances of membrane function. Thereafter, a redistribution of the metal may take place. As the metal penetrates into the cell, additional effects develop and the initially inhibited functions may begin to recover.

In attempting to trace mechanisms of action, it is most important to study the primary rather than the terminal responses, since the inactivation of one sensitive site by a metal usually induces a whole sequence of secondary changes which may ultimately affect the physiological state of the whole cell. Important as these secondary effects may be for the survival of the cell, it is not possible to deduce much information concerning the mode of action of a particular metal from their study, since they may follow the action of almost all nonspecific toxic agents.

In considering the actions of metals on organ systems and on the intact animal, all of the generalizations concerning cells are still valid, but additional complications must be taken into account. In the organs, several cell populations of different susceptibilities may exist in a complex anatomical arrangement. In animals, the effective concentration of metal at the cellular sites of damage and the time of exposure are determined by the patterns of absorption, distribution, deposition, and excretion.

Although the chain of events between the initial chemical insult and the ultimate physiological response may seem most complicated and tangled, yet the same set of biological factors, *viz.*, the chemical, structural, and functional organization, determines the main course of events following heavy metal poisoning. In the present review, it is hoped to develop a group of generalizations concerning these factors, the group as a whole to provide a framework into which diverse kinds of information on heavy metals can be fitted. No attempt has been made to give a comprehensive and encyclopedic survey considering all of the available knowledge. This would be a task of great magnitude.[3] Instead, a small

[3] In the literature there exists a large amount of information about the general pharmacology of heavy metals, based mostly on studies with those few metals which have found

number of selected experiments concerned with a few representative heavy metals will be presented in some detail. Examples have been selected primarily to illustrate certain of the factors governing the interactions between heavy metals and cellular ligands and the metal-induced changes in the physiological state of the cells or animals. The progression will be from the simplest level of organization to the most complex—from molecules to enzymes, to cells, to organs, to animals. The interactions of heavy metals with simple molecules, proteins, and enzymes have been extensively reviewed elsewhere (*v.i.*), and will be only summarized here for purposes of continuity. In the case of enzymes emphasis will be placed not on the actions of specific metals on specific enzymes, but rather on the different ways in which metals can influence enzyme activities. The interactions of metals with cells will be next considered. This will be the most extensive section of the review; firstly, because most of the concepts that can be drawn concerning the general pharmacology of the metals are related to their actions on cells, and secondly, because the topic has not recently been reviewed. The order of presentation will again be from the simplest case to the most complex. Finally, because much less material is available, shorter and less detailed sections will be devoted to the mechanisms of heavy metal interaction at the organ and whole animal levels.

II. CHEMICAL INTERACTIONS BETWEEN HEAVY METAL IONS AND BIOCHEMICAL SUBSTANCES

During the last decade, much progress has been made in the understanding of metal interactions with amino acids, proteins, and other molecules of biological interest (see reviews 30, 38, 56, 109). In fact, such a tremendous amount of information has been accumulated that a discussion of these reactions within the framework of this article will of necessity seem superficial to the chemist and discouragingly complex to the general reader. However, it is not possible to give an adequate survey of pharmacological actions without referring to chemical reactions. Moreover, the chemical behavior of heavy metals allows certain important predictions to be made regarding their mechanism of action. This short chemical section is, therefore, limited strictly to a presentation of some basic information concerning the specificity of metal interactions.

Most heavy metals are capable of forming complexes with ligands containing

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pharmacological uses or have posed large-scale toxicological problems (Hg, Pb, Ag, U). In the case of lead alone, a list of publications appeared in 1922, in which two thousand references were cited (14). A review on the pharmacology of lead in 1934 stated that ten thousand references could be found (32). The data have been contributed by workers in many different fields of the biological and medical sciences and have been obtained on widely different premises. Many of them are to be found in papers dealing with clinical aspects of heavy metal poisoning or with problems of industrial hygiene. Unfortunately, most of the relevant evidence contained in these sources is fragmentary from the point of view of this article. Of greater usefulness are studies of the pharmacologists concerned with the mode of drug action, of the toxicologists who tried to track down the biochemical basis of toxic actions, and of the biochemists and physiologists who have made use of the inhibitory capacity of metals as research tools for the exploration of normal cellular functions.

sulfur, nitrogen, or oxygen as electron donors. The role of sulfur in heavy metal binding is well known and requires no special comment. Nitrogen is the preferred donor in the formation of coordination compounds. Oxygen rarely forms coordination complexes but when present in such dissociable groups as carboxyl and phosphoryl forms strong ionic bonds with heavy metals.

In any living cell, at least the following ligands can be expected to be present:

$$-OH,\ -COOH,\ -PO_3H_2,\ -SH,\ -NH_2,\ -\text{imidazole}.$$

These ligands, which form integral parts of almost any molecule of biological significance, are frequently essential to the normal functioning of the cells.

The knowledge of the relative affinities of heavy metal ions for each type of ligand should permit some predictions concerning the sites of heavy metal action on living cells. Certain rules regarding the complex-forming capacities of these atomic groupings have been established. For example, heavy metals are bound by amines or simple amino acids in the following order of decreasing affinity (38):

$$Hg^{++} > Cu^{++} > Ni^{++} > Pb^{++} > Zn^{++} > Co^{++} > Cd^{++} > Mn^{++} > Mg^{++} > Ca^{++} > Ba^{++},$$

whereas the affinity towards $-COO^-$ groups may be represented by the following series:

$$Cu^{++} > Ni^{++} > Zn^{++} > Co^{++} > Mn^{++} > Mg^{++} > Ca^{++} > Ba^{++}.$$

Precise data about the association constants for the reaction between heavy metals and sulfhydryl groups of organic molecules are not available. However, as an approximate measure, Klotz (57) presented a list of the solubility product constants for various inorganic sulfides:

$$Hg^{++} > Ag^{++} > Pb^{++} > Cd^{++} > Zn^{++} > Ca^{++} > Mg^{++}.$$

Two additional points deserve comment. Firstly, the hydroxyl group of water can participate in complex formation and in the formation of insoluble hydroxides. These complexes may have a very complicated structure and their electrochemical behavior may be quite different from the behavior of simply hydrated ions. Chloride ions, which are always present in biological materials, also form strong complexes with mercury (110), and perhaps with other heavy metals. Consequently, the first complexity in pharmacological studies arises immediately after dissolving the heavy metal in water. Secondly, all the listed biological ligands contain dissociable protons. Heavy metal ions replace these protons in complex formation. Bjerrum (13) demonstrated a simple quantitative relationship between the association constants of hydrogen and heavy metal ions. As a consequence, competition between hydrogen and heavy metal ions must be taken into account.

From the foregoing discussion, the following conclusion can be drawn: heavy metal binding by biological materials is strong but not specific with regard to either the ligands or the heavy metal. The only rules that can be established are

concerned with order of affinities. Addition of increasing amounts of a heavy metal to a solution containing a mixture of molecules with different types of ligands results in successive binding in order of decreasing affinity. The same applies if different classes of ligands are joined together in a single macromolecule. However, not only the affinities but also the relative concentrations of the ligands influence the metal distribution. Thus, even if approximate predictions can be made about the order of affinities, it is not possible to predict the distribution in a living system because of the unknown concentration ratios of different types of ligands.

Heavy metal interaction with organic molecules introduces further and more serious complications. Chelation may introduce specificity patterns that invalidate the lists of relative affinities (70), and catalytic actions of heavy metal ions may lead to the decomposition of organic compounds (6, 116).

Many metabolites like amino acids or dicarboxylic acids are capable of forming chelates. Chelate formation generally leads to a dramatic increase of association constants. Since chelating agents may exhibit high specificity toward metal cations, the rules regarding orders of affinities of heavy metals for ligands can be applied to biological systems only with the utmost caution. For example, if a metal of high affinity toward sulfhydryl groups produces a pharmacological effect, it is not permissible to ascribe the effect to the inactivation of sulfhydryl groups. The observations must be supplemented by studying the effects of additional sulfhydryl reagents (15). The arrangement of ligands within a single macromolecule may also favor chelate formation. For example, chelates of high specificity play an important role in the activation of enzymes by metals (69). Mathematical treatments of metal binding by macromolecules containing chelating sites have been presented in the literature (38, 107).

Heavy metal ions frequently exhibit catalytic activities (6, 116). The pioneer investigations of Warburg on iron-catalyzed oxidations are well known to every biologist (123). Copper catalyzes the oxidation of substances like ascorbic acid or, even more important, the oxidation of sulfhydryl groups and formation of disulfide bridges (38). Several metal ions, especially rare earth metals, can catalyze the hydrolysis of phosphate esters. Recently, a whole review was devoted to this topic (6). It is noteworthy that these catalytic actions proceed with appreciable velocities under physiological conditions. It has been suggested that some pharmacological actions of rare earth metals are explained by a catalytic breakdown of ATP and other phosphate esters within the living cells.

III. THE ACTION OF METALS ON ENZYME SYSTEMS

Most heavy metals interfere with the action of enzymes and other proteins of functional significance. In these cases, function is modified and it becomes meaningful to use the term "poisoning." The toxic action will appear as a reduction in the rate of utilization of substrate. For this reason we must consider the interaction of the metal with the total system which contains all the factors necessary for optimal catalysis, including enzyme, substrate, cofactors, and activators. The binding of metals to any single component of the total enzyme system may affect the over-all function.

A complete analysis of enzyme-metal interactions should include an investigation of the chemistry of metal-binding as well as a study of changes of enzyme activity, and should establish the relationship between the two parameters. Chemical measurements require relatively large amounts of pure enzymes. Functional investigations, on the other hand, can be carried out with traces of the enzyme, sometimes even with relatively impure enzyme preparations. For this reason, most information on the mechanism of metal inactivation of enzymes is derived from studies of inhibition kinetics. The kinetics of enzyme inhibition (2, 71) and the chemical interactions between metals and proteins (56) have been discussed in detail elsewhere. Only a very brief outline will be given here.

A. Inhibition kinetics

Present concepts of enzyme kinetics are based on the assumption (Michaelis-Menten) that the rate of substrate utilization is proportional to the concentration of an enzyme-substrate complex. This complex is formed by mass-law governed reactions between enzyme, coenzyme, substrates, and activators. In the light of this theory, chemical interactions between the heavy metal and any one of the compounds participating in the formation of the enzyme-substrate complex or any intermediary product of the stepwise formation of this complex (including the final complex itself) should alter the rate of substrate consumption. By applying the mass-law, equations can be derived which predict the effects of metal fixation by the various participants of the enzyme reaction.

Analysis of the inhibitory effects may become very difficult in systems containing many metal-binding components. The nature, as well as the degree, of inhibition depends on the relative concentrations of all participants in the reaction. For example, the hexokinase reaction involves the enzyme, two substrates (glucose and ATP), an activating metal (Mg^{++}), and two products (glucose-6-phosphate and ADP). If UO_2^{++} is added to this system, the kinetic pattern almost defies analysis. The uranyl ion combines with ATP, ADP, with hexokinase, and hexokinase-ATP complex, and competes with varying effectiveness with Mg^{++} (45).

