

METALS IN AQUATIC ECOSYSTEMS: MECHANISMS OF UPTAKE, ACCUMULATION AND RELEASE-ECOTOXICOLOGICAL...

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Metals are essential cofactors for bio-chemical processes, including oxidative phosphorylation, gene regulation and free-radical homeostasis. Abnormalities in metal ions concentration in organisms, lead towards the eco-physiological hazards in the aquatics. In aquatic organisms, metal absorption involves transfer of metals to the circulatory system following: uptake by the apical membrane; movement through the cell as well as interaction with intracellular ligands and efflux across the basolateral membrane. Sequestration of metals in an immobilized form occurs throughout the various tissues and organs involved in pathways for metal uptake, transport, utilization and release. Metallothioneins, metal binding proteins, regulate the essential metals (Cu, Zn), detoxify the toxic metals (Cd, Hg) and play key role to the sequestering metals. Plasma proteins play the central role in metal transport in the vertebrates. Besides, blood cells in aquatic invertebrates, hemocytes in mollusks concentrate a variety of metals. Aquatic organisms utilize renal, digestive pathways and diapedesis for the excretion of a variety of metals.

This paper briefly reviews current information about the processes associated with metal uptake, transport, release, accumulation in the aquatic organisms and discuss about eco-toxicological aspect of bio-accumulation and suggest further necessary researches for future endeavor.

Keywords: Metal uptake and release; accumulation; metallothionein; aquatic organisms; eco-toxicology

1. INTRODUCTION

Aquatic ecosystems are naturally exposed to a variety of metals whose chemical forms and concentrations are governed by natural geochemical processes and anthropogenic activities. These metals include both essential elements required to support biological processes and non-essential metals with no known biological function. Cellular functions are critical to processes involved in metal uptake, regulation, utilization and accumulation. Toxicity can be attributed to their dysfunction and the resultant interaction of metals with inappropriate cellular structures. Investigations at the cellular level will be advance our understanding of the mechanisms by which aquatic animals respond to metal exposure.

Attempts to discern general patterns of response to metals in aquatic animals are complicated by the wide variety of species, most of whose fundamental biology is incompletely documented [1, 2]. Here, we focus on mechanisms associated with metal uptake, internal transport, intracellular storage and release and attempt to derive unifying principles for cellular and biochemical responses that underlie recent observations.

Metal ions are essential cofactors for a wealth of biological processes, including oxidative phosphorylation, gene regulation and free-radical homeostasis. Failure to maintain appropriate levels of metal ions in higher animals, lead towards the physiological hazards starting from disorders of metal ions deficiency to hereditary haemochromatosis [3]. Despite their pivotal physiological roles, however, there is no molecular information on how metal ions are actively absorbed by cells. A generalized model of metal sources, transportation to aquatics is shown in Figure 1. Metal uptake, translocation, storage and release in aquatic animals is shown in Figure 2. The gills, digestive tract and integument represent the sites of metal uptake. Metals are subsequently, transported to internal organs for utilization, storage and release. Components of the blood and hemolymph are the carrier for this transport. Cellular mechanisms associated with various organ systems operate in a highly integrated way to co-ordinate metal uptake, transport and release.

2. BIO-AVAILABILITY OF METALS AND FACTORS CONTROLLING METAL UPTAKE IN AQUATIC SYSTEM

Metals are generally of two kinds -- "Borderline" and "Class B". The "Borderline" and "Class B" metals and metalloid ions [3] are of specific interest here. Often referred to as "heavy" or "trace" metals in general nomenclature, include ions such as As^[sup 3+], Cd^[sup 2+], Co^[sup 2+], Cr^[sup 2+], Cu^[sup 2+], Fe^[sup 2+], Fe^[sup 3+], Ga^[sup 3+], In^[sup 3+], Mn^[sup 2+], Ni^[sup 2+], Pb^[sup 2+], Sb^[sup 3+], Sn^[sup 2+],

Sn^[sup 4+], Ti^[sup 2+] and V^[sup 2+] as borderline ions and Ag^[sup 2+], Au^[sup 2+], Bi^[sup 3+], Cu^[sup +], Hg^[sup 2+], Pb^[sup 4+], Pd^[sup 2+], Pt^[sup 2+] and Tl^[sup 3+] as class B ions. Their nitrogen and sulfur seeking properties contribute to their ability to form complexes with such centers in biologically important ligands [4]. Each is potentially toxic, if present at sufficiently high concentrations, and can occur in the aquatic system as anthropogenic contaminants.

The chemical speciation of metals in aquatic system is dependent on the specific physical/chemical factors that prevails in the local environments. Factors such as salinity, dissolved organics, pH, hardness and sedimentary load all influence the prevailing chemical forms of metals in the aquatic systems. These, in turn, influence the metal bio-availability and toxicity.

The relative importance of the bio-availability of other chemical forms of dissolved metals, particularly organically chelated forms, is not as clearly understood [5, 6]. The presence of organic ligands in the surrounding water is reported to increase Cd bio-availability in the mussel *Mytilus edulis* [7]. In fish, complexation with various hydrophobic ligands such xanthates, diethyldithiocarbamates and dithiophosphates can also increase Cd bio-availability [8, 9]. The likely explanation of these observations are related to the enhanced hydrophobic characteristics imparted by the ligands and the resultant increased potential for dissolution of the complex into membrane lipids [10]. Organometallic compounds, such as the organomercurials and organotin are derived from covalent binding of the metal and organic moiety and represent the best known examples of such behavior [11]. Generalizations regarding the influence of ligands of bioavailability of metals must take into consideration the chemical nature of specific metals/ligands complexes. The extensive data for mammals indicate that a number of naturally occurring organic metal-binding substances (L-amino acids, citrate, phosphate, gluconate, oxalate) and synthetic metal chelators (nitroacetate, EDTA) enhance intestinal uptake of Cu and Zn [12].

