FAILURE OF THE BIOTIC LIGAND AND FREE-ION ACTIVITY MODELS TO EXPLAIN ZINC BIOACCUMULATION BY CHLORELLA KESSLERII

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(Received 25 January 2002; Accepted 7 September 2002)

Abstract—Zinc accumulation by Chlorella kesslerii (Chlorophyceae) was studied for [Zn$^{2+}$] ranging from 4 pM to 1 mM. A first-order uptake flux as predicted by the free ion activity model (FIAM) and the biotic ligand model (BLM) was not observed. Furthermore, when algae were preconditioned in slightly limiting (10 pM) versus optimal (1 nM) free zinc concentrations, the internalization flux increased and was nearly constant over the range of [Zn$^{2+}$] examined. This observation was attributed to the synthesis of membrane-bound zinc transporters. Neither solution Zn chemistry nor surface-bound Zn was a good predictor of Zn uptake fluxes. Several hypotheses were examined to explain the failure of the steady-state uptake models. Although zinc excretion and a Zn diffusion limitation were observed, neither result could explain the majority of observed deviation from the models. Bioaccumulation experiments performed with inhibitors (vanadate and carbonyl cyanide m-chlorophenylhydrazone [CCCP]) demonstrated clearly that zinc transport is an energy-dependent process in Chlorella kesslerii. The presence of an active transport signifies that Zn uptake may function independently of the electrochemical Zn gradient and that, in some cases, both uptake fluxes and receptor-bound Zn may be independent of solution chemistry.

Keywords—Zinc  Chlorella  Free ion activity model  Biotic ligand model  Uptake

INTRODUCTION

In waters, trace metals are partitioned among dissolved species and suspended solid phases including particles, colloids, and organisms [1]. Although much work has attempted to relate the solution chemistry of trace metals with their induced biological effects, a thorough understanding of the nature of the interactions between organisms and metals in solution is still lacking, especially for complex media such as natural freshwaters.

The trace metal bioaccumulation process begins with the mass transport of the metal to the vicinity of the organism [2]. The first site of encounter of a metal with an aquatic microorganism is often a protective layer composed of glycoproteins and polysaccharides, including a mucous layer or cell wall [3]. Subsequent to its diffusion through this layer, hydrophilic metal species may cross the biological membrane by a number of mechanisms, but most often a passive transport, driven by the electrochemical gradient between the inside and outside of the organism, is observed [3,4]. The combination of surface-bound (adsorbed) and internalized metal usually accounts for the total metal body burden of the microorganism [5]. In such a general formulation of the trace metal uptake process, four metal fluxes or sources are considered, including diffusive, complexation/dissociation, adsorption/desorption, and internalization [2,6,7]. Depending on the system studied, each of the fluxes has the potential to be rate limiting. The chemical species that are accessible to the organism will be determined, in large part, by the nature of the rate-limiting flux.

Several simplified models are currently used to predict biological effects following metal exposure. In the early 1980s, the free ion activity model (FIAM) [7–10] was developed to explain good correlation between the concentration of the free ion in solution and observed biological effects [11,12]. As an extension of FIAM, the biotic ligand model (BLM) [13] correlates transporter-bound metal or total metal body burdens with induced effects. This simplification provides a direct means to take into account the competitive effects of pH and water hardness. Both the FIAM and the BLM are based on several simplifying assumptions [10]. For example, the models assume that observed acute effects are proportional to metal bound to sensitive sites at the membrane surface (e.g., transporters), which is in turn proportional to the internalization flux of the metal, $j_{\text{int}}$. In addition, both models are based on the attainment of a thermodynamic equilibrium (or steady state). The FIAM and BLM predict a first-order linear relationship between biological effects and either the free metal ion concentration in solution ($[Zn^{2+}]$), FIAM or the transporter-bound metal concentration ($[Zn - R_{\text{cell}}]$), BLM (Eqns. 1–3, 7, 10).

