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Co-Transport of Metal Complexes by the Green Mussel *Perna viridis*

CHIA-YING CHUANG AND WEN-XIONG WANG*

Department of Biology, The Hong Kong University of Science and Technology (HKUST), Clear Water Bay, Kowloon, Hong Kong

We examined the uptake of ligand-bound metals (Cd and Zn) by the green mussel *Perna viridis* using defined artificial seawater. Different free ion concentrations (1 pM to 10 μM) in uptake solutions were created by adding different amounts of total metals (Cd 0.1 nM to 0.1 mM; Zn 0.5 nM to 0.05 mM) and ligands (EDTA, NTA, citric acid). Our results showed that Cd and Zn uptake could not be fully explained by the free Cd and Zn concentrations in the presence of different ligands, indicating that metal−ligand complexes were at least partially available for uptake by the mussels. Total Zn concentrations appeared to be a better predictor of metal uptake than the free Zn ion concentrations in the presence of different ligands. Uptake of lipophilic organic metal complexes was substantially greater than the hydrophilic metal complexes, even though the free ion concentration was comparable or lower. Moreover, the radiolabeled ligand compounds were directly accumulated by the mussels. The accumulation of metal complexes may explain the increased metal uptake with increasing ligand and total metal concentration, even though the free ion metal concentration was constant. Overall, our experimental results indicated that free metal ion cannot fully explain metal uptake since metal complex species were also available to the mussels to some extent, apparently through a co-transport process.

Introduction

Although it is now well accepted that the chemical speciation of trace metals greatly influences their biological availability, no clear consensus exists as to whether metal complexes are bioavailable, especially for bivalves. Hydrophilic ligand−metal complexes play a vital role in metal chelation by enhancing the solubility of metals. A perplexing question is whether this chelation enhances or reduces the availability of metals to aquatic organisms. In the early 1980s, the driving principle behind our understanding of metal bioavailability from chelator-buffered solutions was the free-ion activity model (FIAM) (*1*). The FIAM was developed to explain the good correlation between the concentration of the free ion in solution and the observed biological effects. There is a considerable body of evidence suggesting that metal bioavailability is a function of its free ion activity, rather than the total metal concentration (*2*). The FIAM model has now been evolved to the Biotic Ligand Model (BLM) and there is now substantial emphasis to test and validate the BLM in marine and freshwater systems (*3*).

* Corresponding author phone: (852) 23587346; fax: (852) 23581559. e-mail: wwang@ust.hk.

Dissolved trace metals can be taken up by more than one route across the apical membrane of epithelial cells. Rates of uptake by some routes (e. g., membrane carrier proteins such as transporters and major ion channels) can be modeled by the availability of the free metal ion. Other routes (usually considered less significant but varying with metal, ligand, and species) may involve transport of whole metal complexes across the apical membrane, without a role for the free metal ion. The uptake of charged inorganic metal complexes, as well as that of lipophilic organic metal complexes has also been reported (*4*). Intrinsic proteins span the lipid bilayer, possibly providing pathways through which polar ions or molecules can diffuse or be carried either passively or in association with energy expenditure (*5*). Elucidation of the uptake mechanisms is thus critical for a more general understanding of the bioavailability of metals to aquatic organisms (*6*).

Filter-feeding bivalves, such as mussels, are exposed to large volumes of water for respiratory and feeding purposes, and inevitably the gills and other exchange surfaces are exposed to metals in the water. Marine mussels accumulate these metals in their soft tissues and are thus frequently used in environmental pollution monitoring programs. George and Coombs (*7*) found that the uptake of Cd by the mussels *Mytilus edulis* in the presence of ligands was higher than that without the presence of ligands. In a recent study, Pan and Wang (*8*) determined the organic carbon uptake by the marine mussel *Perna viridis* from different diets (phytoplankton and detritus) and from the dissolved phase (colloidal and low molecular weight organic carbon) and demonstrated the significance of colloidal uptake mechanisms in marine bivalves. The direct absorption of macromolecules, which can often bind with a large fraction of metals in the dissolved phase, has important implication for understanding metal transport in marine bivalves. A better understanding is thus needed for the role of selective uptake of metabolite-bound metals and synthetic chelates by mussels. In this paper we present a large-scale investigation of the effect of complexation by different organic ligands on the uptake of cadmium and zinc by the green mussel *P. viridis*.