The study of inhibition kinetics alone gives only presumptive information on the mode of inhibitory action since, due to extraneous factors, different over-all kinetics may be observed in chemical reactions with the same underlying mechanisms. For example, glucose and fructose compete with each other for the fermentative system of yeast. Yet the kinetics of inhibition of fermentation by UO_2^{++} are entirely different for the two sugars: competitive for fructose and non-competitive for glucose (46). This paradox results from the fact that UO_2^{++} forms a dissociable complex with fructose but not with glucose. An excess of fructose can, therefore, protect the metabolic system by "soaking up" the available UO_2^{++}. The competition kinetics therefore arise from competition between fructose and the metabolic system for UO_2^{++}, and not between fructose and UO_2^{++} for the active center of the enzyme.

Despite the difficulties of interpretation, the study of inhibition kinetics has often provided useful information concerning the probable nature of the actions of heavy metals at the molecular level.

B. Metal interactions with various components of enzyme systems

Metals are potentially able to combine with all of the components of an enzyme system. Chemical knowledge of these interactions is drawn not only from direct studies on enzyme systems but also by inference from studies of the binding of metals to proteins. The following brief summary largely follows the outline presented by Klotz (56).

The possibilities of metal interactions with the enzyme or with the enzyme-coenzyme-substrate complex, are manifold because of the presence of many reactive ligands. The resulting reduction in enzyme activity depends on the accessibility and on the functional significance of the various metal-binding groups. Of primary importance are metal-interactions with those ligands which participate in bond formation with the components of the enzyme-substrate complex (substrates, coenzymes, activators) at the active center of the enzyme. Of somewhat lesser importance are changes brought about through metal-combination with groups linked to the active center (for a discussion of the significance of "linked-functions" see (30)). Even the metal-binding ligands with no functional significance play an indirect role. By combining with metals, they reduce the amount available for reaction with functional ligands, thereby affording protective action.

A good illustration of the factors outlined above is the inactivation of urease by silver and mercury, metals possessing an exceptionally high affinity for sulfhydryl groups. Sumner and Myrbäck (114) found that 90% inhibition of urease activity resulted when fifty atoms of mercury were attached to one molecule of urease. Silver proved to be even more potent, for only fifteen atoms per urease molecule produced 95% inhibition (28). Obviously a large fraction of the atoms of mercury bound to the enzyme is associated with ligands which play no role in enzymatic catalysis. This conclusion is supported by the finding that, of the five sulfhydryl groups which participate in the heavy metal binding (42), not all are of importance to the enzymic activity. A thorough discussion of the situation led Dounce and Lan (28) to the conclusion that one or a few "masked" (*i.e.*, not easily accessible) SH-groups constitute the active center of the enzyme. Thus, in urease inhibition by SH-seeking metals, all the features mentioned above play a role, *viz.*, chemical affinities, functional importance of ligands, diversion to "insensitive" sites, and accessibility of "sensitive" ligands.

The SH-groups of the active center of the urease are part of the protein structure of the enzyme molecule. Frequently, however, the active center is located on a prosthetic group which is more or less firmly attached to the protein moiety of the enzyme. In cytochrome C, the iron porphyrin is linked to the protein by two thiol-ether bridges. These bridges may be broken by a variety of metals—Ag^{+}, Hg^{++}, Pb^{++}, Cu^{++}, Cd^{++} (89). High concentrations of these metals of the order of 10^{-3} M are usually required. It seems unlikely, therefore, that such mechanisms will play a role in heavy metal poisoning of cells, as the toxic concentration of metals is usually much lower.

In many cases, the active center of an enzyme is functional only when an appropriate metal-activator is present. According to current theories (for

recent reviews see 64, 69, 80), the activating metal usually combines reversibly with the enzyme. In many cases, activating metals can be displaced by other metal species which have less or no activating power and thus inhibit the enzyme. This type of competition between activating and non-activating metals is fairly common and deserves no further comment.

Inhibitory effects of heavy metals may also result from interactions with ligands which are not directly involved in the active center of the enzyme. The binding of metal cations to the side-chain residues of the protein may result in a change in electrostatic charge and a shift in the ionization constant of the active center, leading to changes in the catalytic activity (56). Also, the state of aggregation of an enzyme may be affected. The activating effect of Ca^{++} and the inhibitory effect of relatively high concentrations of Cu^{++} or Hg^{++} on α-chymotrypsin have been attributed to changes in the polymeric forms of the enzyme (36).

A second type of indirect action may be a consequence of structural changes in the protein. For example, in the presence of mercury, oxygen uptake by hemoglobin increases, particularly at low O_2-pressures (94). The metal is not attached to the heme-ring but probably exerts its influence by changing the structure of the globin moiety of the hemoglobin molecule.

The formation of a complex between heavy metal ions and a substrate molecule might well produce a compound unacceptable for enzymic catalysis. In general, however, one would expect the substrate concentration to be much higher than that of the heavy metal, so that little inhibitory action would be observed. Indeed it seems likely that the substrate would act as a protective agent, preventing interactions of the heavy metal with the enzyme. On the other hand, coenzymes such as ATP may be present in limiting concentrations. A reduction in its concentration by combination with a heavy metal could limit the rate of an enzymic reaction.

C. Relations between the chemical properties of heavy metals and enzyme inhibition

In the previous discussion of enzyme inhibition by heavy metals, the importance of the chemical nature and of the functional significance of metal-binding ligands was emphasized. This approach was adopted because it is difficult, if not impossible, to classify these interactions in terms of the physicochemical properties of the heavy metals. This point is well illustrated by a comparison of the actions of silver and mercury on invertase.

Both silver and mercury form very stable mercaptides with thiol groups, and do so in preference to reactions with other ligand groups which may be present (38). Thus, one would expect silver ions to inhibit invertase in the same way as mercury compounds, *i.e.*, by combination with the sulfhydryl groups of the enzyme (77). However, a comparison of the inhibition of yeast invertase by silver with that by mercury reveals several dissimilarities (78). The inhibition by silver is non-competitive with the substrate and dependent strongly upon pH; inhibition by mercury is competitive and independent of pH. These facts

exclude the assumption that silver and mercury are bound by the same active groups of the enzyme protein. Indeed, evidence has been presented to the effect that silver is bound by imidazole, and mercury by sulfhydryl groups (77, 78).

IV. INTERACTIONS WITH SURFACE FILMS

Cell membranes contain large quantities of lipids, mainly phosphatides. It is well known that very small amounts of heavy metals produce appreciable changes of surface tension and surface charge of lipid films (1). Alterations of these variables may be expected to lead to marked changes of permeability and metabolic activities of surface enzymes. For example, it has been shown that the activity of phospholipases depends on the surface tension of the phosphatide droplets which serve as substrate (7).

V. ACTION OF METALS ON CELLS

The universal reactivity of the metals and the ubiquity of metal-binding ligands lead one to expect that biological factors, especially those governing the accessibility of metal sensitive loci, rather than the chemical affinities of metals towards the various cellular receptors play a predominant role in metal-toxicology. The cell membrane is the first and most important site of action of metals. Frequently, almost all of the metal applied is rapidly absorbed by the easily accessible ligands of the outer surface of the membrane. The interior of the cell, on the other hand, is protected by the membrane as a diffusion barrier and also by the many inert substances in the cytoplasm that can react with and divert the metal. Fortunately, the same factor of geographic location, which directly exposes the membrane to chemical insult by metals, also facilitates experimental analysis of normal and disturbed membrane function in the intact cell. Knowledge concerning disturbances in the interior of the cell is less complete and much less susceptible to analysis.

Many of the metal-binding ligands are essential to the maintenance of the membrane as a diffusion barrier or are necessary for the functioning of the enzymes of the membrane. The latter play an important role in the digestion of external substrates, in active transport phenomena, and in the synthesis of membrane constituents; they can be expected to occur in all cells, although their presence has been demonstrated in only a few types of cells (*e.g.*, yeast, bacteria, plant root cells, erythrocytes, cells of intestinal and renal epithelia, and muscle cells (97). The general pharmacology of heavy metals is, therefore, largely concerned with pathological changes of functions associated with the cell membrane (100).

Of the several possible organizations of the available experimental material, the one used here is based on a classification in terms of the responses. The effects associated with the cell membrane are differentiated from those associated with the interior of the cell; the responses to which mass-law equations apply are distinguished from those which can be described in terms of population statistics (all-or-none responses); and finally, time-dependent sequences of effects are discussed. As pointed out in the introduction, no attempt is made to

include all available data. Rather, specific examples are described in some detail.

A. Graded responses of the cell membrane

1. The binding of metals—Mn^{++}, UO_2^{++}, and Hg^{++} interaction with the yeast cell membrane. The presence of ionized ligands on the outer surfaces of cells has been established by studies of the electrophoretic behavior of cells (8), the clumping of cells (49), and the binding of metals and cationic dyes (100). However, these observations are qualitative and, without a knowledge of the affinity constants and the distribution of the ligands on the cell membrane, it is impossible to make any quantitative predictions from these data.

The yeast cell lends itself to quantitative studies of cation-binding because the membrane is, under certain conditions, virtually impermeable to ions (98). The interactions on the membrane are, therefore, obscured neither by penetration of the cations into the interior of the cell, nor by the leakage out of the cell of soluble metal-binding substances. An instructive example of the type of information obtainable under such conditions is provided by studies with Mn^{++} (labeled with Mn^{54}) (102). Within a few seconds after the addition of the metal (as $MnCl_2$) it combines reversibly with complexing sites on the outer surface of the cell. The binding data can be evaluated quantitatively by assuming that the distribution of Mn^{++} is governed by the mass-law. In the simplest case, if only one type of Mn^{++}-binding ligand is involved and if all stoichiometrical ratios are unity, the mass-law assumes the following form:

$$\frac{(Mn^{++})(Y^{-})}{(MnY)} = K \tag{1}$$

where (Mn^{++}) is the concentration of free Mn^{++}, (Y^{-}) is the concentration of binding sites, (MnY) is the concentration of bound Mn^{++}, and K is the apparent dissociation constant. Equation 1 can be converted into the more practical form:

$$\frac{(MnY)}{(Mn^{++})} = \frac{Y_t}{K} - \frac{(MnY)}{K} \tag{2}$$

where Y_t is the total number of binding sites. If the ratio of bound to free, $(MnY)/(Mn^{++})$, is plotted against bound, (MnY), a straight line should result with a slope of $1/K$ and an intercept of Y_t/K. The experimental data can be fitted by two straight lines the slopes of which presumably represent two species of binding sites for which Mn^{++} has different affinities. The total concentration of the sites with the higher affinity (the steeper slope) is 1×10^{-3} mol per kg of cells, or about 1×10^{7} sites per cell. The parameters of the shallow slope cannot be accurately determined. The concentration of sites is approximately 5×10^{-3} mol per kg of cells.

