Ligands Influence Zn Transport into Cultured Endothelial Cells (43522)

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Abstract. **Experimental results were obtained that demonstrate the importance of the relative concentrations of Zn ligands and their affinities for Zn to the rate of Zn transport across a biological membrane. The transport rate into cultured endothelial cells became saturated near a Zn concentration of 30 μM in the presence or absence of 14% serum. However, the maximum transport rate identified by the saturation plateau was nearly twice as fast from serum-free medium. The addition of histidine or picolinic acid to the medium with serum resulted in the coincidental shift of Zn from high molecular weight ligands to low molecular weight ligands and increased the overall transport rate. In serum-free medium, adding histidine or picolinic acid slowed the transport rate. This indicates that the rate of Zn transport is influenced by the ligand to which it is associated and that altering the relative proportions of specific ligands influences the Zn transport rate. The rate of Zn transport decreased in a stepwise fashion as the albumin to Zn ratio increased from 0 to 4:1, with further increases having little effect. This suggests that albumin has a special role as modulator of Zn transport into endothelial cells. These studies underscore the importance of controlling the relative concentrations of Zn and its ligands in Zn transport kinetic research and suggest that varying their concentrations in a physiological range may be a method of regulating the distribution of Zn into specific cells and tissues.** [P.S.E.B.M. 1993, Vol 202]

The free Zn cation is very damaging to biological systems and thus is generally associated with other molecules as Zn-ligand complexes, resulting in a free Zn ion concentration that is 10^{-3}-10^{-6} that of the total Zn concentration (1). Albumin-bound Zn accounts for 98% of the exchangeable fraction of Zn in serum (2). It was previously believed that each of albumin's 16 histidine residues could potentially bind a Zn atom (3). However, the concentration of Zn never exceeds that of albumin under physiological conditions. Furthermore, the relative affinities of potential zinc binding sites on albumin are likely to vary considerably. It is now known that Zn favors the formation of tetrahedral complexes and the N terminus tripeptide (Asp-Thr-His) contributes to the formation of the primary zinc-binding site on bovine serum albumin (4). Therefore, only one Zn binding site on albumin is probably occupied under typical physiological conditions. Approximately 2% of the exchangeable Zn in serum is bound to free amino acids, predominantly histidine and cysteine (2). This suggests that the affinity of these low molecular weight molecules for Zn may be greater than any secondary site on albumin. As the Zn concentration exceeds that of albumin or the concentrations of low molecular weight ligands increase, the relative proportion of exchangeable Zn associated with albumin would decline.

In previous studies, we found that the kinetics of Zn transport into cultured endothelial cells was altered when there was significantly more Zn in the medium than could be accounted for by the primary Zn binding site on albumin (5). Using a medium containing fetal bovine serum that contributed 35 μM albumin, Zn transport increased as the Zn concentration increased until a plateau was reached near 30 μM Zn. Subsequent increases in Zn beyond 35 μM increased the transport rate further. We have suggested that Zn transport into this cell type is facilitated by a saturable transporter

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under physiological conditions, but that some other means of transport is involved at elevated Zn concentrations (5). Furthermore, we have proposed that the kinetics of Zn transport are influenced by the ligands to which Zn is bound.

In the present study, we examined the influence of various Zn binding ligands on medium Zn distribution and Zn transport into cultured endothelial cells. Other investigators have observed that chelators influence Zn uptake into cultured cells, but the experimental evidence has been ambiguous and often contradictory. We believe the results presented here will help to clarify the mechanisms underlying these phenomena.

Materials and Methods

Cell Culture. Bovine pulmonary aortic endothelial cells were obtained from American Type Culture Collection (No. CCL 209; Rockville, MD)[3] at Passage 16. Experiments were conducted with cells at Passages 19–25. Cells were subcultured into T25 flasks (Corning Laboratory Science Co., Park Ridge, IL) at a density of 15,000 cells/cm^2 and grown in minimum essential medium ([MEM] No. 320-1095; Gibco Laboratories, Grand Island, NY) with 10% fetal bovine serum ([FBS] Gibco) plus 50 μg of gentamicin and 5 μg of amphotericin B/ml (Sigma Chemical Co., St. Louis, MO). Cultures were incubated in a water-jacketed incubator (Forma Scientific, Marietta, OH) at 37°C, with 90% relative humidity and 5% CO_2. By analysis, the growth medium typically contained 7 μM Zn. The cells were confluent in 5 days; the flasks then contained a monolayer of approximately 3 million cells.