$$Zn^{2+} + R_{\text{cell}} \leftrightarrow Zn \rightarrow R_{\text{cell}} \rightarrow \rightarrow \rightarrow$$ (1)

$$j_{\text{int}} = k_{\text{int}} [Zn - R_{\text{cell}}]$$ (2)

$$j_{\text{int}} = k_{\text{int}} K[R_{\text{cell}}][Zn^{2+}]$$ (3)

where $k_{\text{int}}$ represents the internalization rate constant, $[R_{\text{cell}}]$ is the concentration of free cellular receptors or transporters, and $[Zn - R_{\text{cell}}]$ is the zinc-bound receptor (or transporter) concentration. In short-term bioaccumulation experiments, the attainment of a steady state between the organism and the external media implies that the metal uptake flux is constant and that both dissolved and adsorbed metal concentrations remain unchanged and at equilibrium. In this case, a zero-order internalization kinetics is predicted for $[Zn^{2+}]$ above transporter saturation (Eqn. 3, [14]) while a first-order metal uptake flux should be observed at lower $[Zn^{2+}]$, in agreement with the
FIAM [10]. Although several exceptions to the FIAM model (including the transport of lipophilic complexes [15], the transport of low molar mass metabolizable complexes [16], or the formation of ternary surface complexes [17]) have been documented and reviewed [10], few studies have examined the important assumption of a first-order internalization.

The goal of this study is to determine whether Zn uptake by Chlorella can be predicted by either of the simple models. Because biological effects are most often related to solution chemistry by sigmoidal rather than linear relationships, it is difficult to quantitatively test the models using toxicity data. Instead, a more rigorous quantitative test of the models is to relate metal speciation or receptor-bound metal (i.e., metal bound to the physiologically active metal binding sites leading to transport [18]) to internalization fluxes ([19], Eqns. 1–3). Although most applications of the BLM do not distinguish total metal body burdens or gill adsorption from receptor-bound metal (e.g., [20]), some attempt will be made here to distinguish among the different fractions. The implications of biological regulation on Zn uptake will also be examined in detail.

**MATERIALS AND METHODS**

**Test organisms and growth conditions**

Chlorella vulgaris (University of Toronto Culture Collection, Toronto, Canada, UTCC 266) is a spherical green alga that has recently been renamed Chlorella kessleri [21]. Algae with an average diameter of 3.6 μm were grown in an Organization for Economic Cooperation and Development (Paris, France) media [22] at pH 7 (HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 1 mM) with a calculated free zinc activity of 1 nM (MINEQL+, Ver 3.01a; Procter & Gamble, Cincinnati, OH, USA) that was determined using updated stability constants [23] (NIST Critically Selected Stability Constants of Metal Complexes Database: Version 5.0, Standard Reference Data Program, National Institute of Standard and Technology, Gaithersburg, MD, USA) and an ionic strength correction using the Davies equation. A modified medium was also used to grow the organisms at [Zn2+] = 10 pM by increasing the ethylenediaminetetraacetic acid (EDTA) concentration and compensating for decreases in other free ion concentrations. Algae were grown in an incubation chamber (Multitron, Infors, Switzerland) at 20°C with a 12-h light (50 μmol photons/m²/s)/dark cycle and 100 rpm rotary shaking. Surface areas and cell numbers were determined for each experimental point using a Coulter Multisizer II (Toronto, ON, Canada). Midway through the exponential growth phase, cells were harvested by gentle filtration (3-μm nitrocellulose filter) and washed (three times) in an experimental medium with a similar ionic strength (1.8–3.2 mM) and pH 7 to that of the growth medium.

**Experimental solutions**

All experiments were performed in a simple experimental medium that allowed for the precise determination of equilibrium zinc speciation using thermodynamic calculations (MINEQL+). The PO₄³⁻, Ca²⁺, Mg²⁺, trace metal, and EDTA contents of the growth medium were eliminated to avoid their potential influence on bioaccumulation [24]. These components were replaced by KCl in order to maintain a constant ionic strength. In this manner, when no ligands were used to buffer metal concentrations, Zn²⁺ represented 98% of the total zinc in solution. In order to maintain low [Zn²⁺] in certain experiments, excess nitriotropic acid ([INTA] 5–400 μM) and tiron (50–500 μM) were added to Zn concentrations of 1 to 5 μM. For all experiments performed with tiron and some experiments using NTA, [Zn²⁺] was confirmed using polarography (AC mode; Metrohm, Basle, Switzerland). For experiments performed with [Zn²⁺] < 5 μM, radiolabeled ⁶⁵Zn (specific activity 37 GBq/g; NEN Life Science Products, Boston, MA, USA) was added so as not to exceed 2% of the total nonradiolabeled zinc.