With Hg and Cd, diffusion of the neutrally charged chlorocomplexes HgCl₂ and CdCl₂ across an artificial lipid bi-layer exceeds that of the ionic Hg^[sup 2+] and Cd^[sup 2+] [12, 13]. Thus, even in the absence of organic chelation, the ionic form cannot be categorically stated as the sole biologically significant form of metals. The magnitude of difference between diffusion of the charged and neutral forms is much less with Cd than with Hg, however, and uptake of Cd^[sup 2+] rather than CdCl₂ is generally considered to be more biologically significant on the basis of empirical observations in *vi vo* [13]. These observations have ecological implications because aquatic environments are characterized by differences in the chloride concentration as one moves from fresh water to strictly marine environments. Microenvironments at the sites of metal uptake can differ from the surrounding water and have important consequences on speciation, availability and biological effects of metals [14]. In rainbow trout exposed to Al at low pH, for example, an increase in pH in the branchial microenvironment is attributable to the effect of expired CO₂ and ammonia and results in the reduced solubility of Al and its precipitation on the gill surfaces [15]. This is associated with respiratory ionoregulatory impairment.

In most biological studies, the nature of the external environment with respect to the chemical forms of metals, whether natural or experimental, usually remains a variable that is neither clearly understood nor controlled experimentally. Specific data on speciation are generally lacking for most of the published studies on metal uptake or accumulation. Dissolved and particulate forms follow fundamentally different pathways that need to be more clearly understood. The unique pathways for uptake of ionic and organically chelated forms of dissolved metals need to be identified and characterized [6].

3. METAL UPTAKE PROCESSES

3.1. General Consideration

The absorption of metals by aquatic organism involves transfer of metals to the circulatory system. In animal, transfer of metals to the circulatory system across the epithelial barrier of gills, digestive systems or integument. This transfer across the epithelial cells consist three phenomena: [1] uptake by the apical membrane, the interface with the external environment; [2] movement through the cell and interaction with intracellular ligands and [3] efflux across the basolateral membrane, the interface with the circulatory

system [16] (Fig. 3). Organs that serve as the sites for uptake (e.g., gill, intestine and digestive gland) are also tend to concentrate metals and therefore, exhibit relatively high potentials for bio-accumulation.

Generally, the cellular uptake of metals is a membrane-based phenomenon that can assigned to one of two general schemes, depending on whether the uptake is based on membrane transport or the endocytotic processes of phagocytosis or pinocytosis. Dissolved metals would be expected to be taken up by exposed body surfaces such as the gills. Particulate metals are most commonly ingested and then taken up after solubilization in the gut. They can also phagocytosed and solubilised in endocytic vehicles.

Uptake of metals differs in vertebrates and invertebrates. The invertebrates possess the capability for both extracellular and intracellular digestion, while the vertebrates are considered to rely on the former [17, 18]. So, metal uptake by endocytosis would be expected to be a process of greater significance in the various invertebrate species. Once endocytosed, the particulate metal complexes can be broken down and the metal redistributed to other intracellular ligands. In vertebrates, metal complexes must be broken down to more simple structures in the intestinal lumen before uptake via various membrane-dependent transport pathways can occur.

3.2. Epithelial Uptake of Metals

The gills [19-22] and intestine [23] are the primary sites for uptake of soluble metals from the aquatic environment. In soft-bodied invertebrate species, the body wall may also be an important site of soluble metal uptake. Rapid binding to intracellular ligands [20, 22] and the efflux of metal ions across the basolateral membrane can diminish concentration gradients and therefore, the need for the active transport mechanisms at the apical membrane. Diffusion or carrier-mediated pathways are the more likely candidates for the cellular uptake of soluble metals [16, 24].

Uptake of essential metals, such as Ca, Cu, Fe and Zn, often involves specific pathways and is central for meeting metabolic requirements. For example, Ca channels [25] and specific membrane carriers for Fe and Cu [26] are involved in the uptake of these elements. Their uptake exhibits temperature dependence and in some cases (e.g., Cu and Zn), saturation kinetics consistent with carrier mediated pathways [27-29]. The apparent $K_{0.5}$ of 23 μM is in the toxic range for zinc, indicating that uptake is not specific for Zn. Examination of gills by electron microscopy suggests that the site of Zn uptake in rainbow trout gills is localized in chloride cells [30].

Nonessential, toxicologically significant metals, such as Cd and Hg, are not considered to have specific uptake mechanisms and appear to behave adventitiously, following existing pathways for essential metals. Cadmium, for example, can traverse model membrane in the ionic form [13] and has been shown to be taken up through Ca channels in the pituitary cells [31] and hepatocytes [32] of mammals and the gills of fish [17] and mollusks [33]. With Hg, studies on model membranes suggest the possibilities of diffusion of neutrally charged chloro-complexes as one pathway [12, 34]. In oyster and mussel gills, both Hg and Cd uptake are inhibited by 2-4-dinitrophenol, an uncoupler of oxidative phosphorylation [21, 33, 35]. In these cases, the requirement for energy is probably not for support of active uptake against concentration gradients, but rather for the function of structures involved in the uptake [33]. For example, some Ca channels may be dependent on protein kinases for their function [36].