Kinetics Studies. At 4 days after confluence, the growth medium was removed. The cell monolayer was gently washed with 37°C HEPES buffer (10 mM HEPES, 140 mM NaCl, 7 mM KCl, and 5.6 mM glucose [pH 7.4]) to remove any remaining medium and cellular debris. Then, 3 ml of experimental medium that had been pre-equilibrated overnight in the incubator were added and the flask was returned to the incubator. Endothelial cells under similar conditions accumulate ^{65}Zn at a linear rate for at least 15 min before a plateau begins to develop. This indicates that the amount of ^{65}Zn recycling out of the cell is insignificant and conditions of initial velocity exist during the first 15 min of incubation (5). Therefore, the rate of Zn transport was measured by incubating the cells in experimental medium for 10 min.

The experimental medium contained 14% dialyzed FBS in MEM with 250 nCi/ml of ^{65}Zn ($^{65}ZnCl_2$; Dupont NEN, Boston, MA) plus the various reagents to be investigated. Molecular sieve tubing was used to dialyze the FBS (1000 mol wt, Spectra/Por 6; Spectrum Medical Industries, Los Angeles, CA) for 3 days each (1:40, changed daily) against 10 mM EDTA, 100 mM NaBr, and 150 mM NaCl (6). The EDTA removed protein-bound Zn, enabling us to add back whatever Zn concentration was desired; the NaBr facilitated subsequent removal of the EDTA; the NaCl removed the NaBr and re-established physiological osmolarity in the serum. Dialysis of the serum also reduced the concentration of low molecular weight molecules. Growth of the endothelial cells and their transport of Zn in the undialyzed FBS were indistinguishable from those of cells in dialyzed FBS with Zn replaced by $ZnCl_2$ to the original concentration. The basal medium contained 1 μM Zn and 35 μM albumin by analysis. When the Zn concentration was constant across treatments within an experiment, it was added as $ZnCl_2$ to yield a concentration of 7 μM. These conditions approximated those when 10% undialyzed FBS was added to the medium, and the ratio of Zn to albumin was the same as that which occurs physiologically.

The flasks were gently swirled by an orbital shaker (Mistral Multi-mixer; Lab-line Instruments, Inc., Melrose Park, IL) during incubation to reduce the development of stationary layers of medium near the cell surface. Following the 10-min incubation, the labeling medium was removed and the cell monolayer was bathed for 8 sec in cold (4°C) HEPES/EDTA buffer (10 mM EDTA, 10 mM HEPES, and 150 mM NaCl [pH 7.4]). The cells were then rinsed twice with cold (4°C) HEPES buffer to remove the stop solution. Finally, 2.0 ml of 0.2% sodium dodecyl sulfate in 0.2 N NaOH were added to each flask to denature the cells. The amount of ^{65}Zn in the cell digest was determined with a gamma scintillation detector (Gamma 5500; Beckman Industries, Fullerton, CA). Based upon the specific activity of the medium, the amount of Zn that entered the cells during the incubation was calculated.

Gel Filtration Separation. A column (1 × 18 cm) was prepared with Sephadex G-25-80 beads that exclude molecules of average mol wt greater than 5000. The carboxyl groups on the polydextran chains had been reduced previously with sodium borohydride to lessen ionic binding to the column (7). Samples were prepared daily with 14% dialyzed FBS, 7 μM Zn, and 0.5 μCi of $^{65}ZnCl_2$/ml in MEM and allowed to equilibrate overnight in the incubator. One-half-ml samples were applied to the column and eluted with Tris buffer (0.05 M Trizma-7.5 [pH 7.4]) in 1.0-ml fractions. The fractions were analyzed for radioactivity with a gamma scintillation detector and the percentage of the total ^{65}Zn in each fraction was calculated. Absorbances at 280 nm and 215 nm were measured to identify fractions containing proteins and histidine, respectively.