**Bioaccumulation experiments**

Manipulation of algal cultures was performed under laminar flow conditions. Algal concentrates obtained from the growth media were resuspended in the experimental medium with appropriate [Zn²⁺]. At different times, aliquots of this solution were filtered and the filtrate was analyzed for dissolved zinc. Algae remaining on the filter were washed three times for 45 to 60 s in 5 ml of a 5 mM EDTA solution (pH 7). These wash times were considered optimal because further washing did not increase Zn removal by greater than 5% yet the EDTA contact times were sufficiently rapid to avoid significant efflux or membrane damage [25]. The EDTA filtrate was analyzed for cell surface adsorbed zinc. Cellular zinc was determined after digestion of the filter and EDTA-washed algae in ultrapure concentrated HNO₃ (J.T. Baker, Basel, Switzerland). Dissolved, adsorbed, and cellular zinc fractions were measured with flame atomic absorption spectrometry (Pye-Unicam SP-9, P.H. Stehelin and Company, Switzerland) when not working with isotopes or with a γ counter (Perkin-Elmer, Hombrechtikon, Switzerland) for ⁶⁵Zn measurements. Uptake experiments were performed over short times (25–60 min) in order to minimize biological effects such as exudate production or cell size and number variations.

**Efflux measurements**

One day before attainment of the midexponential growth phase, algae were harvested and resuspended in a modified Organization for Economic Cooperation and Development medium with 0.1 nM or 1 μM Zn²⁺ and a small proportion of ⁶⁵Zn. After a further incubation of 24 h, the cellular, adsorbed, and dissolved zinc fractions were determined. Algae were then washed three times with 5 ml of the experimental medium and resuspended in an experimental solution without ⁶⁵Zn but containing either the same concentration of [Zn²⁺] or 10 μM NTA. Cellular ⁶⁵Zn concentrations were followed over 150 min. A 24-h preincubation time was employed in order to have a high ratio of internalized versus adsorbed zinc.

**Uptake in the presence of inhibitors**

Some experiments were performed with inhibitors. Vana-date (BDH), a P-type ATPase inhibitor, was used at 1 and 20 μM with a 5-min preincubation prior to Zn addition. Carbonyl cyanide m-chlorophenylhydrazone (Sigma, St. Louis, MO, USA), an oxidative and photophosphorylation uncoupler [26], was solubilized in ethanol and used at 20 μM with a 10-min preincubation. The small addition of ethanol to the experimental medium did not affect algal ¹⁴CO₂ assimilation. All experiments were compared with control solutions under identical conditions in the absence of inhibitors.

**General treatment of the results**

Short-term internalization or efflux was quantified using a linear regression of cellular zinc as a function of time. Because
RESULTS AND DISCUSSION

Bioaccumulation experiments

In all experiments, adsorbed and internalized Zn concentrations were small with respect to the total Zn pool, resulting in an essentially constant dissolved Zn concentration (Fig. 1). Adsorbed Zn rapidly attained a constant value, implying that equilibrium had been attained between the cell surface and solution, an important assumption of the FIAM and BLM models [10]. Furthermore, the small linear increase of cellular zinc versus time (Fig. 1) suggested that Zn transport across the membrane was the rate-limiting flux in the overall bioaccumulation process, again in accordance with the FIAM and BLM models.

Internalization data

Zinc accumulation was examined for solution [Zn\(^{2+}\)] ranging from 4 pM to 1 mM. While the higher concentrations were less environmentally pertinent, they were required in order to saturate the cellular transport system. When the slopes (J_m) of the non-EDTA extractable (cellular) Zn time curves were plotted against the free Zn concentrations (Fig. 2), the influence of Zn concentrations in the growth medium was clear. For low concentrations of Zn\(^{2+}\) in the experimental medium (<0.4 \(\mu\)M), the algae preincubated in 10 pM Zn\(^{2+}\) accumulated zinc at a much higher rate than those grown in 1 nM Zn\(^{2+}\). For these algae, internalization fluxes increased only three- to fourfold for a solution [Zn\(^{2+}\)] that varied between 20 pM and 1 mM of Zn\(^{2+}\) (i.e., \(>10^5\)-fold increase). Below [Zn\(^{2+}\)] = 20 pM, Zn uptake fluxes decreased in direct proportion to [Zn\(^{2+}\)] in solution (r\(^2\) = 0.91). Interestingly, the fluxes at these points are below the maximum diffusive limited supply calculated on the basis of Zn\(^{2+}\) alone (discussed later in the text).