Cd and Zn are chemically similar and sometimes share similar uptake pathways (*9, 10*), but Cd is not essential while Zn is essential to mussels. In our study, metal speciation was controlled by varying the concentration of five different organic ligands, including hydrophilic citrate, nitrilotriacetic acid (NTA), ethylenedinitrilotetraacetic acid (EDTA), lipophilic diethyldithiocarbamate (DDC), and 8-hydroxyquinoline (Ox^-). These ligands were selected on the basis of their thermodynamic stability constants and charges of the formed metal complexes that are representative of a wide variety of ligands in natural waters. All of these ligand−metal complexes are relatively small (less than 500 molecular weight) and purposely selected for several reasons. EDTA was chosen as a model ligand because it forms well-characterized, anionic, hydrophilic complexes with the metals of interest and because of its extensive use in metal ion buffer in biological studies. NTA and citrate were chosen because they have weaker complexing capacity with the metals. Ox^- was chosen because it forms neutrally charged, lipophilic complexes with Cd^{2+} and has known stability constants. DDC^- was selected because it forms neutral lipophilic complexes with Cd^{2+} and because of its extensive agricultural use in the class of fungicides such as dithiocarbamates, even though the stability constants with metals are not well established. Metal uptake was studied in solutions of differing complexation capacities

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to reveal the role of the free metal ion and other species in determining the uptake of metals in relation to the type and concentration of the ligands present in solution.

Materials and Methods

Mussels and Metals. Green mussels *P. viridis* (shell length 3−5 cm, dry tissue weight ~0.4 g) were collected from Yuan Shu Au, Tolo Harbor, Hong Kong, and were transported to the laboratory immediately after the collection. Throughout the whole acclimation period, they were maintained in natural coastal seawater collected from the Clear Water Bay, Hong Kong, at 23 °C and 30 psu with continuous aeration, and fed with the diatom *Thalassiosira pseudonana* (clone 3H) at a ration 1−2% of tissue dry weight per day. All the mussels were acclimated under the laboratory conditions for 1 week prior the experiments described below. Radiotracer techniques were employed to measure the rate of dissolved metal uptake. The radioisotopes ^{109}Cd (in 0.1 N HCl, specific activity of 3.96 mCi/mg) and ^{65}Zn (in 0.1 N HCl, specific activity of 45 mCi/mg) were purchased from New England Nuclear, Boston, MA, and Riso National Lab, Denmark, respectively. The ^{14}C−EDTA and ^{14}C−citric acid were purchased from Sigma and Amersham, respectively.

All experiments were performed with artificial seawater AQUIL (*11*) in order to control metal speciation precisely. The solution was filtered through a 0.22 μm Millipore membrane filter prior to use. The addition of ligands (EDTA with a molecular weight (MW) of 372, NTA with a MW of 257, citrate with a MW of 294) or metals (Cd and Zn) to solutions may lead to changes in the availability of other dissolved species. To minimize such potential artifacts, the composition of the exposure solution was adjusted as necessary to maintain the free cation concentrations (Ca^{2+}, Mg^{2+}) and pH at constant levels. Metal speciation in the solution was calculated with the chemical speciation model MINEQL+ Version 4.5 from Environmental Research Software, Hallowell, ME) (*12*) with corrected equilibrium constants (*13*, also see Supporting Information). Cd and Zn uptake experiments were conducted separately.