The chemical nature of the two species of binding ligands has been established principally by studies with UO_2^{++} (101, 104, 105). As in the case of Mn^{++},

binding of UO_2^{++} can be described by a simple mass-law relationship of the form of equation 1. The apparent dissociation constant, K, is 3 to 4 $\times$ 10^{-7} and the total number of binding sites, the same as for Mn^{++}, 1 $\times$ 10^{-3} mol per kg of cells. Uranyl ion forms very stable complexes with substances containing carboxyl groups and especially with those containing phosphoryl groups, but not with sulfhydryl or imidazole groups (28). The UO_2^{++}-binding by the cells was, therefore, compared with that of known carboxyl- and phosphoryl-containing substances by competition studies (105). To a given quantity of yeast and UO_2^{++}, increasing amounts of the test substance were added. The distribution of UO_2^{++} between the yeast groups and the competing agent was determined by using the concentration of agents necessary to give a one-to-one distribution of UO_2^{++} between cells and medium as an end point. Only those agents with multiple phosphate groups in a chelating arrangement can bind UO_2^{++} with the same high affinity as the yeast ligands. Highly polymerized polyphosphates and polymerized ribonucleic acid behaved most like the ligands of the yeast membrane. That the cellular ligands are phosphoryl groups is further indicated by studies of the effects of pH on the stability of the uranium-complexes. The cellular ligands and phosphoryl-substances are similarly influenced by pH, but the carboxyl-substances behave differently. Although yeast cells do contain inorganic polyphosphates, evidence points to ribonucleic acid as the membrane-ligand responsible for the binding. Firstly, the polyphosphate content can be altered markedly or can be virtually depleted without altering the UO_2^{++}-binding. Secondly, cells treated with the enzyme ribonuclease lose binding capacity for the basic dyes (44) which are bound by the same groups as UO_2^{++} (85).

The studies with UO_2^{++} also demonstrate that the phosphoryl binding sites are located on the outer membrane of the cell (104). Two types of evidence can be cited. Firstly, the maximal UO_2^{++}-binding by the membrane ligands is 1 $\times$ 10^{-3} mol per kg of cells. Yet the interior of the cell contains sixty times this amount of phosphate, some two-thirds of which is non-extractable (largely polyphosphate and nucleic acid phosphate). Secondly, the addition of small amounts of inorganic phosphate to the medium will remove uranium from the cell by competition. Yet the interior of the cell already contains fifty times this concentration of inorganic phosphate.

In the studies of Mn^{++}-binding, the data indicated the existence of two species of cation-binding sites in the cell membrane. On the basis of chemical studies with UO_2^{++}, one has been identified as phosphoryl groups. The other can be identified as carboxyl groups by indirect evidence, again with UO_2^{++} as a probing cation. Associated with the binding of UO_2^{++} by phosphoryl-groups is the inhibition of sugar metabolism (see detailed discussion in the next section). If, however, an excess of UO_2^{++} is added above that necessary to saturate the phosphoryl-groups, then a second physiological effect can be observed, the inhibition of the invertase activity of the cell surface (27). This particular enzyme contains no prosthetic group and no phosphate-group, and involves no cofactors (79). Therefore, on the basis of the known interactions of UO_2^{++} with protein ligands (28), the inhibitory effect must be attributed to the binding of UO_2^{++} to carboxyl-

groups. Of the two ligands of the cell surface that can reversibly bind cations, the phosphoryl-groups form the more stable complexes, carboxyl-groups the less stable. Consequently nearly all of the binding at low concentrations of the cation involves the phosphoryl-group. Only after these are saturated does carboxyl binding become a factor.

The binding of cations other than UO_2^{++} and Mn^{++} has been determined by competition studies with Mn^{++} (102). Uranyl ion forms the most stable complexes, followed by other bivalent cations such as Ba^{++}, Hg^{++}, Cu^{++}, Ca^{++}, Sr^{++}, and Mg^{++}. Monovalent cations form relatively unstable complexes.

The phosphoryl- and carboxyl-groups of the membrane cannot account for the binding of all metals. For example, mercury not only binds to phosphoryl sites, as evidenced by competition studies with Mn^{++} and UO_2^{++}, but also combines with at least one other grouping, probably SH. Firstly, the amount of mercury that can be bound (28 mmol/kg cells) is considerably larger than the amount of UO_2^{++} (1 mmol/kg cells) (84). Secondly, the kinetics of mercury binding to and desorption from the cell are different from those for uranium with respect to both rate and temperature dependence. Uranium uptake by and removal from the cell is complete within 2 minutes, and is temperature-independent. Mercury uptake, on the other hand, requires about 15 to 20 minutes for completion; the desorption is slow and temperature-dependent. At 38°C desorption in the presence of excess of cysteine is 90% complete in 3 hours, whereas at 7.2°C it is only 40% complete. Such kinetic behavior is typical of reactions involving the formation and cleavage of covalent bonds. Thirdly, the mercury binding sites of the yeast cell are oxidizable (85). The above evidence, along with the well-known affinity of mercury for SH groups, strongly points to the sulfhydryl grouping as the principal binding site for mercury. Unfortunately, because mercury can penetrate into the interior of the cell, it is not possible to determine what proportion of the binding sites is located on the cell membrane, except in terms of disturbances of functions associated with the membrane.

Imidazole and other nitrogen-containing ligands may also play a role in metal binding by the cell membrane, but no definitive evidence has been presented.

In summary, there is strong evidence for the existence of three types of metal-binding ligands on the yeast cell surface, namely sulfhydryl, phosphoryl, and carboxyl groups. All of these groups are of physiological significance. Metal interactions with the SH-groups lead to a generalized breakdown of the permeability barrier of the cell. The effects produced by combination of carboxyl or phosphoryl groups with metal ions depend on the chemical nature of the metal. Binding of Mn^{++}, Mg^{++}, or Ca^{++} evokes no response (98). The binding of UO_2^{++}, however, can result in two effects, the inhibition of glucose fermentation and the inactivation of the surface enzyme invertase.

2. The inhibition of enzymes of the cell surface—UO_2^{++} and Ag^{+} on yeast invertase. A variety of enzymes is peripherally located (97). These include: enzymes that digest external substrates (such as invertase and phosphatases), enzymes concerned in active transport phenomena, and enzymes involved in membrane synthesis. Such enzymes are probably found in all cells, although experimental

verification is limited to a few (for example, yeast, bacteria, plant root cells, red blood cells, cells of intestinal and renal epithelia, and muscle cells). Because of their location the enzymes of the cell surface are particularly susceptible to heavy metals.

In the yeast cell, invertase functions as a surface-bound, digestive enzyme (27, 97, 128) which hydrolyzes sucrose, a non-penetrating sugar, into utilizable substrates (glucose and fructose). This enzyme can be rapidly inhibited by uranium, silver, and mercury. A comparison of the inhibitory effects of uranium on fermentation and on invertase activity reveals large differences in the susceptibilities of the two functions. Fermentation is completely blocked at concentrations of the metal which have little effect on invertase activity. Low concentrations of UO_2^{++} are preferentially bound by phosphoryl groups (resulting in the inhibition of fermentation), and only if phosphoryl groups are saturated with UO_2^{++} does binding to and inhibition of invertase take place. Stated another way, uranium is diverted from the invertase by phosphoryl sites for which it possesses a higher affinity. The inhibition curve for invertase is thereby displaced toward higher concentrations of the metal (100). In attempting to quantify the kinetics of inhibition, appropriate corrections must be made.

In the case of inhibition of invertase by silver, a similar diversion of the metal to other binding sites has been observed (31). Large amounts of silver are bound by the cell with no concomitant inhibition, or with a slight increase in activity. In fact, the quantity of silver necessary to inactivate the invertase of the intact cell is two hundred times as great as that necessary to inactivate the pure enzyme. This large diversion of silver to inert sites thus leads to difficulties in interpretation of the data.

3. Inhibition of the transfer of sugars through the cell membrane—UO_2^{++} on sugar uptake by yeast. Traces of uranyl ion inhibit fermentation of sugars by the yeast cell almost completely. Yet the inhibition is not due to a blockage of any of the enzymes in the glycolytic pathway (96, 106). For example, UO_2^{++} does not inhibit the fermentation of internally stored carbohydrate, nor does it inhibit the reverse path, the formation of glycogen from alcohol. The oxidative metabolism of sugars is also inhibited, but not that of two- and three-carbon substances. The effects of uranium are thus restricted specifically to preventing sugars from entering any metabolic pathway. The first enzymes in sugar metabolism are the kinases (hexokinase in the case of glucose). Although UO_2^{++} can inhibit the hexokinase reaction in the test tube (45) (and most of the other glycolytic enzymes as well), its action on the intact cell cannot be explained on this basis. For example, Ca^{++}, which is itself inhibitory to the hexokinase reaction, can completely reverse the action of UO_2^{++} on the intact cell. It must be concluded, therefore, that UO_2^{++} acts on a step prior to the hexokinase reaction, presumably the passage of glucose through the cell membrane. Such a conclusion is consistent with the observations described in the preceding section which indicated that uranium is bound only to membrane ligands (104).

The mechanisms by which sugars pass through the cell membrane have been studied in some detail in red blood cells (63, 127), in muscle cells (35), in epithelial

cells of the intestine (25), and in bacterial cells (74), as well as in yeast cells. Certain similarities are found in all of these cells. Sugars enter very slowly by simple diffusion. On the other hand, certain sugars enter quite rapidly by a special mechanism. The specificity pattern does not resemble that for any of the known enzymes for which sugars are substrates, but is somewhat broader, and includes sugars that cannot be metabolized by any known metabolic enzymes. In view of the evidence that the entry mechanism involves a definite chemical specificity, that pairs of sugars compete with each other, that the kinetics of entry follow mass-law behavior (saturation kinetics), and that the entry is susceptible to inhibition by traces of agents (for example, heavy metals), it has been suggested that entry involves an interaction of sugar and a special receptor in the membrane. The term "carrier" (or in bacteria, permease) has been applied as a general name for such receptors. This general topic has been reviewed in detail recently in this journal (62, 127).

In yeast, the capacity of the "carrier" transport system for glucose is less than the capacity of the metabolic systems. Since the carrier system is rate-limiting, glucose does not accumulate within the cell but is utilized as fast as it enters. In the presence of UO_2^{++}, the rate of transfer of sugar into the cell is reduced, with kinetics typical of a non-competitive inhibition (96), implying that the metal and sugar do not bind to the same site on the carrier. Studies relating UO_2^{++} binding to inhibition indicate a direct proportionality (96, 101), *i.e.*, the percentage of inhibition is equal to the percentage of phosphoryl sites occupied by the metal. Consequently, each and every phosphoryl site contributes equally to glucose uptake and when occupied by UO_2^{++} is completely blocked.