Reagents. Unless otherwise stated, all reagents

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that also may be suitable.

were obtained from Sigma. Zn was added as $ZnCl_2$. Picolinic acid was recrystallized from a 1:1 solution of 95% ethanol and toluene and then dissolved in deionized water to produce a stock solution of 50 m*M*. MEM (No. 320-1095) contained 200 μM L-histidine·HCl and 100 μM L-cysteine. Histidine-free MEM was prepared from a Select-amine kit (No. 300-9050AV; Gibco).

Other Procedures. Protein was determined by using the bicinchoninic acid method of Smith *et al.* (8). Albumin was measured by adding bromcresol green and measuring absorbance at 628 nm (9). The Zn concentrations of the culture media were analyzed directly by atomic absorption spectrophotometry (model 503; Perkin Elmer, Norwalk, CT).

Statistical Analyses. The data describing the concentration dependency of the transport rate were analyzed by nonlinear regression techniques (SAS; SAS Institute Inc., Cary, NC) to identify the model that best described the data. To distinguish treatment differences, an analysis of variance was performed with the Crunch Statistical Package (Version 3; Crunch Software Corp., Oakland, CA). Dunnet's test was used to compare treatment groups with a control and Tukey's test was used for pairwise comparisons of multiple groups (10).

Results

Influence of Zn Concentration. The rate of Zn transport into cultured endothelial cells from a serum-free medium was measured at 12 Zn concentrations up to 70 μM (Fig. 1). The data were best described (R^2 = 0.97) by a cubic model. A plateau (J_{max} = 48.2 ± 1.45 pmol zinc/(min × mg protein)) was observed near 30 μM zinc; additional increases in the transport rate were observed as the Zn concentrations rose farther. A subsequent study was conducted to examine the influence of serum in the medium at eight Zn concentrations below 30 μM (Fig. 2). These data were best described by a saturation model in the absence of serum (R^2 = 0.92) and by a rectangular hyperbola (another saturation model, but with a 0-intercept) in the presence of serum (R^2 = 0.96). Without serum, J_{max} = 52.2 ± 2.74 pmol zinc/(min × mg protein); with serum, J_{max} = 30.8 ± 0.82 pmol zinc/(min × mg protein) and K = 3.0 ± 0.35 μM. (The saturation model of the serum-free data does not support an estimate of K.)

Influence of Various Ligands. The effects of adding albumin, cysteine, histidine, or picolinic acid to media containing serum are presented in Figure 3. The addition of 300 μM histidine (final concentration, 500 μM) or 500 μM picolinic acid to the medium resulted in an increase ($P < 0.01$) of Zn transport rate into the cultured endothelial cells. The transport rate was not significantly influenced ($P > 0.05$) by the addition of 300 μM cysteine (final concentration, 500 μM) or 35 μM bovine serum albumin (final concentration, 70 μM albumin). Influences of these four ligands were then further examined individually.

Influence of Albumin. Increasing the bovine serum albumin concentration in serum-free MEM while keeping the Zn concentration constant at either 7.2 or 14.5 μM reduced the rate of Zn transport (Fig. 4). At each

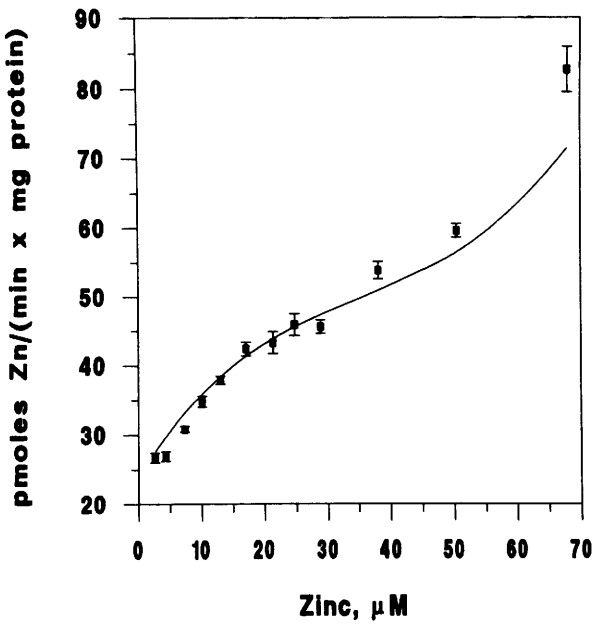

Figure 1. The influence of Zn concentration on the rate of Zn transport into endothelial cells from serum-free medium. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of four flasks; standard errors are indicated by the bars.