For algae preincubated in 1 nM Zn\(^{2+}\), internalization fluxes were nearly constant for [Zn\(^{2+}\)] below 1 nM in the experimental medium. For concentrations above 1 nM, J_m increased 150-fold between 1 nM and 1 mM Zn\(^{2+}\). Neither variation is in agreement with the FIAM, which predicts a first-order relationship between J_m and [Zn\(^{2+}\)] (Eqn. 3) that should be confirmed by a slope of one on the log-log graph shown in Figure 2. Furthermore, the result is coherent with zinc uptake data observed for other microorganisms [27–29]. For example, Sundaa and Huntsman [27] observed a specific zinc uptake rate in an oceanic coccolithophore that was not linearly related to [Zn\(^{2+}\)] in solution. They also measured a higher uptake rate for algae preincubated in 10 pM as compared with 1.6 nM. In their experiments, specific zinc uptake rates increased approximately 130-fold (1.6 nM preincubation) or 11-fold (10 pM preincubation) between 0.1 nM and 0.1 \(\mu\)M Zn\(^{2+}\). Knauer et al. [28] observed a constant uptake for [Zn\(^{2+}\)] < 1 nM and a nonlinear increase of the zinc cellular content versus [Zn\(^{2+}\)] for a Chlorophycee. Finally, for a gram-positive bacteria, *Rhodococcus opacus*, uptake fluxes increased only 14-fold over a 10\(^5\)-fold variation in the free zinc concentration [29]. Those observations and the data given here for *C. kesslerii* demonstrate that Zn uptake is often not first order with respect to [Zn\(^{2+}\)] in solution and therefore cannot be described by the FIAM. The relationship between surface-bound Zn and uptake fluxes will be examined in the next section in order to verify more thoroughly the BLM.

Adsorption data

The EDTA-extractable zinc (operationally equivalent to Zn adsorbed to the algal surface) was not directly proportional to [Zn\(^{2+}\)] in solution (Fig. 3). Furthermore, Zn incubation con-
ditions did not influence adsorption data. These results suggested either a biologically induced modification of the free zinc concentration in solution or an important coulombic effect due to the presence of heterogeneous surface binding sites. Indeed, for trace metal adsorption on environmental surfaces [1], a slope lower than one is often observed and attributed to multiple site effects, with a progressively decreasing binding affinity for an increasing metal:ligand ratio. This effect is likely to be observed due to the negative surface charge of the organism (zeta potential of ~−5.1 mV at pH 7 and I = 10 mM [30]). Although this result implies that a simple model based on a Langmuir-type surface adsorption (such as the BLM) will not be adequate to predict surface concentrations of Zn, rigorous evaluation of the biotic ligand model suggests that receptor-bound Zn rather than total adsorbed Zn should be related to the uptake fluxes (Eqn. 3).

Failure of the FIAM and the BLM

In the following, several hypotheses are examined to explain the failure of the simple models to predict zinc uptake fluxes by C. kessleri: Zinc internalization is not rate limiting; an important algal efflux is present (i.e., non-steady-state conditions); receptor-bound rather than total adsorbed metal concentrations are more representative of uptake fluxes or internalization does not follow first-order uptake kinetics.

Rate-limiting diffusion or dissociation of the Zn complexes

As mentioned above, both the FIAM and the BLM assume that transport of the metal across the biological membrane (i.e., internalization) is the rate-limiting step in the uptake process. In the opposite case, the concentration of the effectively labile species (rather than $[\text{Zn}^{2+}]$) would be expected to be better predictors of uptake fluxes (and therefore biological effect). Complex stability calculations [31] predicted that the Zn-NTA complexes should be inert ($L \ll 1$, Eqn. 4) and therefore could not contribute significantly to the diffusive flux. In this case, the maximum diffusive flux (Eqn. 5; [32]) can be calculated for the free ion concentrations employed here,

$$L = \frac{k_{\text{diff}}}{D_{\text{diff}}} \quad \text{with} \quad \mu = \frac{D_{\text{diff}}}{k_c C_T}$$