Because specific knowledge of the chemical nature and mode of operation of the carrier system is lacking, the mechanism of action of uranium can only be inferred. It might, by forming a chelate with phosphoryl groups of ribonucleic acid, impose a steric hindrance to the access of glucose to the carrier site. On the other hand, there may be no interference with the combination of glucose and carrier site, but the transfer of the carrier-glucose complex across the membrane might be prevented. The latter suggestion implies some role for ribonucleic acid in the functioning of the carrier, even though indirect. The fact that treatment of yeast cells with the enzyme ribonuclease not only reduces their cation-binding capacity but also reduces the rate of sugar metabolism supports this view (115).

In the presence of O_2, the uranium-binding is the same as in its absence, but the physiological effects are somewhat different. When the phosphoryl sites of the membrane are fully occupied by uranium, the anaerobic fermentation of glucose is inhibited by over 90%, but the respiration of glucose is blocked by only 60%. The remainder of the respiration is blocked only if the concentration of UO_2^{++} is sufficiently high to saturate the carboxyl sites of the membrane. Glucose entry under aerobic conditions apparently proceeds through two chemically distinct membrane sites. The kinetics of inhibition are in accord with this conclusion. The kinetics under anaerobic conditions are non-competitive; those under aerobic conditions follow a more complex pattern.

B. All-or-none responses of the cell membrane

In the interpretation of the data in the previous section, the mass-law equations were used to relate either the concentrations of heavy metals or the extent of metal-binding to the physiological effects. Each cell in the population was assumed to respond in a graded fashion to increasing concentrations of metal, with little variation between cells. Yet this assumption does not always obtain. Heavy metals, like drugs, often produce an all-or-none response (16) in which no effect is observed until a certain threshold concentration is attained. The response once elicited is maximal for a given cell. The curves relating dose and effect do not represent the parameters of the chemical reaction of the metal and cellular receptors, but rather the distribution of thresholds in the population. A distribution equation, usually the normal curve, can fit the data, whereas a mass-law equation will not. Sometimes the choice of the biological parameter is the determining factor. For example, a metal may produce a graded response on a particular system. A chain of events is induced by the original insult, terminating perhaps in the death of the cell. If any step in the chain is an all-or-none type of response (as, for example, failure of the cell to divide, or cytolysis) then to the observer, the whole process appears to follow the all-or-none pattern. Of greater interest, from a theoretical point of view, are those all-or-none responses which are direct consequences of the primary chemical insult of the metal. Examples of both situations will be given. For the sake of clarity, they will be labeled "direct" all-or-none responses and "indirect" all-or-none responses.

1. "Indirect" all-or-none responses—gold on hemolysis of red blood cells. An example of a graded effect which terminates in an all-or-none response is the action of traces of gold on the red blood cell (125). The primary response is a graded change in the permeability of the cell to cations. The cells lose K^+ and gain Na^+, the rates increasing with higher concentrations of the metal. Because the gain of Na^+ somewhat exceeds the loss of K^+ (resulting in a net gain of NaCl), the volume increases. When the volume of a particular cell attains a critical size, hemolysis occurs. The critical volume varies for individual cells, depending largely on the original size of the intact cell (90). The hemolysis curve, therefore, represents in large part the distribution of sizes in the original population, even though the primary response is a graded change in membrane properties. For this reason, the response pattern for hemolysis gives no direct information concerning the nature of the chemical interaction between the metal and the membrane. Thus, simple morphological differences between cells are responsible for the nature of the response of the population.

Many kinds of chemical disturbances can finally result in the same final injury, for example the failure of cell division (mortality) (16). The same pattern of all-or-none response will be observed regardless of the particular nature of the initiating injury. The only information usually derived from such studies is the relative toxicity of the agents, and the extent of the biological variation in a given population of cells. Little information is obtained concerning the physicochemical nature of the interaction of metals with cellular receptors. For this reason it is important in evaluating mechanisms, to study responses directly

associated with the damaged receptors, or to have some preknowledge of the sequence of events between the receptors and the measured response.

2. "Direct" all-or-none responses. a. Lead on permeability of red blood cells. In the normal functional state, the human red cell membrane maintains large concentration gradients of sodium and potassium. Passive ion movements down the electrochemical potential gradient, which tend to diminish the concentration difference between plasma and cells, are balanced by active transport processes in the opposite direction. The transport system derives energy from ATP generated by glycolysis (34). The dependence of potassium influx on the external K^+ concentration can be quantitatively described in terms of the sum of two processes, one with saturation kinetics and one with simple diffusion kinetics. At physiological concentrations of intra- and extracellular K^+, the rate of K^+-exchange across the membrane of human erythrocytes, as measured with K^{42}, amounts to about 1.5 to 2 mEq/l cell/hr.

If minute amounts of lead are added to a red cell suspension (10^{-7} mol/g of cells) two effects can be observed: a rapid net loss of K^+ (51, 81), and a drastic reduction of intracellular ATP (65). The effect on potassium permeability is quite specific. The cells lose KCl with little concomitant sodium uptake (51, 83, 121). As a consequence, the osmotic content of the erythrocytes diminishes, the cells shrink, and a marked increase in osmotic resistance occurs.

Only a very small fraction of the lead-induced potassium loss can be related to the deficiency of cellular ATP, and its loss as a substrate for active transport. If the interruption of the energy supply and the absence of the active transport component were the sole cause of the potassium loss, its rate of loss should not exceed the rate of K^+-accumulation in metabolically intact cells (*i.e.*, 1.5 to 2 mEq/l cell/hr). Yet, net potassium loss after lead poisoning proceeds at least twenty to forty times as rapidly. Clearly, lead produces not only an inhibition of active K^+-transport (in rabbit cells an initial stimulation of active transport was observed) (51) but also a drastic and specific increase in the permeability of the membrane. The latter observation suggests that the site of action of lead is the cell membrane itself.

The localization of the lead-effect to the membrane structure has been confirmed by experiments with isolated erythrocyte membranes (65). It is possible to remove intracellular enzymes and substrates from red cells by osmotic hemolysis, without destroying the integrity of the cell membrane. Such isolated membranes ("ghosts") have similar properties to the membranes of intact cells. They are only slowly permeable to sodium and potassium. If lead is added to ATP-poor ghosts, the rate of potassium diffusion through the membrane is greatly enhanced.

The relationship between the dose of lead and the effect on potassium permeability shows the following features (87): 1) with high concentrations of lead (10^{-7} mol/g of cells) all of the cellular K^+ leaks out into the medium; 2) with lower concentrations of the metal the effects are intermediate; 3) at any particular lead concentration, the potassium loss progresses rapidly for about 60 minutes, but thereafter slows down. After this time, little or no further loss occurs, even

though only a small fraction of the cellular potassium may have diffused out of the cells.

Primarily, higher concentrations of the metal are associated with a higher total K^+-loss rather than with a higher rate of loss. Similarly, a variation of pH at a constant lead concentration alters the total K^+-loss but leaves the half-value time of K^+-loss unaffected.

From such observations it seems probable that the observed potassium loss represents an all-or-none response in a fraction of the cells of the population, each cell having a unique threshold for lead. In those cells in which the threshold is exceeded, a complete loss of intracellular K^+ occurs within about 60 minutes. In the total cell population the increased potassium loss associated with increasing lead concentrations would represent an increasing proportion of cells the thresholds of which have been reached.

Proof of the all-or-none relationship was obtained by physically separating the leaky cells from the normal cells, taking advantage of differences in osmotic resistance. Leaky cells, which have lost KCl, shrink and as a consequence survive in hypotonic solutions. Intact cells, on the other hand, disintegrate because of osmotic hemolysis. The number of lead-treated cells with increased osmotic resistance is approximately proportional to the observed K^+-loss. Furthermore, analysis of the shrunken cells after separation from the hemolyzed cells reveals a reduced potassium content, whereas the potassium content of the other cells is normal (87). This finding is in agreement with microscopic observations. After lead treatment, two different cell sizes coexist in the same population (81, 119); the smaller cells presumably are those which have lost K^+.

Lead uptake by red cells has been repeatedly measured (11, 21, 37, 75). Attempts to correlate lead binding with physiological effects reveal that a large fraction of the lead is diverted to binding sites which play no role in the maintenance of the normal membrane resistance to K^+ (37). For example, no tendency toward saturation of binding sites is observed over the whole concentration range between minimal and maximal physiological response. Binding to the "inert sites" continues at concentrations higher than those necessary for maximal effect.

Desorption experiments lead to the same conclusion (37). A surplus of a complexing agent in the medium [*e.g.*, ethylenediaminetetraacetic acid (EDTA)] prevents lead uptake by the cells. On the other hand, if the cells are allowed to equilibrate with lead before the addition of EDTA, only a partial desorption of the metal can be induced by the complexing agent, the amount of non-desorbable lead being temperature-dependent. The non-desorbable lead is not concerned with the change in the resistance of the membrane to K^+. At 20°C, restoration of the normal membrane resistance occurs with EDTA despite the fact that the complexing agent removes only 50% of the previously bound lead.

In contrast to the reversibility of the permeability response, the removal of virtually all of the bound lead by EDTA does not restore the ability of the cells to accumulate potassium against the concentration gradient (86).

At present, the nature of the chemical reactions between lead and the red cell

membrane is still unknown. Sulfhydryl groups do not seem to be involved because the effects and binding of lead are entirely different from those of the sulfhydryl-seeking metals such as mercury (see details in the next section). Aub and coworkers (4) suggested that lead was precipitated in the membrane of the red blood cell in the form of colloidal lead phosphate. With the electron microscope, the deposited lead can be seen on the outer surface in large aggregates and within the membrane in the form of small aggregates (52). The larger aggregates on the outer surface arise from adsorption of preformed lead phosphate colloid, whereas the small aggregates deposit within the membrane structures under conditions where an excess of lead over phosphate is present in the medium. Presumably, as Pb^{++} diffuses into the membrane precipitation with cellular components occurs (4). These observations are in agreement with the finding that the rate-determining step in the process of lead fixation by red blood cells, in phosphate-containing and phosphate-free media, follows the same kinetic pattern as does lead precipitation by phosphate in test tube experiments (21).

The membrane of the cell seems to possess a special affinity for lead. Not only is colloidal lead adsorbed on, or deposited within the membrane of the intact cell, but the freshly isolated membrane fraction (stroma or ghost) of the cell will compete with equal strength with intact cells for lead (120). The suggestion has been made that lipids of the membranes are important in lead-binding (32, 52), and indeed lipid-free stroma does not bind lead effectively (120). Furthermore, Solomon (113) made the interesting observation that red cells after having passed through a column of alumina become insensitive to the action of lead. Apparently, in the outer cell surface there is a loosely bound substance which is involved in the reactions leading to the response of erythrocytes to lead. Alumina has been observed to remove cholesterol, phospholipid, and lipoprotein from the red cell (68).