Transmural transport across epithelial cells involves intracellular ligands responsible for moving metals from the apical to basolateral membranes. These ligands may serve as specific transporter molecules, whose characteristics are not well understood. Early studies implicated metallothionein in the intestinal absorption of Zn, Cu and Cd in mammals. Induction of intestinal metallothionein by elevated dietary levels of these metals was initially reported to increase absorption and enhance accumulation in internal organs such as liver and kidneys [11, 37, 38]. However, the high affinity of metals for metallothionein and the increased turnover rates conferred by this affinity have been difficult to reconcile with a transmural transporter function. Metallothionein-bound metals tend to accumulate in cells of the intestine, resulting in elevated total metal concentrations. Moreover, there is evidence that intestinal metallothionein induction will decrease the release of Cd from the intestine and its accumulation in the internal organs such as the liver [39]. Nevertheless, the concomitant induction of intestinal metallothionein, enhanced metal binding by

this protein and increased metal concentrations by organs such as the kidney have been used as evidence that metallothionein-bound metal complexes are released from the intestine and subsequently transported to internal organs [11, 37, 38].

Identification of cysteine-rich intestinal protein (CRIP) [40] as a transmembrane Zn-transporting protein in mammalian intestinal epithelial cells [41] has shed new light on intracellular mechanisms associated with transmembrane metal transport and casts doubt that metallothionein is an intracellular metal transporter. CRIP binds newly taken-up Zn and facilitates its intercellular transport to the serosal side of the intestinal epithelium by delivering the metal to outward transport mechanisms in the basolateral membrane [42]. The role of CRIP in intracellular Zn transport and its relationship to metallothionein is depicted in Figure 4. CRIP has been proposed to work in conjunction with metallothionein. Metallothionein can successfully compete with CRIP by virtue of its induction by Zn and its greater metal affinity. Binding of Zn to metallothionein tends to reduce binding to CRIP, retard transmembrane transport of the metal to the blood and increase metal retention by the intestinal cells. These observations are consistent with a role of metallothionein in intracellular metal sequestration rather than metal transport. The coordinated behavior of CRIP and metallothionein may effectively regulate the absorption of Zn.

In winter flounder intestinal mucosa, the cytosolic proteins include multiple systems for binding Zn^[sup 2+] [43]. These may include a protein similar to CRIP in addition to the Zn-inducible, intestinal metallothionein previously characterized in fish [44].

Once taken up by epithelial cells and transported to the basolateral membrane, the mechanisms for efflux of metals from epithelial cells to blood or hemolymph have yet to be clearly delineated. Zinc transport across the basolateral membrane of trout gills is proposed to be mediated by Ca-ATPase [28]. In the Tilapia intestine, Cd transport across the basolateral membrane follows pathways for Ca that involve Ca-ATPase and Cd-Ca exchange [23]. Because little unbound metal is expected to occur within cells, transporter molecules such as CRIP would be expected to present metal ions to efflux mechanisms that would, in turn, supply metals to transport molecules (e.g., albumin's in mammalian plasma) in the circulatory system.

3.3. Metal Uptake through Endocytosis

It is well known that endocytosis is used by invertebrates for uptake of materials from the environment [45]. The digestive cells of most invertebrates are capable of phagocytosis and intracellular digestion of food particles. Additionally, external organs, such as gills, are also capable of phagocytosis of particles from the environment. Metals associated with particles have been shown to be accumulated by endocytotic mechanisms in various invertebrate species [45]. Once metals are brought into a cell by endocytosis, they are incorporated into lysosomal vesicles and subsequently follow intracellular pathways that are only partially understood [46]. Pathways for absorption into the blood may include transfer of the particles or vesicles to blood cells or release of vesicle contents directly into the blood plasma. However, such pathways remain to be elucidated. Although considered to be a viable mechanism, there are only a few examples implicating endocytosis in the uptake of metals from the environment [6]. Colloidal ferric hydroxide [47] and Pb [45], for example, are taken up by the endocytosis by mussel gills. More likely, endocytosis is the mechanism that is used in assimilating metals from food in the digestive tract of invertebrates, although specific examples to support such a hypothesis are lacking. Quantitative assessments of the contribution of endocytosis as a pathway for metal uptake are currently lacking.

4. CELLULAR STORAGE AND SEQUESTRATION

4.1. General Considerations

Sequestration of metals in an immobilized form occurs throughout the various tissues and organs involved in pathways for metal uptake, transport, utilization and release. Sequestration by any one compartment in the chain may be temporary, a single step in the sequence of events that starts at the sites of uptake (gills, intestine, integument) and proceeds to the sites of detoxification and either long term storage or excretion (i.e., liver or equivalent organ and kidney). Two of the best-studied intracellular structures involved in metal sequestration and storage are the metallothioneins and the intracellular vesicle-bound granules.

Undoubtedly, they interact in coordinated functions in those tissues where both are present and where each has been shown to play a significant role in metal binding and sequestration.

4.2. Metallothioneins

Metallothioneins are a family of low-molecular-mass, metal-binding proteins believed to function in the regulation of the essential metals Cu and Zn and in the detoxification of these and nonessential metals such as Cd and Hg [48, 49]. Their role in sequestering metals are well established and their induction by metal exposure are associated with conferring protection against metal toxicity. Studies on the regulation of metallothionein gene expression have firmly established that induction by metal is a direct response to increase in the intracellular metal concentration and mediated through the action of transacting metal-binding regulatory factors [50].