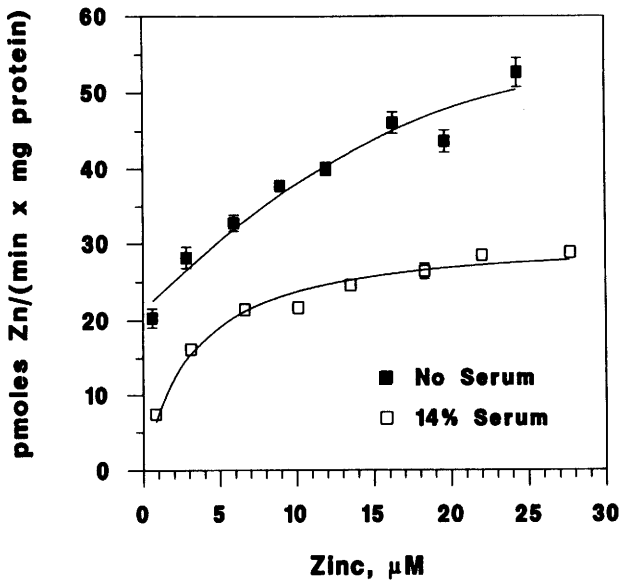

Figure 2. The influence of Zn concentration on the rate of Zn transport into endothelial cells from minimum essential medium containing 0 or 14% fetal bovine serum. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of four flasks; standard errors are indicated by the bars, which may be small enough to be obscured by the symbols.

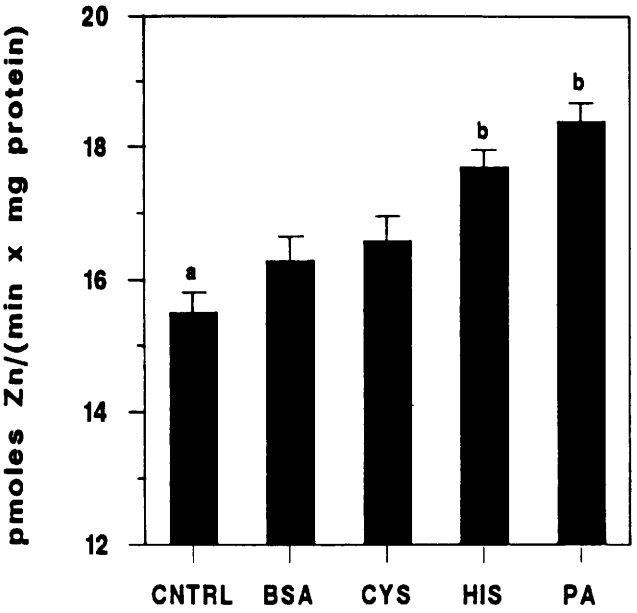

Figure 3. The influence of various ligands on the rate of Zn transport into endothelial cells from media containing 14% fetal bovine serum in minimum essential medium. The Zn concentration was 7.6 μM. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of six flasks; standard errors are indicated by the bars. The transport rates were different ($P < 0.01$; indicated by different superscripts on the bars) from the control when 300 μM additional histidine (HIS) or 500 μM picolinic acid (PA) were added to the medium, but not ($P > 0.05$) when 35 μM additional albumin (BSA) or 300 μM cysteine (CYS) was added, as determined by Dunnet's test.