No explanation has yet been presented to explain the pharmacological responses in terms of lead-binding by particular membrane components, or in terms of the deposition of colloidal lead. Adsorption of preformed colloidal lead by red cells leads to no toxic response (22), but formation of colloid within the membrane cannot be excluded as a factor in toxicity. In a negative sense, certain ideas have proved inadequate. Thus, it has been suggested that the deposition of colloidal lead in the membrane might lead to damage because of a reduction in surface charge-density (22). However, amounts of lead capable of inducing K^+-loss produce no change in electrophoretic mobility of the cells (89). It has also been postulated that the effects on the membrane were caused by the local liberation of acid during the precipitation of lead phosphate (4), but later studies discounted this explanation (73). Finally, complexes of lead with intracellular diphosphoglyceric acid (DPGA) are held to be responsible for the toxicity of lead (73). In fact, in centrifuged hemolysates of lead-poisoned erythrocytes, considerable amounts of lead have been found, part of it bound to non-dialyzable substances and the rest in a dialyzable form (93). On the other hand, experiments with DPGA-depleted ghosts showed that the action of lead does not depend on the presence of DPGA but is a consequence of a direct interaction with the membrane

(65). Lead provides a good example of some of the difficulties that may arise in determining the mechanism of action of a metal on a cellular function. Despite the many studies of the chemical interactions and of the physiological responses, the full story remains to be told.

b. Mercury on permeability of yeast cells. Yeast cells contain about 0.15 mol of potassium per kg of cells. If the cells are suspended in distilled water, a small fraction of the intracellular K^+ leaks out until a steady state distribution is attained. In the steady state large concentration gradients (as large as 2000 to 1) are maintained. The rate of K^+-efflux has been estimated at about 8 mmol/kg cells/hr, or about 5% of total cellular K^+ per hour (84).

If mercury is added to a cell suspension, rapid potassium loss occurs. At a mercury-dose of about 15 mmol/kg cells, the rate of efflux is increased to about 100 mmol/l cell/hr, or almost 15 times that of the control (98). However, the defect in the membrane produced by mercury is not specific for potassium. It represents a rather general breakdown of the permeability barrier. Thus, for example, the normally anion-impermeable yeast cell membrane, in the presence of mercury, allows the penetration of anions in both directions. This effect is comparable to the hemolysis of red cells, except that the presence of a rigid cell wall prevents a complete disintegration of the cellular structure (84).

The kinetics of potassium release in mercury poisoned yeast cells are very similar to the kinetics of potassium loss in red blood cells treated with lead (p. 201). Potassium loss reaches a maximal value after about one hour, regardless of the concentration of the metal. This indicates that the time constant for K^+-loss is independent of the metal concentration, whereas the total K^+-loss is directly dependent. The total loss plotted against the logarithm of the mercury concentration gives a symmetrical S-shaped curve that corresponds to the integrated form of a "normal" distribution (84). The explanation for the observed response to mercury is the same as for the action of Pb^{++} on red blood cells: that individual cells respond in an "all-or-none" fashion. This suggestion is supported by the following observations: 1) If cells exposed to mercury are treated with appropriate concentrations of a dye, such as methylene blue, a fraction of the cells will stain, whereas unexposed cells do not stain. An almost perfect correspondence is found between the fraction of the cells that is stained and the fractional loss of K^+ from the population. 2) A similar relationship can be demonstrated for K^+-loss and the ability of the cells to form colonies. It is apparent, then, that the cells which can be stained and the cells which cannot divide are the cells which have lost all of their potassium (100).

No simple relationship between mercury-binding and pharmacological effects could be found (84). The binding curve shows two phases, with an inflection point at a level of 18 mmol of bound metal per kg of cells. Below the inflection point, mercury alone is bound, but above it, mercury and chloride are bound, presumably as a mercury-halide complex. The response (K^+-loss) occurs entirely within the first phase of binding, but the binding curve and the response do not match. No response is found until 2 mmol/kg is bound, and the maximal response is observed at 12 mmol/kg, whereas the binding curve continues without inflec-

tion to 18 mmol/kg. Obviously, only a portion of the binding ligands is physiologically responsive, and diversion of metal by inert sites is an important factor.

c. The mechanism of all-or-none responses. Two questions concerning all-or-none responses deserve discussion. Firstly, why do cells show a threshold behavior at all? Secondly, why do the threshold values differ between individual cells in a population? As far as the first question is concerned, in the examples given, the metal is not acting on enzyme systems, but rather on sites maintaining structural integrity. For example, in the case of the action of mercury on yeast (p. 197), it has been suggested that the metal combines with sulfhydryl groups in the membrane. No single one of these ligands plays any measurable functional role. However, when many of these groups are cross-linked by reaction with the metal, the stress on the membrane is sufficient to destroy it as a permeability barrier (84). In other words, if groups of ligands acting in unison are essential for a particular function, all-or-none responses may occur.

With regard to the second question, variation of resistance between individual cells of a population is not surprising. Cell populations usually comprise cells in all stages of development, and within each age group a certain scatter of physiological activities is to be expected. Yet it is difficult to express these physiological variations in terms of physicochemical parameters. It is conceivable that metal distribution between individual cells is unequal, which might cause the variation of the effects observed. Unequal distribution of metal will be found only if (a) binding occurs more quickly than mixing of cells and metal at the beginning of the experiment, and (b) redistribution of the metal between the different cells of a population proceeds more slowly than the development of the pharmacological response. In the case of lead poisoning of human red cells, this possibility could be excluded (86), and it appears unlikely to play a role in other cases of metal poisoning (23, 24).

Even if mixing were achieved instantaneously, a statistical variation of the number of metal atoms bound per cell would occur. Several theories of disinfection are based on this assumption (3, 49, 91, 92, 129). Significant differences of metal distribution between cells, due to statistical variation, may be expected only if the number of sensitive ligands as well as the number of metal atoms per cell is very small, perhaps less than one hundred. In the experiments discussed above, such an explanation seems to be improbable, since several million metal atoms per cell are required in order to produce the observed responses.

In most experiments all cells are equally exposed to the metal and significant differences of binding due to statistical variation can be excluded (16). Consequently, differences of response must be attributed to diversities in biochemical and morphological properties of the cells.

The chemical differences most likely to occur between individual cells of the same population will be a variation of the concentrations of sensitive and of insensitive metal-binding ligands. If the metal is reversibly bound, and if the stoichiometrical ratio in the reaction involved is equal to one, the mass-law predicts that an equal percentage of these ligands in each cell combines with the metal. This is true irrespective of the concentrations of all types of metal-binding

ligands present. For this reason, variation of the threshold value for a metal cannot be caused by variations in the percentage of occupied ligands. Therefore, different thresholds can occur only if the physiological effect depends on the absolute number of ligands which are combined with the metal.

C. The action of metals in the interior of the cell

Although the cellular membrane acts as a diffusion barrier, certain metals will eventually penetrate. However, little direct experimental knowledge of intracellular metal distribution and specific actions of metals on metabolic sequences is available. Any presentation of the scattered examples of intracellular actions seems of little use at this time, since no general conclusions can be drawn. Indeed, because of the vast number of different complexing substances within the cell interior, it seems hopeless even to try to predict any chemical reactions following the entrance of the heavy metal. This is easily illustrated by the following considerations. The majority of all enzymes, especially those carrying SH-groups, can be inhibited by metals. One would expect, therefore, that almost any branch of metabolism in living cells is susceptible to the action of heavy metals. Yet, different metabolic pathways may exhibit marked differences of sensitivity towards a given metal. For example, in liver homogenates, lead inhibits the Krebs cycle but not the glycolytic system (5); in the kidney, cadmium produces rather specific changes of protein synthesis, apparently without affecting the basic metabolism of the cells (33).

One group of effects which deserves some comment is the frequently observed stimulation of certain cellular activities by concentrations of metals below the inhibitory level. This phenomenon has been known for many decades as the Schultz-Arndt Rule (16). One example has already been referred to in the section on inhibition of invertase by silver. The activation of respiration by low concentrations of mercury and other sulfhydryl reagents has been studied in some detail. For example, the respiration of sea urchin sperm can be enhanced by the addition of traces of mercury; a maximal rate is reached at intermediate concentrations and increasing inhibition is seen with higher concentrations (10). The stimulation-inhibition relationship is explained as follows. Within the cells small amounts of soluble substances containing free sulfhydryl groups exert an inhibitory effect on the activity of sulfhydryl enzymes which control the rate of respiration. The removal of free sulfhydryl groups by combination with mercury leads to an acceleration of respiration. At high concentrations of mercury, however, the metal becomes attached to the enzyme molecules themselves and inhibition of oxygen-uptake follows. It is not yet clear whether such an explanation can be applied to all cases of stimulation by low concentrations of otherwise toxic metals.

Similar stimulation of activities has also been observed in impure preparations of enzymes, with similar explanations (16). On the other hand, some heavy metals form complexes with inert proteins or amino acids; these complexes exert a strongly activating influence. For example, the activity of 2,3-diphosphoglycerase may be increased up to eight-fold by mercury or silver combined with certain amino acids or even with serum albumin (108).

D. Time dependence of metal actions

1. The membrane as a diffusion barrier to metals. The examples presented in the previous sections were confined to a description of experimental situations in which the metal distribution reached a steady state after a short time. Moreover, the physiological response remained at a relatively constant level for most of the subsequent experimental period. Such time-independent responses frequently obtain if an impermeable cell membrane prevents the metal from entering the cell interior, or if a rapid and irreversible change in the membrane occurs. If the cell membrane is permeable to the toxic metal ions, complicated time-dependent situations may develop. The sequence of reactions is always from the outside toward the inside, with the first responses associated with the membrane and the later responses with the interior of the cell.

a. Copper and mercury on glycerol permeability of red cells. Marked differences of permeability to glycerol are found in red cells of different species. Two types of behavior have been described. Glycerol penetrates only very slowly into erythrocytes of pig, ox, dog, and sheep. The penetration process has a high temperature coefficient and is insensitive to changes of pH. The addition of heavy metals does not interfere with the glycerol uptake. The erythrocytes of man and of the rodents, on the other hand, are readily permeable to glycerol. The temperature coefficient is low and the rate of penetration is very sensitive to changes of pH. The glycerol entry into the cells can be prevented by traces of copper or mercury (47, 48).

The effect of copper is independent of time. Only a very small amount of the metal becomes attached to the cell membrane and no penetration occurs (72). With mercury, on the other hand, the situation is more complicated. The inhibition of glycerol uptake is only temporary. After a lag period, the cell membrane suddenly regains its original permeability, and glycerol uptake proceeds as fast as in the absence of the metal. The duration of the lag period increases with increasing mercury concentration.