The capacity for metallothionein induction is greatest in tissues that are active in metal uptake, storage and excretion. In aquatic animals, metallothioneins have been identified in the small intestine [44], liver [51-53] and gills [54] of fish and in the digestive gland [55, 56] and gills [21, 57-59] of mollusks and crustaceans. This induction results in relatively high concentrations of metallothionein-bound metals in these organs and in cases such as Cd, results in a slower turnover of the metal. Thus in the mammalian intestine, binding to metallothionein is now considered to retard transfer of metals to the blood, [39, 42, 60] with the intestine serving as a filter for the subsequent translocation to internal organs. Bindings of metals to induced metallothioneins in the gut and gills of aquatic animals probably has an effect on metal absorption similar to that noted above for the mammalian intestine. Binding of metals to metallothionein enhances bio-accumulation in these organs, as well as in the liver and kidney. Whether a protein analogous to CRIP may co-occur with metallothionein and function as a metal transporter in the intestines and gills of aquatic animals remains to be determined.

It is clear that the protein has a central role in regulating the bioavailability of intercellular Cu and Zn for essential cell function. Metallothionein can donate Zn or Cu to metalloproteins, such as carbonic anhydrase, pyridoxal kinase and hemocyanin, [61-63], and activate them. Thionein, the apoprotein form, can remove metals from Zn-finger proteins [64, 65]. Inactivation of these transacting regulatory factors through this later mechanism has been proposed to function in the regulation of gene expression. In mammals, fish and invertebrates metallothionein synthesis is developmentally regulated [66-68] and possibly, involved in the regulation of gene expression by controlling Zn availability to regulatory factors. Its synthesis is also regulated in the higher invertebrates by a number of factors apart from metals [50]. The normal function of metallothionein are critical to the cell and are superimposed on any toxicological response.

Increased rate levels of metallothionein and metals bound to metallothionein appear to be characteristic of exposure to Cd, Cu and possibly, Zn in aquatic animals. Although yet to be confirmed, the cellular interactions involving metallothioneins can be expected to follow two general lines, the first being the interception and binding of metal ions that are initially taken up by the second being the removal of metals from nonthionein ligands that include cellular targets of toxicity. The later may represent a 'rescue' function for structures that have been reversibly impaired by inappropriate metal binding [69]. It still remains to be determined whether metallothionein induction may interfere with the normal regulation of metal-dependent systems. In this context, metal-induced thionein synthesis may result in an increased pool of ligands that can inappropriately remove essential metals from active sites of other molecules and possibly interfere with cellular function.

Measurement of metallothionein induction has been proposed as a cellular indicator of metal exposure and toxicity in aquatic animals [59]. This induction confers enhanced metal tolerance to both cells [70-72] and intact individuals [73-77]. Because the organism is protected, it does not succumb as readily to metal toxicity and coupled with the relatively long turnover time for metallothionein-bound metals, higher burdens of metals can be accumulated than would otherwise occur. Thus, one of the correlates of metallothionein induction is that an increased metal burden can be tolerated by the individuals. A possible consequence of this increased capacity for sequestration is an increase in the potential for trophic transfer of metals. Such compensatory mechanisms for responding to metal exposure may have as yet undermined ecological or public health consequences, the latter when the species affected is a human food source.

The ready manipulation of the metallothionein gene has made possible prospects for constructing transgenic organisms possessing a metallothionein promoter and an appropriate receptor for easy quantification of gene expression. For medaka, transgenic fry with a trout metallothionein-A or mouse metallothionein-I promoter and chloramphenicol acetyltransferase (CAT) reporter have been developed through recombinant techniques [78]. Metal exposure of these individuals results in a high CAT activity that is mediated initially through the metallothionein promoter. Further development and refinement of this and similar transgenic systems are expected to lead to a greater understanding of metallothionein function and the possibility for metallothionein-based molecular monitors for metals in the aquatic environment.

4.3. Intercellular Deposits

All aquatic animals contain a wide variety of membrane-bound intracellular deposits, many of which bind metals. Classified as either 'granules' or 'concretions', these structures are generally associated with the digestive or excretory tissues of invertebrates (i.e., mid gut, digestive gland, hepatopancreas, malpighian tubules and kidney). They are also found in the connective tissue of both vertebrates and invertebrates (e.g., Asifofuschin granules), as well as in specialized cells of some organisms (e.g., Cu-containing pore cells in gastropods). The metal content of these various granule types varies considerably.

Brown [69] identified three types of Cu⁺, Fe⁺, and Ca-containing granules. Copper-containing granules are composed of phosphorus and Cu, but little additional metal besides some Fe. These rather homogeneous granules are limited to arthropods and may be involved in Cu regulation. Ca-containing concretions can be subdivided into the Ca carbonate and Ca phosphate types [46, 69]. Calcium carbonate granules are often found in the connective tissue of arthropods, gastropods and apparently, serve as an easily mobilized store of Ca. These granules may be formed by the mineralization of an organic template produced by the Golgi [79]. Calcium carbonate granules are probably not important for the physiological regulation of heavy metals, although some metals such as Pb may substitute for Ca ions to a limited degree [46].