albumin concentration investigated, the transport rate was greater at the higher Zn concentration, although the difference was narrowed and became statistically insignificant when the albumin concentration reached 37 μM. Next, the transport rate was measured over a wider range of albumin concentrations, while keeping the Zn concentration constant at 10 μM (Fig. 5). The stepwise pattern of albumin's influence on the uptake rate resembled a titration curve in both studies. In Figure 4, the transport rates were similar ($P > 0.10$) whether 3.7 or 9.1 μM albumin were added to the medium containing either Zn concentration. The addition of 18.5 or 37.2 μM albumin also resulted in similar ($P > 0.10$) Zn transport rates when the Zn concentration was 7.2 μM. In Figure 5, the transport rate was not affected ($P > 0.10$) by the addition of 1.7 or 4.1 μM albumin, as compared with no albumin; however, the presence of 6.9 μM albumin, or more, slowed the transport rate to less ($P < 0.05$) than it was in the absence of albumin. The transport rates at 6.8, 10.3, and 12.7 μM albumin were similar ($P > 0.10$) to each other but greater than ($P < 0.05$) the rates at 22.0, 27.4, and 30.8 μM albumin. Zn transport at the latter concentrations of albumin were similar ($P > 0.10$) to each other but greater than ($P < 0.05$) the rates at 41.6 and 50.8 μM albumin, which were similar ($P > 0.10$).

Influence of Histidine. As shown in Figure 6, increasing the histidine concentration in histidine-free medium without serum resulted in a dose-dependent

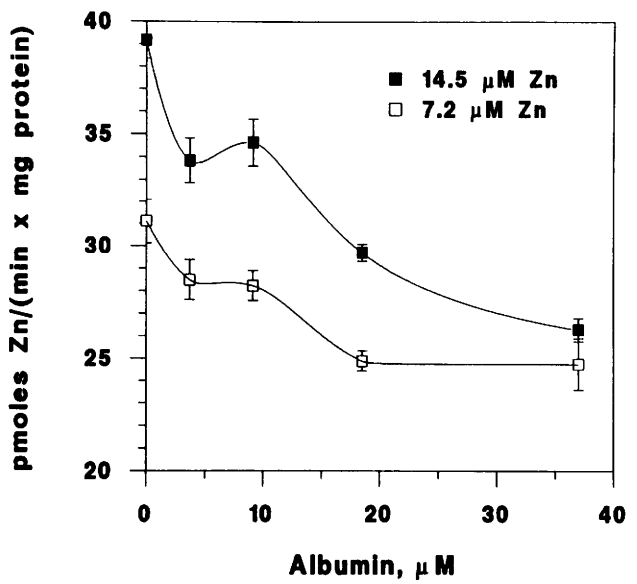

Figure 4. The influence of bovine serum albumin concentration on the rate of Zn transport into endothelial cells from serum-free minimum essential medium. The concentrations of Zn and albumin were determined by analysis. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of five flasks; standard errors are indicated by the bars, which may be small enough to be obscured by the symbols.

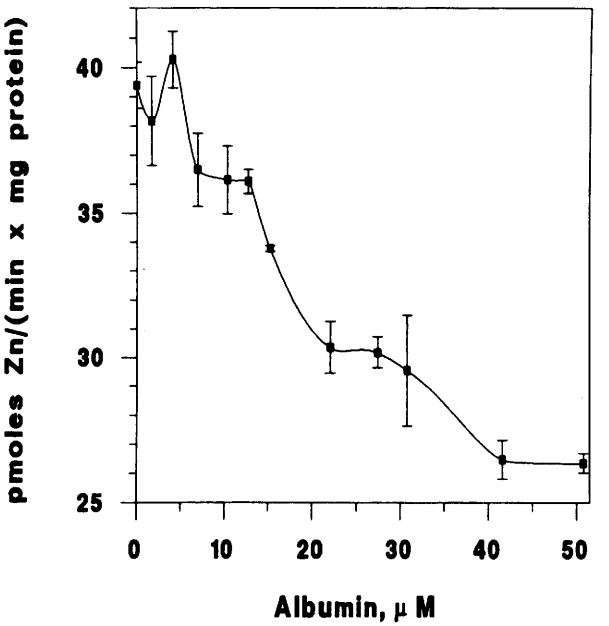

Figure 5. The influence of bovine serum albumin concentration on the rate of Zn transport into endothelial cells from serum-free minimum essential medium. The Zn concentration of 10 μM was maintained. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of four flasks; standard errors are indicated by the bars.