Mercury is temporarily fixed to ligands in the membrane that control glycerol permeability. Subsequently, it moves into the interior of the cell, and combines with the many complexing substances. As long as some mercury is bound to the membrane, inhibition of glycerol permeability is observed, but as soon as all of the mercury has passed through the membrane, the inhibition disappears. At very high mercury concentrations, the complexing ligands of the cell interior become saturated. The excess metal remains attached to the cell membrane and a permanent, time-independent reduction of glycerol permeability is produced (124).

b. Copper and mercury on glucose uptake and respiration of muscle cells. Mercury inhibits the uptake of glucose by the rat diaphragm. The inhibition reaches a concentration-dependent maximal value in less than twenty minutes. After a lag period of over thirty minutes, a second effect gradually develops: respiration becomes progressively inhibited, provided relatively high concentrations of mercury are applied. Maximal effects are not observed even after one and one-half hours. Upon addition of slowly penetrating complexing agents like BAL

or cysteine or after application of non-penetrating serum albumin, the inhibition of glucose transport can be reversed whereas that of respiration cannot. In contrast, the respiration of muscle homogenates is reduced almost immediately on addition of the metal, and the inhibition can be just as rapidly reversed by the addition of cysteine. These observations suggest that the metal first interacts with functional groups at the cell surface, therefore inhibiting the transport of sugar. Subsequently it slowly enters the cells and the respiration is progressively inhibited (26).

This interpretation of the observed responses was confirmed by studies of the binding of mercury (26). Uptake of the metal by the excised diaphragm proceeds fairly rapidly for about twenty minutes. Thereafter, the rate of mercury uptake proceeds at a much lower rate constant. The time-sequence involves a rapid diffusion through the interstitial spaces and immediate binding on the cell surface, followed by a slow penetration through the membrane to the respiratory sites within the interior of the cell.

Copper (as cupric chloride) behaves in a manner similar to mercury. Sugar uptake by the diaphragm is rapidly inhibited. The lag period preceding the inhibition of respiration is longer, but the extent of inhibition is greater.

2. Reaction sequences—lead on the red cell. It has already been mentioned that the potassium permeability of human red cells is greatly increased by traces of lead. In the course of several hours, however, a "recovery" of the cell membrane occurs. The recovery process does not require addition of complexing agents or any other variation of the experimental conditions (37). The fixation of the lead to the cells is completed within about ten minutes and the increase in permeability is detectable in a few minutes, but it takes four to six hours for the cells to recover. If the recovery process were caused by slow penetration and subsequent inactivation of the metal by intracellular complexing agents as occurs with mercury (p. 207), a second dose of lead would produce the same sequence of actions as did the first dose. However, the addition of a second dose of lead to cells having recovered from the action of a first dose, does not produce any increase in potassium permeability. The cells have become refractory. Only if the cells are treated with complexing agents (EDTA) do they regain their sensitivity to lead. Clearly the mechanism of recovery after lead insult is quite different from that after mercury.

It has been suggested (87) that in the presence of lead a sequence of chemical reactions takes place in the membrane; this sequence resembles the catalytic action of certain metals on disulfide bonds. In the first reaction, SS-bridges are broken open to give a S-Pb-complex, and a leaky membrane. Thereafter the metal slowly forms a linkage of the type S-Pb-S, with recovery of normal permeability, and a refractoriness to further lead treatment. Complexing agents remove the lead from the mercaptide, thus re-establishing the normal state of the membrane. However, in view of the evidence presented previously that lead may not bind to SH groups, this mechanism may require modification to include similar reaction sequences of lead with other ligands.

A somewhat similar response is the "hardening" or "tanning" of the red cell

membrane by relatively high concentrations of metal. Such tanned cells can be suspended in distilled water without hemolysis, but they become sensitive to osmotic hemolysis once again if the metal is removed by the addition of suitable complexing agents (125). The recovery from lead poisoning is not, however, the same as "tanning" or "hardening." The lead-treated cells still possess many features of normal erythrocytes. For example, the typically rapid anion exchange across the cell membrane can still occur; a lowering of the ionic strength of the medium, which produces potassium loss in normal red cells, causes the same response in recovered erythrocytes. These observations lead to the conclusion that the recovery from lead is due to a specific chemical reaction sequence at a restricted number of sites within the cell membrane and not to a generalized "hardening" of the membrane.

VI. THE ACTIONS OF HEAVY METALS ON EPITHELIAL TISSUES

In epithelial tissues such as frog skin, small intestine, and kidney, several populations of cells are found in a definite geometrical arrangement. Obviously the actions of heavy metals are more complicated than in the case of homogeneous populations of cells. Nevertheless similarities are found because the absorptive and secretory functions of the epithelia represent specialized use of mechanisms present in nearly all cells.

The ability of a variety of epithelial tissues to transport solutions of NaCl from the mucosal to serosal sides is due to an active transport of Na^+ ions, followed passively by Cl^- (59, 117). Recent evidence also suggests that water moves passively, responding to osmotic and perhaps electroosmotic forces set up as a result of the salt transfer. The active transport of sodium across the epithelia is produced by essentially the same mechanism responsible for the active extrusion of Na^+ from single cells. If more Na^+ ions are extruded from the serosal side of the epithelial cells than from the mucosal side, a net transfer of Na^+ will take place across the tissue. Similarly, it has recently been suggested that glucose transport by the jejunal segments of small intestine (25) is due to active transport of the sugar across the mucosal membrane of the columnar epithelial cells, accumulation to a high concentration within the cells, and passive diffusion through the serosal membrane into the interstitial spaces. Consequently, it might be anticipated that any observed inhibition by heavy metals of the net transport of salt solutions or of glucose across epithelia can be explained in terms of mechanisms similar to those described in the section on cells.

The action of mercury on the membrane of the columnar epithelial cells of the jejunum has similarities to its action on the membranes of yeast, muscle, and red blood cells. Immediately following the addition of the metal (as mercuric chloride) to the mucosal side, rapid responses are observed in the electrical potential across the intestine, the loss of cellular K^+, and the cessation of glucose uptake (18). Each of these responses is associated with the action of mercury on the membrane facing the lumen. After a delay period, other functions are inhibited. These include active Na^+- and glucose-transfer into the serosal solution and the production of lactic acid. It has been suggested that the delayed effects

may result from the cessation of glucose uptake and consequent reduction in metabolism. Alternatively the delayed effects may be associated with the diffusion of mercury into the cell as described for muscle cells. Similar disturbances in electrolyte equilibrium have been reported for the action of mercury on kidney slices (55).

In addition to the direct interaction between heavy metal and cell, there are often superimposed effects arising from the geometrical arrangement of the cells. A simple example is the case where the metal produces one kind of response when added to the mucosal side of an epithelial tissue and a different response when introduced on the serosal side. When mercury at 10^{-4} M (present as an organic mercurial, mersalyl) is in contact with the mucosal surface of frog skin, the transport of salts and water is reduced because of the specific inhibition of the sodium-transfer system (66). Since the effects are noted at mercury levels which do not produce inhibition of cellular metabolism, it seems likely that the toxic action of mercury is mediated through a direct effect on the outward-facing membrane of the epithelial cells. On the other hand, when mersalyl is present on the serosal side in the same concentration, sodium transport is accelerated. This response has been attributed to an interaction of mercury with the inward-facing membrane of the epithelial cells (117).

The diuretic action of mercuric chloride and organic mercurial compounds provides a more sophisticated example of how a complex structural arrangement of cells may considerably modify the direct cell-metal interaction as seen in single cell suspensions. Since mercurial diuretics have been discussed in recent review articles (54, 76) only the more salient points will be mentioned. It is generally accepted (59) that organic mercurials produce an increase in urinary volume by an inhibition of the reabsorption of NaCl by the kidney tubule. There is still, however, considerable discussion on the detailed mechanism of action. On the one hand, evidence has been presented that the organic mercurial acts as an intact molecule by a "lock and key" interaction with cellular components (54). On the other hand, the release of free mercuric ion from the organic compound may result in the diuretic effect (76). Also, there is debate concerning the action of mercury on K^+ secretion—in some cases it is increased and in others, decreased (59). Of most importance in the present discussion is the remarkable specificity of the organic mercurial in therapeutic dosages; it affects only the absorption of NaCl and leaves unaffected the renal handling of bicarbonate, ammonia, and other constituents (59).

Perhaps the most important single factor responsible for the specific effect is the localized deposition of the organic mercurial in the kidney. Radioautographs indicate that mercury is deposited predominantly, if not entirely, in the renal cortex (17). Two mechanisms may contribute. In the first place, organic mercurials are cyclic, weak acids which, like diodrast and phenol red, are secreted in the proximal region of the renal tubule (53). In the second place, the hemodynamics of the kidney are favorable to cortical deposition. The cortex is the first area of the kidney with which mercury in the arterial blood will equilibrate. Moreover, since mercury is present in blood as a non-ultrafilterable protein-

complex (53), it will be retained in the blood stream during the glomerular filtration process. Consequently, the concentration of the mercurial in the blood stream just distal to the glomerulus will be increased by 20% or so, depending upon the fraction of plasma filtered. Finally, as discussed in the "counter current" hypothesis for production of hypertonic urine, the distribution of blood vessels in the medulla is such as to produce a low effective blood flow in the region of the loop of Henle and the distal tubule (39).

The secretory power of the proximal cells may have particular significance in regard to the site of action of the mercurial. Thus, it has been suggested (59) that the mercurial is first carried across the epithelial cells and released into the lumen of the tubule. At this point it interacts with the mucosal surface of proximal cells to produce inhibition of Na^+-transport. This suggestion is supported by the finding that albuminuria in rats (67) and induced hemoglobinuria in dogs (40) exert a protective effect against the mercurial. Moreover, this mechanism finds a parallel in the action of mercury on frog skin where inhibition of NaCl transport is produced only when mercury is applied to the outside of the skin (equivalent to luminal side) (66).

Lesions produced by heavy metals on epithelia can be remarkably specific, with respect both to the metal and to the function affected. Copper (as $CuSO_4$) at 10^{-5} M in the solution bathing the outside surface of frog skin completely inhibits chloride-transport without changing sodium transport (118). Mercury inhibits sodium-transport by frog skin and kidney as discussed previously. Changes in the urinary excretion of amino acids by men exposed to heavy metals provide a third example of specific lesions (20). The urinary excretion of seventeen different amino acids was followed in four groups of workmen variously exposed to mercury, uranium, lead, and cadmium. A fifth group, comprising workmen not exposed to a toxic hazard, served as controls. Two points of particular interest arise from the data. In the first place, varying degrees of amino-aciduria could be produced by the different heavy metals, *e.g.*, uranium and cadmium were far more potent than mercury or lead. Secondly, each metal affected the pattern of urinary amino acid excretion in a different way. Uranium and cadmium exhibited specificity towards threonine and serine, lead increased alanine output, and mercury was most effective on glycine. It seems likely that the metals interfere with specific resorptive mechanisms of the kidney.