Calcium phosphate granules are ubiquitous throughout the invertebrate phyla and are thought to play a major role in metal detoxification (and possibly elimination) [46, 79]. These granules are primarily composed of Ca and Mg phosphates, but they can incorporate high concentrations of such metals as Al, Ag, Ba, Co, Fe, Mn, Pb, Sn and Zn. The ratio of (Ca + Mg): P varies considerably between species and individuals [80-81]. The amount incorporated of each metal is highly variable, even within the same species, and probably relates to variable environmental metal concentrations [46, 82]. These granules are generally have a low percentage (< 10%) of organic matter [82], although granules in the kidney of *M. edulis* are an exception to this pattern, with 46% metal [69]. Metal-rich Ca phosphate granules are generally associated with digestive and excretory tissues [69]. The granules are usually 0.2 to 3 μm in diameter in most species, although 40-nm diameter particles have been identified in the hemocytes of a number of marine bivalves [83]. Exceptionally, large granules of 10 to 15 μm and up to 1.9 mm diameter have been reported in some bivalve species [82]. These large granules are extracellular and can continue to grow after exocytosis or holocrine or apocrine secretion. The granules can be composed of pyrophosphate or orthophosphate, depending on species [84]. Both types are virtually insoluble in saline, making them excellent 'sinks' for immobilizing metals in a nontoxic, unavailable form.

The initiation of intercellular granule formation is not well understood. No single mechanism may account for the diversity of granule types observed in aquatic organisms [85]. In some species, an organic matrix or template may first be produced by the endoplasmic reticulum or Golgi and housed within a membrane-limited vacuole. This template can then be mineralized [85, 86]. Further evidence supports the view that the cellular lysosomal system is involved in most instances of granule formation [46, 69]. Lysosomal systems are present in virtually all vertebrate cells and are particularly well developed in digestive and excretory cells such as those of the molluscan digestive gland and kidney and arthropod hepatopancreas. Particulate material, such as food, foreign particles, membranes, cellular organelles and cytoplasmic proteins are endocytosed and incorporated into secondary and tertiary lysosomes. Metals associated with these materials would be similarly incorporated. In mussel kidney, the breakdown of metallothionein/metal complexes results in incorporation of Cu, Cd, Hg and Zn into lysosomal granules [87]. Similarly, ferritin, complexes are

broken down in the lysosomal system of mammalian liver cells and produce hemosiderin and residual bodies rich in Fe [88]. In lysosomally formed Ca phosphate granules, lipofuscin is generally the organic material left after membrane decomposition. Lipofuscin may prove to be the primary organic constituent of all granules that are produced via the lysosomal system, regardless of the type of tissue where the granules are produced or the metal content of the granules. In the bivalve *Mercenaria mercenaria*, lipofuscin was identified histochemically in both kidney granules with high metal concentrations and digestive gland granules with much lower metal contents [82, 88]. Any metals bound to the membrane would be incorporated into residual body during lipofuscin formation. Both Cd and Zn bind weakly and reversibly to lipofuscin by a passive adsorptive process [89].

Accretion of metals to the granule periphery has been reported both *in vivo* and *in vitro* [87, 89, 90]. The concentric rings observed in granule sections by electron microscopy have been interpreted as alternate periods of surface accretion and resorption [91]. Known changes in pH inside the secondary and tertiary lysosomes may also favor metal deposition onto the granule surface [92]. Active transport of vesicles from the endoplasmic reticulum across the vacuolar membrane and precipitation of vesicular contents onto the growing granule surface have also been proposed as a mechanism of granule accretion [93]. Accretion of metal ions onto vesicle-bound granules may also occur by a surface absorption/corrosion process that involves the binding of metals to sites where Ca has been dissociated during a H⁺-induced dissolution of the granule surface [94] or where Mg has been released from the granules [95].

4.4. Other Mechanisms

The only other well-characterized system for the intracellular storage and sequestration of metals involves ferritin, a 450-kDa protein that is ubiquitous in vertebrate (and probably invertebrate) tissues [96]. In the invertebrates, ferritin has been isolated from body tissues of crustaceans, chitons, gastropods, cephalopods and bivalves [97-99]. Circulating ferritin has been measured in the blood plasma of both chitons and limpets [100, 101].

5. INTERNAL TRANSPORT OF METALS

5.1. General Consideration

The delivery of metals to internal organs and the subsequent redistribution among organs [102-105] occur as a result of metal transport via the circulatory systems of invertebrates, such as mollusks and arthropods, which possess an open circulatory system and other invertebrate which possess a closed circulatory system. The nomenclature used to describe analogous components of the respective systems are specific for each. However, for convenience and in recognition of function similarities, we use a general terminology when describing structures that comprise the circulatory system [106]. Thus, for the invertebrates, the term 'blood' and 'hemolymph' are used synonymously, as are 'blood cells' and 'hemocytes'. Vertebrate circulatory structures referred to 'blood' and 'blood cells'. 'Plasma' is the cell-free blood or hemolymph.

It has been known for some time that the plasma proteins of vertebrates play the dominant role in metal transport [107]. The blood plasma contains diverse proteins that transport a wide range of metals. Binding of metals to plasma proteins can be either specific (e.g., Fe by transferrin and Cu by ceruloplasmin) or nonspecific (e.g., Ca, Ni and Zn by serum albumin). Although metals can be taken up by mammalian erythrocytes and leukocytes, these types of blood cells are not considered carriers of metal.

In invertebrates, blood cells and hemocytes have traditionally been considered the primary vehicles for transporting metals. Many types of these cells are highly mobile, phagocytic and capable passing between cells of tissue layers [106]. Certain cell types can harbor high concentrations of metals [107-110]. It is believed that these cells are capable of carrying metals and transferring their metal loads to other tissues, presumably through exocytosis. However, recent evidence indicates that invertebrate plasma proteins may play a much greater role in metal transport than was previously thought, one that is analogous to the situation in vertebrates. Invertebrate plasma proteins bind metals [111-113] and may be responsible for the bulk of the metal transport that occurs in the circulatory system of these animals.