$$J_{\text{diff}} = \frac{D_{\text{diff}} [\text{Zn}^{2+}]}{r}$$

where $D_{\text{diff}}$ represents the diffusion coefficient of the complex species $\text{ZnY}$. $C_T$ is the concentration of ligand $Y$, $\mu$ is the dissociation reaction layer thickness, $r$ is the measured average radius of C. kessleri (1.9 µm), and $k_c$ and $k_d$ are association and dissociation rate constants for $\text{ZnY}$, respectively. Note that Equation 4 is valid only in the case of $\delta > r > \mu$, where $\delta$ is the diffusion layer thickness (20–30 µm) and $\mu$ = 0.9 µm for $[\text{Zn}^{2+}]$ = 20 pM. A value of $D_{\text{diff}}$ of $3.8 \times 10^{-8} \text{ m}^2/\text{min}$ was employed [33]. Under these extremely restrictive conditions, only algae grown under the more limiting conditions, 10 pM Zn$^{2+}$, were found to be potentially limited by diffusion ($J_{\text{int}} > J_{\text{diff}}$) and only for $[\text{Zn}^{2+}] < 20 \text{ pM}$. Complex lability considerations are only relevant for the diffusion-limited points because, for the other data, the flux of free ions is sufficient to meet the biological uptake requirements of the organism.

In the marine phytoplankton literature, diffusion limitation of essential metals has been hypothesized for situations in which the transport system is stimulated in response to metal depletion [34]. In our case, given that observed $J_{\text{int}}$ values are 10 times higher than the predicted diffusion limitation at low $[\text{Zn}^{2+}]$ (<20 pM), it is likely that Zn complexes are indeed able to contribute to uptake fluxes. Because the lowest free zinc concentrations were obtained for a >10-fold excess of Zn complexes with respect to the free ion, the observed $J_{\text{int}}$ may be partially due to an induced lability of the Zn-NTA complexes due to an extremely high biological demand. For the majority of the data, it is nonetheless unlikely that rate-limiting diffusive fluxes are responsible for the noncompliance of the FIAM (Fig. 2).

Algal efflux

Efflux measurements were performed to ensure that zinc efflux was negligible with respect to zinc uptake so that steady-state conditions could be assumed. As in the bioaccumulation experiments, algae were preexposed to 1 nM or 10 pM Zn$^{2+}$. Efflux rate constants were subsequently determined for several resuspension solutions (Table 1). No significant effect (analysis of variance, $p > 0.05$) of zinc concentration in the culture or the experimental or resuspension solutions was observed. An overall average first-order efflux rate constant of $(2.6 \pm 1.0) \times 10^{-3} /\text{min}$ was determined. The efflux rate depended only on zinc concentrations inside the algae prior to experiments and appeared to be a constitutive phenomenon. Based on the criterion that $J_{\text{int}}$ should be less than $10 \times J_{\text{eff}}$, no efflux was significant with respect to the observed internalization fluxes of $^{65}\text{Zn}$ at any point in these short-term experiments. This result is in agreement with that of Wolterbeek et al. [35], who found a $^{65}\text{Zn}$ efflux rate constant of $3.5 \times 10^{-3} /\text{min}$ that was irrespective of the Zn treatment that was applied following a long-term exposure (75 h) of the green alga Selenastrum capricornutum.

Determination of receptor-bound metal

In both the FIAM and BLM models, solution speciation and biological effects are predicted to be related to the metal bound to sensitive sites rather than total adsorbed metal. Measurements relating total body burdens to toxicological effects do not distinguish among adsorbed, cellular, or receptor-bound metal, with the underlying assumption that all accumulated metal will contribute to biological effects [13]. On the other hand, internalization flux measurements can only reasonably be related to transporter bound metal because binding to peripheral adsorption sites or cell wall components should not
lead to biological uptake or biological effects. In this case, the biotic ligand is best represented by Zn transport sites [18].