Free Ion Dependence of Metal Uptake. To demonstrate whether metal bioavailability was enhanced in the presence of low molecular weight organic ligands, it was necessary to determine the concentration−response curve. Two different experiments were conducted to determine the free ion dependence of metal uptake by the mussels. The first experiment was carried out by preparing different total metal concentrations (10^{-10} to 10^{-5} M) with three different organic ligands (EDTA, NTA, citric acid) added to create free ion concentrations ranging from 10^{-12} M to 10^{-7} M for Cd^{2+} and 10^{-10} M to 10^{-5} M for Zn^{2+}. In the second experiment, we fixed the free ion concentration at 10^{-10} M for Cd^{2+} and 10^{-8} M for Zn^{2+} by adding different amounts of total metal and ligand into the uptake solution. Biouptake in the presence of synthetic ligands was then performed at pH 8.0 in AQUIL. Radioisotope additions were 29.6 kBq L^{-1} for Cd or 38.5 kBq L^{-1} for Zn. Following radioactive additions, 0.5 N NaOH was added to AQUIL to maintain the pH because the metals were carried in 0.1 N HCl solution. The radioisotopes and stable metals were equilibrated overnight before the uptake experiments. Six mussels were exposed individually to radioisotopes plus stable metals in 200 mL of 0.2 μm filtered synthetic seawater for 1 h. The experiments started when the mussels opened their shell valves. An aliquot of exposure water was also taken for radioactivity measurements before and after the exposure. Following the exposure, the mussels were removed and placed in nonradioactive filtered seawater for 5 min to remove the weakly bound radioisotopes. They were then dissected and the soft tissues were radioassayed. The tissues were dried at 80 °C and the dry weights were determined. Metal influx rate (I_w, ng g^{-1} h^{-1}) was calculated as

$$I_w = A_{tissue}/(SA \times W \times t)$$

where A_{tissue} is the radioactivity in the soft tissue after exposure, SA is the specific activity of metal in the seawater, W is the dry weight of the soft tissue (g), and t is the duration of exposure (1 h).

Co-Transport of ^{14}C-Labeled Ligands and Metals. Two radiolabeled ligands (^{14}C−citric acid and ^{14}C−EDTA), ^{109}Cd, and ^{65}Zn were used to trace the ligand and metal distributions in the mussel tissues after exposure. First, we investigated the uptake of ^{14}C-labeled ligands as a function of time. The ligand may have complexed with metals or hydrogen ions once they were added to the water. Mussels were exposed to 45 nM ^{14}C−citric acid and 47 nM ^{14}C−EDTA, respectively, for 3 h. There were a total of 6 replicated beakers, each containing 3 individual mussels. At each time point of exposure (1, 2, and 3 h), one individual mussel from each beaker ($n = 6$) was transferred to the rinsing solution for 5 min and the gills, digestive glands, and soft tissues were dissected and weighed, treated with Tris-buffer, and homogenized by ultrasonication. The ^{14}C radioactivity of each body part was then assayed. The concentration factor was calculated as the radioactivity in the mussel's tissue (ccpm g^{-1}) to the radioactivity in the water (ccpm mL^{-1}).

To examine the co-transport of the ^{14}C-labeled ligands and metals, individual mussel uptake was quantified in solutions containing different concentrations of radiolabeled ligands (10^{-6} and 10^{-5} M for ^{14}C−EDTA; 10^{-4} and 10^{-3} M for ^{14}C−citric acid) and both ^{109}Cd and ^{65}Zn (10^{-7} M). The exposure lasted for 2 h; afterward the mussels were transferred to the rinsing solution for 5 min and the gills, digestive glands, and soft tissues were dissected and weighed. The tissues were first counted for radioactivity of ^{109}Cd and ^{65}Zn, and then treated with Tris-buffer and homogenized by ultrasonication. The ^{14}C radioactivity of each body part was then assayed. Interferences of gamma emission were calibrated by internal spikes of ^{109}Cd and ^{65}Zn standards into the mussel tissue samples before liquid scientillation counting.

Lipophilic Ligand. To investigate whether the mussels accumulated the lipophilic metal complexes and whether there was any difference in metal uptake (not ligand uptake) when the metals were complexed, two lipophilic ligands were used in this experiment (DDC with a MW of 225 and Ox^- with a MW of 145), and EDTA was used as the control. The total metal concentrations were 10^{-7} M for both Cd and Zn, and the total ligand concentrations were 10^{-5} M. The solution for uptake was designed such that the free metal ion concentration (calculated by using the equilibrium program MINEQL+) in the presence of lipophilic ligands was comparable to (for Cd^{2+}) or much lower than (for Zn^{2+}) when complexed to EDTA. However, the stability constants for complexes with DDC are not well-known, thus the same concentration of DDC as Ox^- and EDTA was added for the Cd and Zn uptake experiment (10^{-5} M). The uptake experimental procedures were followed as described above.