5.2. Blood and Hemocytes

Studies on the role of blood in metal transport in fish are scarce. Thus, in lieu of specific information, mammalian systems currently serve as models for deducing the role of blood cells in metal transport in fish. Mammalian blood cells can accumulate metals. Erythrocytes, for example, readily take up Pb, Zn and Cd [114, 115]. With Cd, 90% of the total Cd loading by blood cells may be bound by metallothionein [116]. Vertebrate blood cells do not appear to be involved in metal transport. Metals in these cells are not readily available for exchange with external receptors [114].

Considerably more data are available on metals in blood cells of aquatic invertebrates, especially in tunicates and mollusks, in comparison with fish. In tunicates, V, Fe, or a mixture of both metals, depending on species, are concentrated in blood cells [108, 109]. These metals are localized in membrane-limited vacuoles in signet ring, compartment, granulocyte and at much lower concentrations, morula cells [106, 117]. In mollusks, hemocytes are known to concentrate a variety of metals [46, 112, 118] which are sequestered by several intracellular ligands. Metal concentrations in hemocytes are much higher than in the surrounding plasma. However, because hemocytes comprise only a small percentage of both the weight and the volume of bivalve blood (e.g., 2.1% of wet weight for *Mytilus edulis*) [119], the actual metal burden contained in the blood cells, expressed as mass of metal, is usually a small fraction of the total body burden and is often much less than the load carried by the plasma [81, 110].

Special mechanisms for concentrating exceptionally high levels of Cu and Zn have been observed in hemocytes of some species of oysters. As a result of this specialization, a higher percentage of the whole blood Cu and Zn load is localized in these cells. In *Ostrea edulis*, 70 to 75% of the hemolymph Cu and 42% to 77% of the hemolymph Zn occur in hemocytes [107]. In the extreme case, 'green-sick' oysters accumulate such high levels of Cu in their hemocytes and tissues that they have acquired a greenish coloration [107]. Crassostreid oysters contain hemocytes that concentrate either Cu or Zn [120]. *O. edulis* also possess separate Cu⁺ or Zn⁺ containing hemocytes [107] with concentrations up to 400 mM Cu and up to 1.2M Zn [121]. A third type of hemocyte that sequesters both Cu and Zn also exists in *O. edulis*, *O. angasi* and *Crassostrea gigas* [121].

Metals in invertebrate hemocytes are often localized in membrane-limited vesicles or vacuoles. These vesicles appear to be part of the cellular lysosomal-system in hemocytes of mollusks and arthropods and so-called 'leukocytes' of tunicates [46, 81, 106]. However, in the colored, vacuolated blood cells of tunicates (i.e., in the signet ring, morula and compartment cells, the vacuoles appear to be specialized repositories of either V or Fe, rather than part of the lysosomal system [106]. In addition to vacuolar storage, metals in hemocytes may be bound to cytoplasmic proteins. Copper, zinc and cadmium are bound to cytoplasmic molecules < 3 kDa and metallothioneins in the hemocytes of mussels [122, 123] and oysters [124].

Turnover rates of metals in invertebrate blood cells vary by species and metal. In the crab *Scylla serrata* injected with Cu-chloride, much of the Cu accumulated by hemocytes 2 hr after injection is released within the next 2-hr period [125]. In contrast, in hemocytes of the hard clam *Mercenaria mercenaria*, elimination of cadmium is very slow and that of Ag is negligible [119]. Slow release of metals from hemocytes would argue against a role in metal transport. Currently, there is no direct evidence to support the hypothesis that metals are transferred from hemocytes to other cells. The prevailing view that metals are transported by bivalve hemocytes and transferred to tissues such as the kidney and digestive gland for eventual elimination is based on proximity of hemocytes to renal and hepatic tissues in histological sections rather than on direct observations of such transfer (Fig. 5).

5.3. Plasma

Plasma proteins play the dominant role in metal transport in mammals [39]. This is probably the case for fish, where even the most primitive of species contain plasma proteins that are homologous to those of mammals [126]. In plasma of winter flounder, binding of Cu and Zn to plasma proteins is both metal and gender related [127]. Copper is bound to proteins of 170 kDa in both sexes. With Zn, about 95% is bound to proteins of 76 kDa in both sexes. In males, the remainder of the plasma Zn is bound to proteins of 186-

kDa, while, in females, the remainder is distributed between the 186-kDa proteins and others of 340 to 370 kDa. The larger proteins in females are hypothesized to be circulating vitellogenin. A novel 66-kDa Zn binding protein has been reported in plasma of albacore tuna [128]. This is increasing evidence that plasma proteins in invertebrates may also have an important function in metal transport.

The role of plasma proteins in Fe transport in mammals is well characterized. Iron must be rapidly bound to proteins in order to protect the organism from the Fe³⁺-catalyzed Haber-Weiss reaction, which reduces Fe³⁺ to Fe²⁺ and produces free OH radicals via the Fenton reaction [129]. In vertebrates, transferrin binds and transports Fe to tissues requiring the metal or to the liver for storage as a ferritin-bound complex [130]. Transferrin can also bind other metals. For example, Cd binds to transferrin at two sites with K_d 's of 10^5 to 10^6 M⁻¹ [131]. Iron in the Fe³⁺ form is strongly bound by both transferrin and ferritin with K_d 's $\geq 10^{22}$ M⁻¹. Thus, a strong redox environment with pH below 5.5 is required to dissociate the Fe from transferrin to ferritin for storage [132]. Transferrin has yet to be identified in any aquatic invertebrate, a "transferrin-like" protein of 41 kDa has been identified in an ascidian [133].