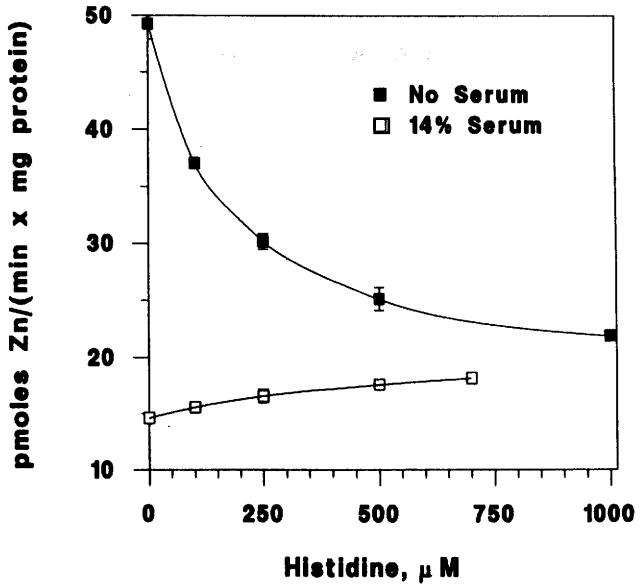

Figure 6. The influence of histidine concentration on the rate of Zn transport into endothelial cells from histidine-free minimum essential medium containing 0 or 14% serum. The Zn concentration was maintained at 7.5 μM. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of five flasks; standard errors are indicated by the bars, which may be small enough to be obscured by the symbols.

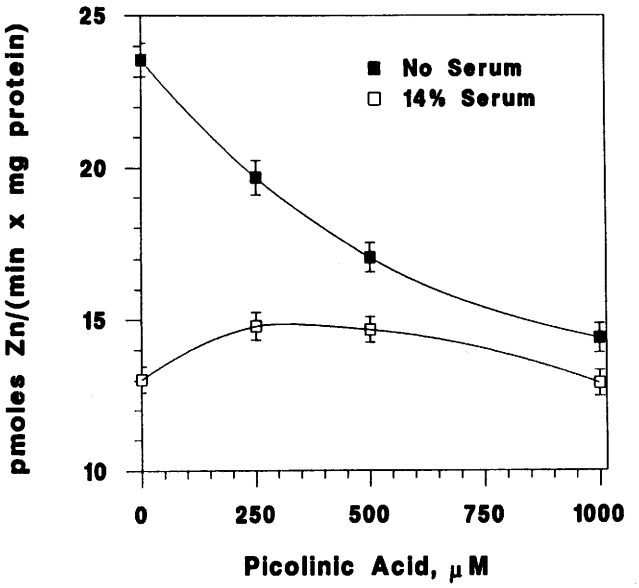

Figure 7. The influence of picolinic acid concentration on the rate of Zn transport into endothelial cells from minimum essential medium containing 0 or 14% serum. The Zn concentration was maintained at 7.7 μM. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of six flasks; standard errors are indicated by the bars.

decrease in the Zn transport rate. The rate at each histidine concentration in the absence of serum was different ($P < 0.001$) from all others. When the medium contained 14% serum (35 μM albumin, by analysis), the Zn transport rate was severely depressed when compared with that when no albumin was present. However, with the addition of 250 μM histidine, the transport rate was significantly increased. Furthermore, the addition of histidine to 700 μM caused a 24% increase ($P < 0.001$) in the transport rate.

Influence of Picolinic Acid. As shown in Figure 7, The addition of picolinic acid to the serum-free medium resulted in a dose-dependent decrease in the Zn transport rate. The rate at each picolinic acid concentration in the absence of serum was different ($P < 0.001$) from all others. However, when the medium contained 14% serum (35 μM albumin, by analysis), the transport rate increased ($P < 0.05$) by 11% and 12% as the picolinic acid concentration was increased to 250 and 500 μM, respectively; this effect was abrogated ($P > 0.10$) at 1000 μM picolinic acid.

Influence of Citric Acid. As shown in Figure 8, The addition of citric acid to the medium had no effect ($P > 0.10$) on the rate of Zn transport. However, the transport rate was consistently higher ($P < 0.001$) without serum in the medium.