Analytical Measurements and Statistical Analysis. ^{109}Cd and ^{65}Zn radioactivity was measured by a Wallac gamma counter. All counts were related to standards for each isotope. The gamma emissions of ^{109}Cd were determined at 88 keV and those of ^{65}Zn were determined at 1115 keV. Counting times in all samples were adjusted so that the propagated counting errors were typically <5%. To count the ^{14}C activity in the samples, scintillants (Fisher Chemicals, UK) were added and mixed with the samples. The radioactivity of ^{14}C was measured by a Wallac 1414 liquid scintillation counter using the external standard ratio method and calibrated for quenching. Statistical difference among different treatments was performed by one-way ANOVA or t-test and significant difference was accepted at $p < 0.05$.

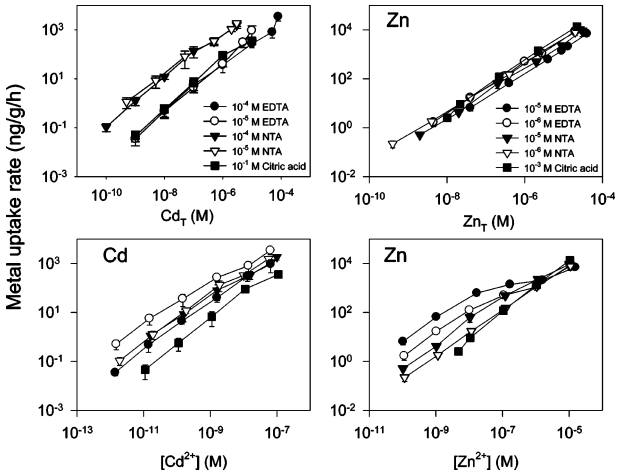

FIGURE 1. Influx of Cd and Zn in the mussels in relation to the total nominal metal (top panels) and the modeled free metal ion (bottom panels) concentrations. The mussels were exposed to different total metal and ligand concentrations while the free metal ion concentrations were varying. Mean ± SD (n = 6).

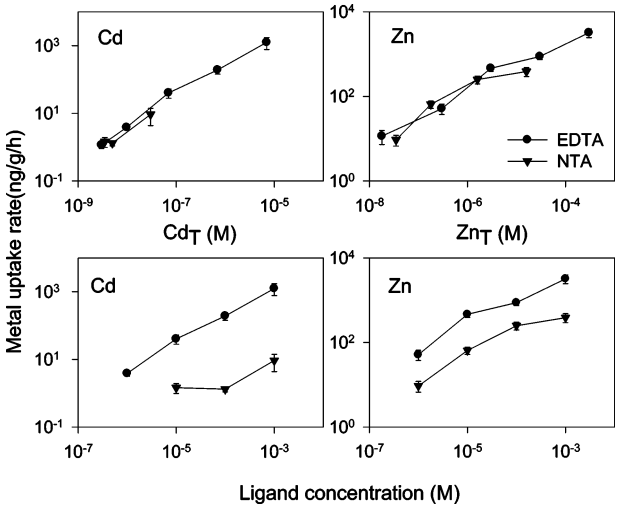

FIGURE 2. Influx of Cd and Zn in the mussels in relation to the total metal (top panels) and the ligand (bottom panels) concentrations. The mussels were exposed to different total metal and ligand concentrations at a fixed free ion concentration (10^{-10} M for Cd^{2+} and 10^{-8} M for Zn^{2+}). Mean ± SD (n = 6).

Results and Discussion

Free Ion Dependence of Metal Uptake. Figure 1 shows the metal uptake rates in mussels exposed at different total metal concentrations added with different hydrophilic ligand types and concentrations. At different ligand concentrations, the uptake rate increased linearly ($p < 0.001$) with the exposed dissolved metal concentration. At the same dissolved Cd concentrations, uptake rates were comparable at different NTA or EDTA concentrations, but Cd uptake was over 1 order of magnitude higher in NTA-buffered solution than in EDTA or citric acid buffered solution. For EDTA, the Cd uptake rate was better predicted by the total Cd concentration than by $[Cd^{2+}]$, whereas both total Cd and $[Cd^{2+}]$ accounted for the uptake at two different NTA concentrations. For Zn, uptake was better predicted by the total Zn concentration for different ligands. The free ion accounted for the uptake rate for different ligands when the $[Zn^{2+}] > 10^{-6}$ M, but failed to predict uptake at $[Zn^{2+}] < 10^{-6}$ M. Thus, in addition to the labile concentrations, Cd and Zn uptake were also related to the different ligands and total metal concentrations, indicating that metal−ligand complexes were at least partially available for uptake by the mussels. These data strongly suggested that ligands had clear effects on metal uptake and free ion cannot fully explain the metal uptake in buffer solution with the presence of different ligands. Mussels exposed to metals in a complexing medium accumulated more metals than expected on the basis of free metal. Moreover, the less than 1 slope (on the log−log plot) at higher Zn^{2+} concentrations ($>10^{-8}$ M) indicated that the mussels regulated the Zn uptake (i.e., increased less proportionally) or the uptake sites were saturated.