VII. THE ACTIONS OF METALS ON ANIMALS

With cells or tissues, studied *in vitro*, the metal is applied in a known concentration and in a chemically defined medium. Difficulties in interpretation are predominantly concerned with the complexities of the biological systems. With the whole animal, on the other hand, additional factors must be considered. Not only are the variety and anatomical arrangement of potentially sensitive cells difficult to cope with, but the concentration of the metal in the immediate neighborhood of the sensitive cells and the time of exposure are determined by a complex of factors including dosage, route of administration, absorption, distribution, and excretion. A brief outline of studies with two heavy metals,

uranium and mercury, will be presented in order to demonstrate the difficulties of deducing generalizations.

A. Uranium

Although soluble uranium compounds, such as uranyl nitrate ($UO_2(NO_3)_2$), have been known for many years as potent nephrotoxic agents, only in recent years, with the widespread use of uranium in atomic energy installations, have detailed studies been made of many aspects of its toxicological actions. A full discussion is to be found in the monographs edited by Voegtlin and Hodge (122).

The distribution pattern of injected (intravenous) uranium is relatively simple. In the blood, almost no uranium exists as free uranyl ion. Despite its insolubility in water at pH 7.4 (due to formation of hydroxides) it remains in solution in blood, about half as a soluble, filterable complex with bicarbonate, and half as a soluble complex with serum albumin. Most of the metal is rapidly cleared from the blood. A large amount (up to 25% of the dose) deposits in the bone, forming a complex with the phosphate groups in competition with Ca^{++}. The metal in the bone, although firmly bound, is held in reversible equilibrium with that in the blood. It acts, therefore, as a reservoir. The only soft tissue in which appreciable amounts of uranium accumulate is the kidney. The level is appreciably higher than in other tissues, with as much as 25% of the dose localized in a few hours.

Excretion of soluble uranium is almost exclusively by way of the urine. The data suggest that the portion of the metal which is present in the blood as a bicarbonate-complex is filterable at the glomerulus. Of the filtered metal some is fixed by the tubular cells and the rest (about 50% of the dose) is excreted in twenty-four hours.

Soluble uranium compounds are relatively toxic. The LD50 for rats is 1 mg/kg, and for rabbits 0.1 mg/kg. Death is caused by renal failure. The characteristic pattern involves the first appearance of signs in one-half to three days. These become increasingly severe, with a crisis, sometimes complete renal shut-down, between five and ten days. Finally, the recovery period in surviving animals lasts until about twenty-one days. Histologically, the damage is restricted primarily to the cells of the distal portion of the proximal convoluted tubules. Changes in appearance occur in a few days, followed, during the renal crisis, by a cellular breakdown resulting in excretion of casts into the tubular lumen. During the recovery period regeneration of tubular cells of somewhat altered appearance occurs.

Physiologically, the first sign of uranium action is reduced tubular function. The reabsorption of glucose and of amino acids is decreased, for example, within ten to twenty minutes. Associated with the loss of tubular reabsorptive function is a marked diuresis, followed, during the stage of cellular necrosis, by blockage of the tubular lumen and by anuria. Associated with the early stages of cellular breakdown is the appearance in the urine of intracellular enzymes such as catalase. The minimal dose of uranium which will produce a physiological response (increased secretion of amino acids) is 0.02 mg/kg in rabbits (12).

The highly specific action of uranium in attacking only the tubular cells of the distal end of the proximal convoluted tubule can be explained as follows: In the blood, the free UO_2^{++} concentration is exceedingly low because of high concentration of HCO_3^- and because of the high pH, 7.4. These conditions are unfavorable for reactions with tissues (except with bone, because of the high affinity of the crystal surfaces for UO_2^{++}). The filterable uranium of the blood, in the form of the $UO_2(HCO_3)^+$ complex, passes through the glomerulus. As the glomerular urine passes through the proximal tubule, water and salt resorption and acidification take place, thereby increasing the concentration of UO_2^{++} and favoring the binding of the metal to cellular elements of the distal tubule. For this reason any agent which leads to alkalinization of the urine will help to prevent uranium poisoning. For example, animals given large amounts of bicarbonate may be more resistant by a factor of 10 to the lethal action of the metal. Similar observations can be made in studies *in vitro* with yeast cells. At pH 7.0, in the presence of complexing-anions such as bicarbonate little UO_2^{++} is bound to the cells. On acidification and in the absence of complexing-anions, even traces of UO_2^{++} are taken up by the cells. The initial response of the kidney cells is also similar to that of the yeast cells (see p. 195) with an immediate inhibition of the membrane functions involved in transport phenomena, such as sugar and amino acid absorption. It seems justified, therefore, to draw analogies between the clearly defined action of uranium on yeast described previously and the less clearly defined actions on the tubular cells. As in the yeast cell, a reaction of UO_2^{++} with membrane sites (either phosphoryl or carboxyl groups) is presumably responsible for the reduced resorptive activity, the diuresis, and the ultimate deterioration and death of the cells. The dose-response relationship in the case of the yeast cell can be described by simple derivations of the mass-action law. A similar relationship would presumably obtain with respect to the tubular cells if the local concentration of the free UO_2^{++} were considered. Unfortunately, the local concentration is not known. It depends on several factors: 1) the concentration of uranium in the glomerular urine (equal to the concentration of filterable uranium in blood); 2) the concentrating action associated with reabsorption of fluid in the proximal tubule (cells in general are impermeable to uranium, so the concentration in the tubular urine may be five times as high as in the other tissues, assuming 85% resorption of fluid) and 3) changes in local conditions, particularly in bicarbonate concentration and pH, may result in a large increase in the concentration of free UO_2^{++}. In fact, such changes in the local chemical environment are perhaps the major factor determining the specific localization of uranium damage to the distal end of the proximal convoluted tubule. An alternative but less satisfactory explanation would be a unique uranium sensitivity of the cellular systems in this part of the kidney.

Most of the dose-response relationships in the uranium literature are mortality data. Here the chosen response is by nature a threshold phenomenon (see discussion of indirect all-or-none responses). Typical of most mortality data, the experimental curves can be fitted by the normal distribution on a graph of log-dose against response. Although it would be possible to measure a graded re-

sponse, such as the inhibition of the reabsorption of amino acids or sugars, as a function of dose, the complicating factors in the intact animal would probably completely obscure the physicochemical relationship between the metal and the sensitive sites in the kidney cells.

B. Mercury

Although toxic responses to mercury have been recognized from early historical times, and although numerous studies have been made, analysis of its interactions in the intact animal in physicochemical terms is most difficult. The toxic responses are of two kinds: an acute response resulting primarily from renal dysfunction, and a chronic response sometimes appearing after years of exposure traceable to effects on the central nervous system. Almost nothing is known of the etiology of the chronic disease, and it will not be discussed further (99).

The distribution pattern of mercury (given intravenously, as $HgCl_2$) is relatively simple. In the blood, less than 1% is filterable; about half is associated with the red blood cells, and the remaining half forms a complex with serum

significance because a seemingly insignificant primary defect may be amplified through a sequence of reactions into considerable biological damage.

A. Specific alterations in membrane permeability and transport

1. Changes in ion distribution. The maintenance of ionic gradients across cell membranes involves a balance between active transport systems, which pump ions against the electrochemical gradient, and passive movements down the electrochemical gradient. Both the metabolically-forced ion accumulation and the passive ion fluxes may be altered by heavy metals. For example, lead and mercury inhibit the active K^+-accumulation, and increase the passive K^+-outflow in human erythrocytes (p. 201). Similar disturbances in the metabolism of Na^+ and K^+ have been observed in kidney slices treated with mercury (55). As a result the ionic balance between the cells and the environment is disturbed.

The transport of ions across absorbing or excreting epithelial membranes are also sensitive to the action of heavy metal ions. The effects of mercury and of organic mercurials on the absorptive and secretory function of the kidney are well known. Another particularly instructive example is the inhibition of active sodium and passive chloride movements across frog skin by mercury (or the organic mercurial, mersalyl) and copper, respectively. At very low concentrations, mercury completely blocks active sodium-transport without interfering with the respiration of the tissue or the passive anion movements (66). Copper, on the other hand, has no effect on active sodium-transport but prevents passive chloride movements across the skin (118).

2. Fluid movements. In epithelial tissues such as frog skin, intestine, and kidney, the movement of fluid is dependent on the transfer of electrolytes (59). Any inhibition of ion-transporting systems is therefore reflected in a reduced fluid movement, *e.g.*, the diuretic action of mercury (inorganic or organic) on kidney. The inhibition of active transport of ions is also associated with water uptake by cells. In the presence of mercury, kidney and liver slices release potassium but gain sodium at a faster rate, and there is an appreciable increase in cell volume (55, 95). In mercury or gold poisoned red cells, swelling may proceed

Despite the fact that less than 1% of the mercury is filterable, studies with a perfused kidney indicate that as much as 8 to 12% of the mercury can be cleared from blood in a single passage (17). For this reason, the fixation of mercury must be assumed to occur largely from outside the tubule, rather than from inside following glomerular filtration. Some evidence on localization obtained from radioautographic techniques has shown that most of the deposited mercury is found in the renal cortex (17).

More information is available concerning the specific renal effects of mercury in the form of organic compounds (the mercurial diuretics) than in the form of inorganic salts of mercury. Although it is not clear whether organic compounds act by liberation of inorganic mercury, or act as the original undissociated molecule, it seems safe to assume that in either case the mercury will combine with the same kinds of receptors (sulfhydryl) to produce similar responses (see discussion on organic mercurials). The main difference is perhaps one of the local effective concentration of metal. The inorganic mercury, in addition to its diuretic action, produces more drastic renal responses of a toxic nature.

Quantitative analysis of the action of mercury in physicochemical terms is complicated enough in isolated cellular systems and becomes even more difficult with the additional factors within the whole animals. Nevertheless, the studies with enzymes and with intact cells can serve as a model for the qualitative action of mercury on the kidney cells.

VIII. THE NATURE OF THE METAL-INDUCED RESPONSES

In the previous sections, the responses of biological systems to heavy metals were considered in terms of inferences that could be made concerning the site of the original chemical insult. Yet knowledge of the spectrum of responses may be useful in itself, for it sets the limits for any attempt to categorize and to predict the pharmacological actions of the metals.

At the cost of some repetition, a listing and brief discussion of the most commonly seen responses will be given. Since a metal can disturb any existing biological activity, theoretically as many kinds of responses can be found as there are activities. Nevertheless, because of the factors of anatomical structure and the nature and accessibility of biological components, certain types of responses predominate. One important distinction which can be made, provided that adequate information is at hand, is the distinction between primary effects and secondary effects. This has already been pointed out in the discussion on direct and indirect all-or-none responses but applies equally well to all responses. The primary response is the reduction in activity of the site with which the metal binds, whereas the secondary response is the reduction in activity of another system, dependent in some manner on the binding site. In the first case, the response is determined by the factors governing the metal distribution, and by the relationship of binding site and function. In the second case, the course of events is determined by the interrelationships between various cellular functions. Such secondary reactions are frequently of utmost pharmacological

significance because a seemingly insignificant primary defect may be amplified through a sequence of reactions into considerable biological damage.