Zn Distribution in Medium. The addition of ligands to the medium caused a redistribution of Zn among the components. ^{65}Zn in samples of labeled culture media eluted from a Sephadex column in three peaks

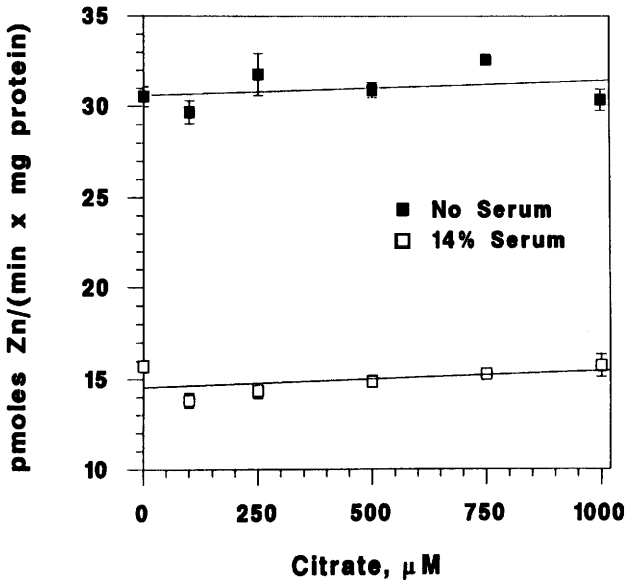

Figure 8. The influence of citric acid concentration on the rate of Zn transport into endothelial cells from minimum essential medium containing 0 or 14% serum. The Zn concentration was maintained at 6.7 μM. The transport rates were calculated from the amount of ^{65}Zn taken up by the cells during a 10-min incubation. Each value represents the mean of four flasks; standard errors are indicated by the bars, which may be small enough to be obscured by the symbols.

and were designated high, intermediate, and low molecular weight ligands (HMWL, IMWL, and LMWL, respectively). Figure 9 shows the elution profile of medium (14% FBS in MEM) with 500 μM additional histidine. This was from a representative example and demonstrates the three Zn peaks. The HMWL peak

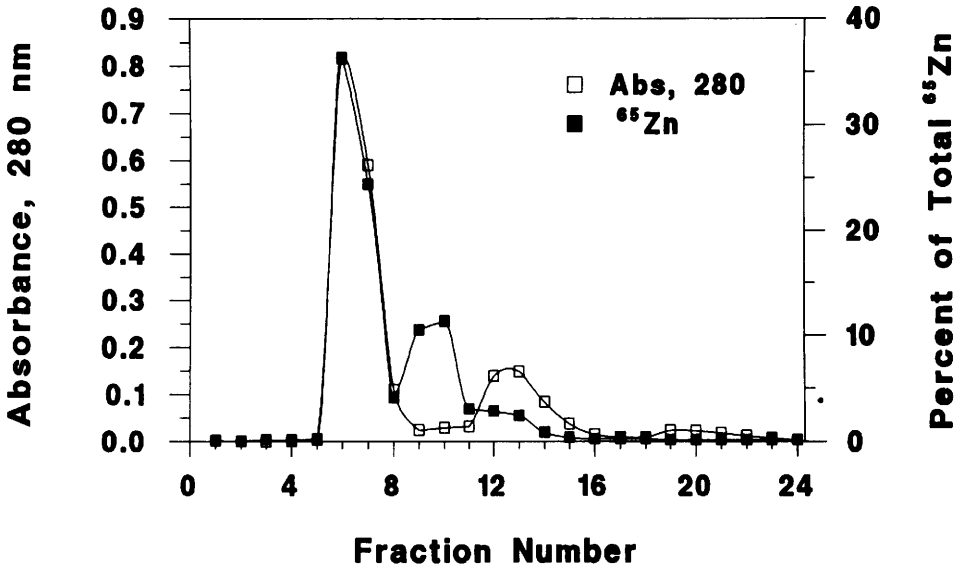

Figure 9. Sephadex G-25-80 elution profile of culturing medium containing 14% serum, 7 μM zinc, and 500 μM additional histidine in minimum essential medium. Each point represents the mean of three samples for the percentage of ^{65}Zn recovered in each fraction and the mean of two samples for the absorbance at 280 nm.

includes Fractions 6 and 7 and corresponds to the elution of high molecular weight proteins as demonstrated by the peak absorbance at 280 nm. The IMWL peak includes Fractions 9 and 10. The LMWL peak includes Fractions 12 and 13 and corresponds to the elution of low molecular weight molecules such as histidine and other amino acids, as demonstrated by peak absorbance at 280 nm and 215 nm (not shown). The distribution of ^{65}Zn among the three ligand groups is presented in Table I. Increasing the concentration of LMWL by adding 500 μM histidine or picolinic acid caused a significant ($P < 0.01$) shift of ^{65}Zn from HMWL to LMWL. The addition of 500 μM cysteine or citric acid had no effect ($P > 0.05$) on Zn distribution.