Metal uptake in mussels may not be diffusion-limited due to the large volume of water filtered (*14*), which may explain why the total metal concentration was a better predictor of uptake. We further checked the ability of the FIAM to predict the metal uptake by fixing the free ion concentration in the presence of different ligands. Consistent with the previous experiment (Figure 1), Cd and Zn uptake by green mussels was dependent on the nature of the metal-complexing ligands and the total metal concentration, which was not predicted by the FIAM (Figure 2). If uptake was solely due to the free metal ion concentration, we would expect to see straight lines with zero slope in Figure 2. Even though the free ion concentration was fixed, uptake of Cd and Zn increased with both total metal and ligand concentration, again strongly suggesting that metal−ligand complex was directly available for uptake by the mussels.

In contrast to the FIAM, our study found that the total metal concentration may better predict the uptake rate in mussels than the free metals alone. Metal uptake not only increased with the free metal concentration, but also was dependent on the ligand type and concentrations. Our results also showed significantly higher Cd and Zn accumulation in mussels with increasing chelation ($p < 0.05$). Indeed, the effects of ligands on metal uptake may well prove to be metal- or organism-specific. For example, George and Coombs (*7*) measured the long-term (21-day) uptake of Cd by the mussel *Mytilus edulis*. Experiments were first run in filtered seawater in the absence of added ligands and then in the presence of "excess" ligands (concentrations unspecified). Four ligands were investigated (humic acid, alginate, pectinate, and EDTA). In all four cases they reported that Cd accumulation increased 2-fold as compared to the trials in filtered seawater. Moreover, Vercauteren and Blust (*15*) determined the effects of complexation of organic ligands (EDTA, NTA, histidine, citrate, and glycine) on the uptake of Zn by the mussel *M. edulis*, in synthetic seawater (salinity 35‰, pH 8.0, 15 °C) over 24 h. They showed that in the presence of different ligands, the $[Zn^{2+}]$ alone did not explain the majority of variability in the Zn uptake rate. The correlations between $[ZnHis^+]$ and Zn uptake rate were significantly ($p < 0.05$) positive, indicating that $ZnHis^+$ promoted the Zn uptake by the mussels. We thus hypothesize that a fraction of the metal−ligand complexes (e.g., $CdEDTA^{2-}$, $ZnNTA^-$) species in the medium may be transported across the plasmalemma of the cell. Such metal−ligand complexes may be a significant mode of metal transport in a medium where the free metal ion activities are very low.

Ligand and Metal Co-Transportation. As a complement to the metal uptake experiments, an additional assay was performed with radiolabeled ^{14}C−citric acid and ^{14}C−EDTA to directly quantify the uptake of ligands in kinetic experiments. For both ^{14}C−citric acid and ^{14}C−EDTA, uptake in the digestive gland, soft tissue, and gill was linear within the 3 h exposure period (Figure 3). These results unequivocally demonstrated that mussels were able to directly accumulate the ligand compounds in their tissues.