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3. Changes in transmembrane potentials. Changes in ion permeabilities are usually associated with changes in transmembrane potentials. In frog skin, active sodium transport generates an electric potential difference between the two surfaces of the membrane (sodium battery). The sodium potential provides the driving force for a passive chloride movement. If the sodium pump is made inactive by mercury, the potential falls to zero. On the other hand, if the membrane is made impermeable to anions by copper, the potential rises. Similar observations have been made with the rat intestine (p. 209), and with the synovial membrane in the knee joint of the dog (50).

Other actions of heavy metals which influence bioelectric phenomena include the blocking of nerve conduction by copper and other SH-reagents (111) and the inhibition of junctional transmission by lead at the motor endplate (58).

B. Blockage of substrate supply

Various metal ions are known to inhibit enzymes located on the surface of the cell (p. 197) (*e.g.*, phosphatases of yeast cells and erythrocytes, or the invertase of yeast cells). These surface enzymes fulfill important functions as digestive enzymes (97). They split non-penetrating substances (*e.g.*, phosphoric acid esters or sucrose) into substrates which can pass through the cell membrane. Inhibiting metals can shut off the supply of such products.

Perhaps of greater importance to cellular nutrition are the specific transfer-systems in the membrane which are responsible for the penetration of important substrates such as sugars or amino acids. Many of these transfer-processes are sensitive to metal ions. For example, glucose uptake by erythrocytes (61, 126) and muscle can be completely blocked by low concentrations of copper and mercury, and glucose transfer into yeast cells is very sensitive to uranium.

C. The breakdown of the permeability barrier

Yeast cells, bacteria, and plant cells are surrounded by a rigid cell wall which presents osmotic swelling and disruption of the cell. Nevertheless, mercury produces a complete disintegration of the permeability barrier of the cell which leads to a depletion of ions, substrates, and soluble enzymes, and eventually results in cellular death (p. 204).

D. Changes in surface charge of cells

Heavy metals, by combining with surface ligands, can change the net surface charge. In fact, at a certain metal concentration, characteristic for each species of metal ion and for each type of cell, a reversal of charge occurs.[4] Blood cells remain dispersed in the plasma, partly because of their negative charge. This suggests that although the reduction of surface charge may be a necessary prerequisite for the cells to come in close contact with each other, the aggregation itself may be due principally to direct cross-linking rather than to a simple reduction of charge or potential.

Concentration gradients of ions exist within the electric double layer surrounding the charged cell surface. In the immediate neighborhood of the cell surface, therefore, the ion concentrations are different from those of the bulk phase. The surface concentrations can be estimated from electrophoresis data. The concentration of monovalent cations in the immediate surface of red cells, for example, is about two to three times (0.3 pH unit) higher than in the bulk phase of the surrounding saline solution (pH 7.2). Alteration of the surface charge will shift the distribution coefficient for any charged particle (including H^+) (88). It has been suggested that the inhibition of glycerol permeability of red cells by copper and other heavy metals is related to changes of surface pH.

[4] By observing a series of metals the so-called "charge reversal spectra" can be constructed. By comparing these spectra with those of known biochemical substances like cholesterol, lecithin, and others, information concerning the chemical composition of the surface layers of various types of blood cells (polymorphonuclear leucocytes, lymphocytes, erythrocytes, platelets) and bacteria (*E. coli*) has been obtained (7, 8).

This contention is supported by the observation that changes in the pH of the medium influence glycerol permeability in a way similar to the heavy metal ions (124).

At the present time, it cannot be unequivocally decided whether or not alterations of surface charge and secondary changes following them, play a significant role in metal toxicity. Changes of surface charge should produce the same pattern of responses for all metals, which is indeed contrary to most observed effects of metals on cells.

E. Actions within the cell

Although the cellular membrane acts as a diffusion barrier to metals, nevertheless certain metals will eventually penetrate. The disturbances resulting from metals inside cells have not been extensively investigated, but it is known that different metabolic pathways may exhibit marked differences of sensitivity toward a given metal. For example, in liver homogenates, lead inhibits the Krebs cycle but not the glycolytic activity (5). Potentially, all enzyme systems are susceptible to metals, but factors of diversion to "inert sites" and of accessibility of "sensitive sites" preclude predictions. The inhibition of respiration in muscle by mercury and copper has been discussed, but the metals may also activate the metabolism (p. 206). Structures within the cell are certainly of importance. By inference, inhibition of the Krebs cycle by tin can be attributed to the action of metals on mitochondria (9), and alterations in protein synthesis by cadmium (33) could be attributed to action on microsomes. Membranes within the cell, as well as the outer membrane, may be attacked by metals. With the alga, *Nitella*, mercury acts on the outer membrane (cell membrane or plasmalemma) if the medium is water. On the other hand, if KCl is present, the outer membrane is protected (presumably by the formation of mercury-chloride complexes) but the mercury penetrates the cytoplasmic layer and injures the vacuolar membrane (tonoplast) (82).

F. Organ specific responses

In the bodies of higher animals, the blood and interstitial fluid may provide a medium of relatively uniform composition, exposing all tissues to approximately the same concentrations of absorbed metals. Nevertheless, for each metal, a certain organ is often more susceptible than the others. Even within the organ, certain cells are damaged and others are not. The differences in susceptibility can be attributed to two factors: 1) certain cells are inherently more sensitive to the metal, and 2) local changes in the effective concentration of the metal may result from activities within the organ.

The susceptibility of the tubular cells of the kidney to mercury and uranium has been attributed to local factors, which lead to a high rate of deposition of the metals. In the case of lead, the effects on hematopoiesis can also be correlated with local deposition in the reticuloendothelial system. It seems likely that the distribution in this case is associated with the colloidal state of lead in blood (22). The effects of cadmium on the liver and the kidney are also related to local distribution (112).

In some cases, no obvious correlation can be seen between the localized distribution of the metal and the specific toxic effect on certain organs. For example, prolonged exposure to mercury can produce lesions of the central nervous system, yet the concentration of the metal in brain is never higher than that in the bulk of the soft tissues (103).

G. Generalized responses

In many cases, the responses are nonspecific, in the sense that many kinds of primary lesions could lead to the same end result. Examples are the failure of colony formation in microorganisms or tissue culture cells, and weight loss or mortality in animals. These responses are obviously secondary in the sense that they represent the termination of sequences of reactions induced by the primary metal-lesion. The relationship of response to lesion is not predictable at the present time, but the response itself is no less important from a pharmacological point of view.

IX. GENERAL DISCUSSION AND CONCLUSIONS

Ultimately, useful generalizations must allow predictions of behavior of the metals in biological systems. Because the heavy metals constitute a chemically heterogeneous group of elements, it is doubtful whether generalizations based on their chemical properties would be helpful. In fact, an examination of available information suggests that unpredictability rather than predictability is the general rule, with each metal a more or less unique situation. For example, it might be expected that elements in a given group of the periodic table would behave similarly, or that elements with a high affinity for particular ligands, such as sulfhydryl groups, might behave similarly. However, this is not always the case either at the level of the intact animal, the tissue, the cell, or even the enzyme where the chemical situation is more clearly defined. A few illustrations can be given, some of which have been described in more detail elsewhere in the review.

Zinc, cadmium, and mercury are in the same group of the periodic table. In the whole animal zinc is relatively non-toxic; in fact it is an essential element, whereas cadmium and mercury are very toxic. However, the patterns of toxicity and metabolism are not at all similar. Cadmium, for example, induces the excretion of a unique abnormal protein, not seen with any other poison (33). Mercury produces K^+-leakage in yeast and red blood cells, but zinc and cadmium do not (84, 121). In enzyme systems, zinc is, in some cases, an activator or essential cofactor, whereas cadmium and especially mercury are inhibitors.

Mercury, copper, lead, and silver have high affinities for sulfhydryl groups, yet sometimes they behave quite differently in biological systems. For example, mercury reduces the membrane potential of the frog skin or synovial membrane by inactivating the sodium-pump, whereas copper increases the potential by reducing the permeability to anions (66, 118); mercury induces K^+-leakage in yeast cells, but lead does not (84); lead and mercury both induce K^+-leakage in red blood cells, but copper does not (121); silver and copper both inhibit the activity of the enzyme invertase, but the mechanisms of action are completely

different (77); lead produces K^+-loss in human and rabbit red blood cells, but not in horse and pig, whereas mercury produces K^+-loss from the cells of all species (51).

The simplest biological response, the inhibition of the activity of an enzyme, can be described in terms of metal affinities for particular ligands and other physicochemical parameters. Even at the level of the cell, some responses can be related to metal-fixation by particular enzymes and can therefore be expressed in terms of simple chemical parameters, but for the other responses, the basic chemistry of the interaction is obscured to a greater or lesser degree. The universal chemical reactivity of the heavy metals leads to interactions with such ubiquitous ligands as sulfhydryl, carboxyl, amino, imidazole, or phosphoryl groups. Thus, many different cellular functions will be inhibited by the same metal and large quantities of the metal will be diverted to physiologically inert binding sites. Since the metal potentially can interact with almost any accessible ligand, the location of the various binding sites within the cellular structure, as well as the presence of diffusion barriers, decisively influences the nature and time course of metal actions on cells. Thus, the structural and functional organization of the cell, modified to a degree by the chemical properties of the metal, is the predominant factor in determining the patterns of toxicity, with inhibition of functions associated with the outer cell surface being of special importance.

In tissues and whole animals, additional complications are imposed by the nature of biological organization, so that analyses of chemical mechanisms of metal action are indeed difficult. The metal-sensitivities of different populations of cells may be quite different. In addition, the exposure of the different cells to the metal depends on anatomical arrangements and on local environmental conditions. For example, in the kidney, the pH in tubular urine is different from that in the blood, and proteins are largely absent, favoring interactions between metals and receptors in the tubular cells. At the level of the whole animal, the basic chemistry is almost lost in a sea of complications: the dynamics of absorption, distribution, and excretion. For the present point of view, it can be considered that the state of biological organization distorts the primary response of the "sensitive" cellular sites to some degree, regardless of the metal studied. In fact, the useful generalizations that can be made are concerned with the nature of such distortions, rather than with the chemical reactivities of specific metals.

The role of cellular structure and tissue organization is no less important for other groups of chemical agents than it is for the heavy metals. They too must act from the outside of the cell toward the inside; their toxic action is modified, as in the case of heavy metals, by the structural and functional properties of the cell; their concentration in the neighborhood of the sensitive cells is influenced by phenomena of absorption, distribution, excretion, and local conditions. Only the relative importance of the different factors will be altered. Some organic chemical agents may be much more specific in their action. They may act only on one or a few kinds of specialized receptors. At the same time the possibility of diversion is far less. Perhaps the best known examples are the highly specific

and toxic cholinesterase inhibitors. On the other hand, with organic substances, metabolic destruction, which, in a sense, is another form of diversion, commonly occurs.

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