Slaveykova and Wilkinson [19] have recently shown that the small positive intercept of cellular metal versus time curves could be employed as an estimate of receptor-bound Pb for C. vulgaris. In a similar manner to their study, intercepts from the zinc uptake curves were therefore plotted against solution [Zn^{2+}] for C. vulgaris (Fig. 4). Contrary to the single curve observed for total adsorbed zinc in Figure 3, receptor-bound zinc estimated in this manner was clearly dependent on the growth conditions, similar to the flux data in Figure 2. The increased uptake fluxes resulting from the preexposure to low [Zn^{2+}] in the growth media can be interpreted in terms of increased receptor numbers or an increased receptor affinity leading to higher concentrations of [Zn-R_{cell}]. Using this estimation, saturation of the zinc transporters occurred at approximately 10^{-10} mol/cm^2, independent of the growth conditions (Fig. 4). Because the forward rate constant for the association of the metal to the receptors is likely controlled by the loss of water in the Zn^{2+} inner hydration shell (Eigen mechanism), an increased affinity is unlikely because desorption kinetics (including transfer of the metal receptor complex across the membrane) would also be slowed. On the other hand, the coinciding plateaus on the uptake flux curves (Fig. 2) do not intuitively support an increase in total receptor number. Because cellular regulation of specific transporters in response to the cellular trace metal status is generally explained by a modification of receptor numbers [4], it is possible to speculate that the increased uptake flux observed following preincubation at low [Zn^{2+}] (10 pM) was due to the production of high-affinity receptors, with insignificant numbers with respect to the total receptor concentration. In that case, an increased production of the high-affinity receptor would be expected to increase the uptake flux at low [Zn^{2+}] but have no significant effect at high [Zn^{2+}] (Fig. 2). It should nonetheless be noted that Zn to R_{cell} concentrations estimated using these assumptions correspond to a high surface coverage that is difficult to reconcile with a large receptor and a 1:1 metal:ligand stoichiometry.

Sunda and Huntsman [27] have observed a similar induction of zinc uptake following a low Zn^{2+} preincubation. They interpreted their results to indicate that zinc internalization by *Emiliana huxleyi* involved two receptors. Although, the nonlinear relationship observed between [Zn-R_{cell}] and [Zn^{2+}] (Fig. 4) could also be explained by the presence of at least two Zn uptake receptors, the results observed here cannot be unambiguously interpreted by a multiple receptor model. Application of a simple adsorption model such as a Langmuir isotherm to determine constants for the BLM model [13] is not possible because there is no direct linear relationship between [Zn^{2+}] and [Zn-R_{cell}] below saturation values. The use of a more complex model to take into account surface heterogeneity and charge effects [e.g. 1,36], although possible, was not undertaken here.

**Non–first-order uptake process**

Zinc transport studies were conducted in order to determine whether *Chlorella* indeed possesses several pathways for zinc transport. Because a large quantity of genetic material codes for transport proteins (~12% for prokaryotes and 33% for yeast [37]), transport of essential trace metals is likely to be regulated in a very complex way.

Most models of trace metal uptake and receptor-driven transport processes, including the FIAM and BLM, incorporate first-order uptake kinetics. Indeed, receptor-facilitated (passive) transport across membranes is most often a first-order process [38] that is driven by the electrochemical potential at the biological interface. This contrasts with the active transport of macronutrients or trace metals [39], which is an energy-dependent process that can be driven against the electrochemical gradient.

In order to gain greater insight into the actual uptake process, the effects of two inhibitors were verified. Vanadate is a

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**Table 1. Efflux rate constants (k_{eff}) and the corresponding efflux rates (J_{eff}) determined as a function of the zinc concentration inside the algae prior to manipulation ([Zn]_{cell}). A first-order efflux with respect to internalized zinc is assumed:**

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Preincubation [Zn^{2+}] M (OECD modified)</th>
<th>Resuspension solution</th>
<th>k_{eff} ± SD [min^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-9} M Zn^{2+}</td>
<td>10^{-6}</td>
<td>10^{-6} M Zn^{2+}</td>
<td>(3.7 ± 2.1) × 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>10^{-10}</td>
<td>10^{-10} M Zn^{2+}</td>
<td>(1.4 ± 0.4) × 10^{-3}</td>
</tr>
<tr>
<td>10^{-11} M Zn^{2+}</td>
<td>10^{-6}</td>
<td>10^{-5} M NTA</td>
<td>(3.3 ± 1.5) × 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>10^{-10}</td>
<td>10^{-5} M NTA</td>
<td>(2.8 ± 0.1) × 10^{-3}</td>
</tr>
</tbody>
</table>

*NTA = nitrilotriacetic acid; OECD = Organization for Economic Cooperation and Development.*