Active uptake indicates a transport specific for the complex or its analogue. EDTA is commonly used in chelator-buffered solutions and its uptake probably occurred via some ion

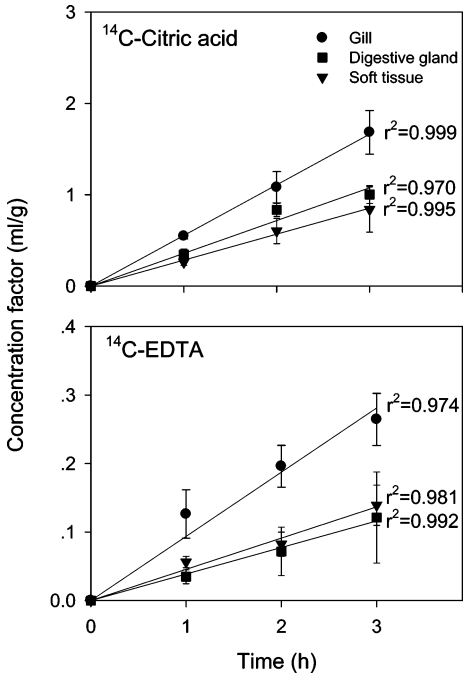

FIGURE 3. Accumulation of radiolabeled citric acid and EDTA in the gills, digestive glands, and soft tissues of mussels over 3 h of exposure. The accumulation was quantified as the concentration factor of radiolabeled compounds. Mean ± SD (n = 6).

channels, because EDTA is a small molecule. Although EDTA uptake occurred, the amount was smaller as compared to the citrate uptake under the same experimental conditions (Figure 3). The ^{14}C radioactivity in the gill, digestive gland, and soft tissue were 10−100-fold greater for citrate uptake than for EDTA uptake (data not shown). Transporters for citrate occurred in the bacteria *E. coli*, where it acted as a siderophore (*16*), and in *Bacillus subtilis*, where divalent metals were required for movement of the metal−citrate complex into the cells (*17*). A citrate transporter occurs in mitochondria of all plant cells where the tricarboxylate moves from the mitochondria to the cytoplasm (*18*).

The percentages of radiolabeled ligands ^{109}Cd and ^{65}Zn distributed in different parts of mussel tissues after simultaneous exposure to these radiolabeled compounds are shown in Figure 4. Two different ligand concentrations were tested while the total Cd and Zn concentrations were maintained at 10^{-7} M. Overall, a much higher fraction (>30%) of Cd and Zn was detected in the digestive gland than in the gills and soft tissues. For ^{14}C−citric acid, the distributions in the digestive glands under all exposure conditions were >50%, followed by its distribution in the gills while the soft tissues contained the least fraction of radiolabeled citric acid. For ^{14}C−EDTA, the distribution in the digestive glands was 20−30%, and significant accumulation in the gills and other soft tissues was also evident. Given the general comparable distribution of radiolabeled ligands and metals in different mussel's tissues, the exposure routes of metals and ligands may be similar and metal−ligand complexes may have been transported by the mussels.

The diffusion of metal−ligand complexes across cell membranes is thought unlikely because charged complexes cannot cross the membrane except through transporters and ion channels (*19*). However, our study demonstrated that metal−ligand complexes were apparently accumulated by the mussels over the short-term exposure periods. Such accumulation of metal complex also explained the increasing metal uptake with increasing ligand concentration and total metal concentration, even though the free ion metal concentration was maintained constant (Figures 1 and 2). In an

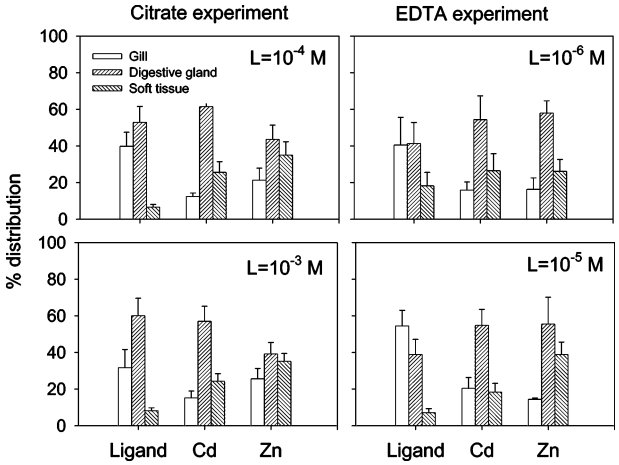

FIGURE 4. Relative distribution of radiolabeled ligands, Cd, and Zn in the gills, digestive glands, and soft tissues of mussels after 2 h of exposure. Left panels for ^{14}C−citric acid experiment, and right panels for ^{14}C−EDTA experiment. Two concentrations for each ligand were examined. Mean ± SD (n = 6).