Ceruloplasmin, a 150-kDa μ -globulin, is a specific Cu transport protein in mammals [11, 29]. Ceruloplasmin is secreted by the liver and binds approximately 90 to 95% of the Cu present in the plasma tightly [11]. The remainder is primarily bound to serum albumin. Although the relative importance of ceruloplasmin and serum albumin has been the subject of controversy, the primary role of serum albumin appears to be transport of newly absorbed Cu from the gut to the liver. The lifetime of any Cu-serum albumin association is thus relatively short. Copper is subsequently released from the liver tightly bound to ceruloplasmin [29]. Additional vertebrate plasma proteins that may be involved in metal transport currently include the Cu- and Zn-binding proteins in winter flounder; [127] the 66-kDa protein that binds three molecules of Zn per molecule of protein in albacore tuna plasma [128] and mammalian high molecular weight Cu-containing plasma protein of 270 kDa [134]. Non-protein transport ligands include free amino acids [46].

The examples presented above for vertebrates alert us to the possibility that both specific and non-specific metal-transport proteins may be present in the plasma of aquatic invertebrates. To date, evidence that plasma protein plays a major role in metal transport in invertebrates is still limited. Serum albumin and possibly other plasma proteins identified in mammals, do not occur in invertebrates [126]. However, it is likely that analogous proteins exist in these organisms, and future efforts may demonstrate this. Metal binding to respiratory pigments, apart from that needed to activate the proteins, appears to be a general phenomenon in aquatic invertebrates [91]. Zinc, cadmium and mercury bind to arthropod hemocyanin, [111, 129-138] which has been proposed as the primary Zn transport proteins in the decapod crustaceans [111, 138]. Other high molecular weight plasma proteins that bind Cu, Pb and Fe in crabs [139] and Ca, Cd, Co, Cu, Fe, Mn, Sr and Zn in crayfish [140] may prove to be subunits of hemocyanin [97]. Hemoglobin contained in coelomocytes of the polychaete *Glycera dibranchiata* binds Cd [141].

The evidence presented above indicates that plasma proteins may play a central role in metal transport in aquatic invertebrates and reflects a situation analogous to that in mammals. In view of the greater carrying capacity usually observed in invertebrate plasma, in comparison with blood cells or hemocytes, the plasma may be a far more important component of the metal transport mechanism in invertebrates than has been previously been recognized.

6. EXCRETION OF METALS

6.1. Conception of Metal Excretion

Aquatic organisms utilize a variety of mechanisms to eliminate metals from their bodies. The overall process is a species-specific, organ and tissue-specific and metal and ligand-specific process. In a single individual, the kinetics of metal release are expected to be very complex and to reflect the diverse compartments from which metals must be mobilized. Additionally, physical and chemical parameters, such as temperature and salinity, may affect the rate of release in aquatic animals [142-145]. However, due to the inherent variability in biological systems and the fact that whole body release rates are usually reported,

the observed kinetics of metal release in aquatic species are usually described by a two-compartment model [142, 146]. The two compartment model provides a useful framework for the development of hypothesis based on more complex interactions. In this model, metals are accumulated into a rapidly exchanging compartment, from which metals are easily mobilized and slowly exchanging compartment, in which metals are tightly bound. The amount of metal in each of these depends on a variety of factors, the most important of which is the length of time that has been available for sequestration into slowly exchanging compartments. The easily mobilized metals include those that are adsorbed to external surfaces, complexed to external mucus or complexed to low-affinity intracellular and extra-cellular ligands. Release from these sites may take hours (e.g., Cd) [146], days (e.g., Zn), or weeks (e.g., Ni, Cu, Pb)[147-149]. The tightly bound metals include those that are sequestered by calcified concretions, metallothionein, ferritin and ceruloplasm. Release from these sites depends on the turnover rate of the metal and the ligand and can take months, seasons or years.

The rate at which metals are released appears to be directly related to the rate of accumulation [150,151]. Exposure to high metals concentrations for short periods of time is expected to result in rapid accumulation by the easily mobilized, labile compartment. Low-level, chronic exposure seen in the natural environmental favors slow filling of the tightly bound compartment and release rates are correspondingly slower.

6.2. Pathways of Metals Release

A. Renal Pathways

The renal pathway is the primary route for the excretion of a variety of metals, such as Co, Cd, Sn, Ni, Cr, Mn, Zn and Cu in mammals [152]. Urinary monitoring for free metal ion is used for the clinical diagnosis of Pb, Cd and Hg poisoning [153]. Similarly, the presence of elevated urinary levels of metallothionein, metallothionein-bound metals and proteinuria are indicative of toxic metal induced renal damage [154-156].