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**Fig. 4. Relationship between the estimate of receptor-bound Zn and [Zn^{2+}] in solution. Full symbols are experimental results for algae preincubated in 10^{-9} M Zn^{2+} with various metal buffers (● nitritriacetic acid, ■ Tiron, and □ no buffer). Open symbols correspond to experimental results for algae preincubated with 10^{-11} M Zn^{2+} using the same metal buffers. The line represents the slope of a first-order relationship. Error bars represent standard deviations (n = 4–6).**
specific inhibitor of a P-type ATPase [40] that has been shown to have no general metabolic effect on C. vulgaris at 1 μM [41]. On the other hand, carbonyl cyanide m-chlorophenylhydrazone (CCCP) inhibits electron transport by uncoupling oxidative and photophosphorylation reactions [26]. It has been shown to decrease the cellular ATP (adenosine triphosphate) content of Chlorella fusca by 75% following a 10-min preincubation [42] at the concentration employed here. Algae were precultured as in previous experiments (10 pM and 1 nM). Uptake fluxes were determined in the presence or absence of inhibitors for an exposition of either 1 nM or 5 μM of Zn2+. CCCP strongly inhibited zinc transport, independent of the growth conditions (Fig. 5), implying that zinc transport required the production of cellular energy. On the other hand, both concentrations of vanadate partially inhibited Zn transport for algae precultured at 100 nM Zn2+ (Fig. 5). For organisms grown in 1 nM Zn2+, only 20 μM of vanadate inhibited uptake and only at the higher [Zn2+]. This result suggested that a P-type ATPase might be partly responsible for zinc uptake in C. kesslerii. Furthermore, because the effect of vanadate is stronger for algae grown in 10 μM Zn2+ for low [Zn2+] in solution, the ATPase transport pathway might be more important at the lower concentrations, where Zn transport fluxes are uploaded. Active zinc transport has also been demonstrated for Escherichia coli [39], where an ATPase for Zn uptake has been isolated.

The observation that CCCP inhibited Zn uptake fluxes to a much greater extent than was observed in the presence of vanadate coupled with a lack of first-order uptake kinetics suggests the existence of at least two types of energy-dependent zinc receptors. High- and low-affinity zinc transporters have been observed in yeast (Saccharomyces cerevisiae) [43], where the stability of the high-affinity transport system was generally 10 times higher than the low-affinity pathway but increased more than 100-fold for zinc-limited growth conditions. Furthermore, Eide [43] has demonstrated that other unidentified pathways for zinc transport might exist because mutants missing both known transport systems were able to survive. The active transport of zinc implies that uptake is independent of any electrochemical gradient and explains why uptake fluxes may not be explained by the simple BLM or FIAM. The result is reasonable given that several active transporters have recently been found in bacteria and fungi [39,40,43]. It also provides an explanation about why several studies have not observed a direct proportionality between [Zn2+] and bioaccumulation or toxicity [27–29,43,44]. For zinc and perhaps other essential metals, the lack of a first-order uptake process may be a common observation that would preclude the direct use of chemical speciation measurements to directly predict biological availability.

CONCLUSIONS

Although a zinc efflux and a diffusion limitation were observed for C. kessleri, these explanations were not sufficient to explain the failure of the FIAM and the BLM over the range of [Zn2+] examined here (4 pM to 1 mM). Zinc efflux appears to be a phenomenon related to internalized [Zn]. Diffusion limitation occurred only for extremely low [Zn2+] and corresponding high internalization fluxes. Algae grown in slightly limiting conditions of Zn2+ (10 pM) were apparently able to increase the number of high-affinity receptors under Zn2+-limiting conditions. At least two zinc transport pathways were hypothesized to be present, one of which was related to a P-type ATPase and the other (or both) that required cellular energy production. Internalization by the active transporters was independent of the electrochemical gradient and thus first-order uptake was not observed. In this case, the FIAM and BLM cannot be expected to relate chemical speciation to biological uptake or effects. Because similar transport pathways have been observed for zinc and other trace metals for several organisms, the first-order uptake assumption underlying the FIAM and the BLM models should be verified in much more detail.

Acknowledgement—We thank M. Tuveri, P. Salaun, and L. Tomaszewski for technical assistance and V. Slaveyková, C. Rossier, H. van Leeuwen, and J. Buñel for helpful comments on previous versions of the manuscript. Financial support was provided by the European Union (EVK1-CT-2001-00086) and by the Swiss National Funds (20-61648.00).

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