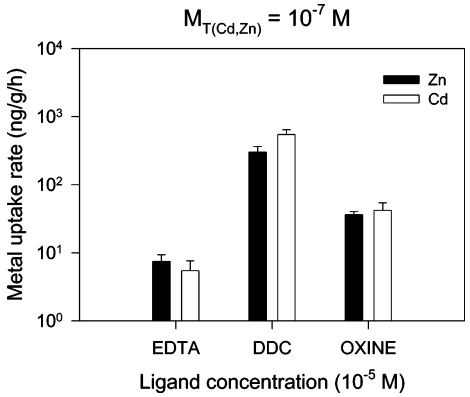

FIGURE 5. Influx of Cd and Zn in the mussels in the presence of different ligands. The calculated [Cd^{2+}] in the EDTA and Ox^- treatments was 1.14 × 10^{-10} M. The calculated [Zn^{2+}] in the EDTA and Ox^- treatments was 2.50 × 10^{-10} M and 7.61 × 10^{-13} M, respectively. The [Cd^{2+}] and [Zn^{2+}] concentrations in the DDC treatment were not calculated since the stability constants were not well-known. Mean ± SD (n = 6).

earlier study, Pan and Wang (*8*) demonstrated that the mussels were able to directly accumulate the macromolecules (the carbohydrate macromolecular dextran with size raging from 3 to 2000 kDa) in their digestive glands. Such mechanisms may also operate for the co-transport of metal complexes in marine mussels. Wang and Guo (*20*) also showed that colloid-bound metals were bioavailable to both mussels and clams, but the influences of colloidal binding on metal uptake varied among metals and between the two bivalves.

Uptake of Lipophillic Metals. In our lipophilic ligand uptake experiments, the highest uptake rate (10−100-fold higher) was found for metals (both Cd and Zn) bound with DDC, followed by metals bound with Ox^- and EDTA (Figure 5). Such a difference of metal influx among different ligands was probably caused by the different uptake routes for different ligand−metal complexes. The uptake in the presence of lipophilic organic metal complexes was substantially greater than in the treatments of hydrophilic metal complexes, even though the M^{2+} concentration was comparable (for Cd^{2+}) or several orders of magnitude lower (for Zn^{2+}). Thus, metals in the form of these lipophilic complexes must enter the cells via a different route than the surface transport model. A plausible route was via the direct passive diffusion of the lipophilic metal complexes.

A number of exceptions to the FIAM have been reported (*2* and references therein). One key assumption of the pertaining FIAM is that the transport of the metal in solution is fast as compared to the actual internalization rate (which then becomes rate-limiting or diffusion limited). Exceptions have been found with the presence of lipophilic metal ligand complexes, $M-L_n^0$, which are able to traverse the biological membranes without first dissociating (*4, 21*). Phinney and Bruland (*22*) also demonstrated that the addition of synthetic organic ligands DDC and Ox^- facilitated the transport of ambient Cu and Ni into the coastal diatom *Thalassiosira weissflogii*. In their study, the steady-state cellular Cu concentrations were over 10 times and 6 times greater, for DDC and Ox treatments, than in the controls. Moreover, there have been some reports of enhanced metal availability in the presence of low molecular weight, hydrophilic ligands of biogenic origin (primary metabolites such as citrate and glycine, *7, 15, 23*).

In this study, we showed conclusively that the uptake of Cd and Zn by mussels was enhanced in the presence of low molecular weight synthetic organic ligands. These data indicated that the bioavailability of divalent metals in the presence of citrate or some lipophilic ligands may well diverge from the predictions of the FIAM. We propose that the synthetic ligands may play a vital role in the uptake of physiologically important metals as well as toxic metals in marine mussels and probably other bivalves. It is likely that the specialized receptors may be present on the basal membranes of mussel tissues. Following the binding of the metal−ligand complex to these cell receptors, Cd and Zn may be dissociated from the complex and internalized in an unbound form, or the complex itself may be internalized as a whole entity. Furthermore, our data suggested that passive diffusion of lipophilic metal complex also operates in the uptake of metals by the mussels. The complexity of metal transport as influenced by the functional physiology of bivalves clearly needs to be considered.

Acknowledgments

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Supporting Information Available

Stability constants used in the calculation of free metal ion concentrations with MINEQL+ software and the K_{ow} of ligands used in the experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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