Unlike mammals, whose excretory systems are reasonably well characterized, a consensus on the sites of ultrafiltration in aquatic invertebrates does not exist. The antennal and maxillary glands of crustaceans, the branchial heart appendages of copepods have all been identified as sites of ultrafiltration on the basis of morphological evidence [157]. In bivalves, identification of this sites remains controversial. Ultrastructural studies, using low-molecular-weight markers such as colloidal gold, have identified pericardial glands as potential sites of ultrafiltration [158]. It is generally recognized that the primary urine of bivalves travels from the pericardial cavity, which surrounds the heart, to the kidney proper, via the renopericardial ducts. Ultrafiltration in bivalve mollusks allows passages of molecules smaller than 45 to 83 kDA [119, 159]. Because plasma proteins that are smaller than this range are not prevalent, the vast majority of protein-bound metals will probably not pass into the primary urine. Additionally, experiments on *Mercinaria mercinaria* using [97] Cd have shown that 5% or less of the plasma-borne radionuclide is associated with very low-mass substances (< 1kDa), such as amino acids. Thus, the transport of metals to the kidney of bivalves via ultrafiltration may not be a major pathways for metal excretion.

B. Digestive Pathways

A second route for release of metals is through elimination with the feces. To date, no studies have specifically examined fish. In aquatic invertebrates, metals may be released by the discharge of digestive tissue residual bodies. In mollusks and crustaceans, these membrane-limited granules occur as a product of intracellular digestion by lysosomes in digestive gland and hepatopancreas cells. They are prominent features of these cells. The residual bodies can contain a variety of metals, including Ca and Mg, an organic matrix and phosphate ions. Thus, they may be considered to be one of the group of Ca concretions discussed previously. They are released from digestive gland cells by exocytosis during the later part of the normal digestive cycle [160] and are eliminated as part of the feces. It is unlikely that metals accumulated in these residual bodies would be reabsorbed during passage through the gut [161-163].

Lipofuscin granules are also present in a wide range of aquatic invertebrates and vertebrates. These fluorescent granules are thought to be formed by the lysosomal degradation of membranes and are often

found dispersed in pockets within connective tissues or intercalated between epithelial cells. Because they are formed by lysosomal action, they have characteristics similar to the residual bodies formed during intracellular digestion of ingested material. A variety of metals have been shown to be associated with such granules in the digestive gland. For example, Ca, Cd, Cr, Cu, Fe, Mn, Ni and Zn occur in digestive gland granules of *M. mercenaria* [82]. Since more lipofuscin granules are found in older animals, these granules are thought to accumulate with age rather than be eliminated.

C. Diapedesis

Based on histological evidence, several investigators have described a process referred to as "diapedesis", which is defined as a one-way migration of molluscan hemocytes from internal tissues, through epithelial layers and into either the gut lumen or the surrounding water [163, 164]. This process is considered to be involved with elimination. Metals sequestered by hemocytes would be eliminated from the body during this process. Unfortunately, it is very difficult to determine the importance of this route of metal release, hemocytes do not as a general rule accumulate a significant portion of the body burden of metals. The oyster, for which diapedesis has been described, is an exception. More information on the turnover rate of hemocytes and the contribution of diapedesis to this turnover is required before definitive conclusions regarding the significance of diapedesis in regulating metal levels can be made.

7. CONCLUSIONS

Processes associated with metal uptake, transport and release are integrally linked to both environmental conditions and the intrinsic biological functions of organisms. Studies, to date, have identified the importance of the chemical speciation of metals in controlling metal uptake and a number of the biological mechanisms involved in metal regulation and metabolism.

It is clear that metal absorption is a membrane-dependent phenomena associated with the influx and efflux of metals across epithelial barriers. For example, metals often are taken up via pathways that exist for essential nutrients. Thus, while it is known, for example, that Cd can be taken up through Ca channels, [31] the existence of such channels has been reported in uptake organs of both fish [17] and invertebrates [33]. Similarly, cellular mechanisms associated with transmural transport and the function of blood and hemolymph in metal transport by the circulatory system have yet to be studied in sufficient detail to provide an accurate view of the actual processes that are involved. Probably, the need for such information for designing toxicological studies will stimulate greater efforts in elucidating the basic biology of underlying fundamental processes.

Much less is known about the mechanisms of metal release [165] from aquatic animals than is known about metal uptake and accumulation. Of the numerous studies on aquatic invertebrates that have documented metal release, few have examined the actual mechanisms involved in this process. Renal pathways, digestive pathways and diapedesis have received the most attention, although much of the information is still anecdotal. Some metals may also be released with gametes during spawning [162]. It is not clear which of the proposed pathways of metal loss in invertebrates are of greatest significance. While it is clear that much remains to be learned, it is equally clear that knowledge of the various mechanisms involved in metal uptake, accumulation and release in aquatic organisms has advanced considerably over the past few decades. This knowledge is fragmentary, however, concentrating on a few groups of organisms (food chain components), a limited number of metals (e.g., Cd, Cu, Zn) and a handful of metal ligands. Much is known, for example, about vertebrate and invertebrate metallothioneins, the presence and role of other intracellular metal-binding proteins and transmural transporters have only received attention. The most exciting areas of future research will deal with the integration and co-ordination of these separately described steps, not only to elucidate toxicological responses to metals, but ultimately to understand the routine regulation of physiologically important elements.

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DIAGRAM: FIGURE 1 A generalized model of metal sources, transportation, absorption, sinking and percolation to a sewage fed aquatic system at Calcutta (India).

DIAGRAM: FIGURE 2 Generalized scheme for metal uptake, absorption, translocation, storage and release in aquatic animals.

DIAGRAM: FIGURE 3 Schematic diagram of metal transfer through epithelial cells.

DIAGRAM: FIGURE 4 Model for metal absorption through the gills and intestine/digestive glands based on the behavior of CRIP in intracellular Zn transport and its relationship to metallothionein.

DIAGRAM: FIGURE 5 Pathways for metal transport to the kidney or other organ in invertebrates.

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