Table I. Distribution of ^{65}Zn among High, Intermediate, and Low Molecular Weight Ligands in Culturing Medium[a]

	HMWL	IMWL	LMWL
Control[b]	68.1 ± 1.3	21.7 ± 1.3	0.9 ± 0.0
+His	60.9 ± 0.8[c]	22.0 ± 0.7	6.7 ± 0.5[c]
+Cys	70.0 ± 1.1	17.8 ± 1.3	2.0 ± 0.2
+PA	53.9 ± 1.4[c]	18.6 ± 0.8	16.6 ± 0.9[c]
+CA	65.0 ± 1.5	23.0 ± 1.1	1.3 ± 0.4

[a] Treatments were designed to examine the influence of increasing the concentration of the low molecular weight ligand fraction with 500 μM histidine (His), cysteine (Cys), picolinic acid (PA), or citric acid (CA). Each value is the mean ± SE for the percentage of total ^{65}Zn that eluted in a fraction. Four samples of each medium (except the +His medium, which had $n = 3$) were eluted on a Sephadex G-25-80 gel filtration column.

[b] The control was the "experimental medium" that was composed of 14% fetal bovine serum in minimum essential medium with 6.8 μM Zn and 35 μM albumin, by analysis.

[c] Means are significantly different ($P < 0.01$) from the mean of the control group for the column as determined by Dunnet's test.

Discussion

We examined the influences of various physiological Zn ligands on Zn transport into cultured endothelial cells. The results demonstrate that the nature and concentration of ligands in the medium significantly affect the rate of Zn transport; this is an indication that ligands in blood would produce a similar effect. The influences of Zn ligands on the transport, accumulation, or absorption of Zn have been examined previously, but without much agreement among investigators. Zn transport was reported to be inhibited by albumin, histidine, and cysteine (11–14) or citric acid and picolinic acid (15–17) and promoted by albumin, histidine, cysteine (18, 19), and picolinic acid (20, 21). These various, and often conflicting, observations have contributed to our understanding of the zinc transport mechanism, but they also underscore our ignorance of the transport process and how zinc transport is regulated. Such knowledge would enhance our understanding of the directional fluxes of Zn between organs and the redistribution of Zn pools. The present work provides a more comprehensive description of how ligands influence the Zn transport process.

The Zn transport rate was saturable near 30 μM Zn whether or not serum was in the medium, but the rate at saturation was considerably higher in medium without serum. The transport rate from serum-free medium with 1 μM Zn was similar to the rate when the Zn concentration approached the albumin concentration in medium with serum. Also, the transport rate increased as the Zn concentration increased from 1 μM

Zn in the serum-free medium in a pattern similar to that observed previously (5) when the Zn concentration increased beyond the albumin concentration (35 μM). Therefore, the kinetics of Zn transport in the absence of serum resembles the kinetics when the Zn concentration exceeds the albumin concentration in medium with serum. This suggests that the kinetics of Zn transport is different when Zn is associated with albumin than when it is not.

The models that best described the data obtained with serum-free media (Figs. 1 and 2) indicate a nonzero intercept. This is probably not an artifact caused by nonspecific binding of ^{65}Zn, because the stop solution contained 10 mM EDTA. When statistical models were forced to include a 0-intercept, the experimental data were not accurately described. Our experimental protocol did not allow us to determine transport kinetics below 1 μM Zn, so we cannot be certain what the pattern is in the 0–1 μM Zn range; it could be quite complex. Although a 0-intercept is predictable, this point was not included in the analyses.

What determines the kinetics of Zn not associated with albumin? Apparently, it is the presence of other potential Zn ligands. The results suggested that the rate at which Zn was transported into an endothelial cell depended upon the ligand with which it was associated, and the overall rate of Zn transport depended upon the concentration of Zn associated with each ligand. We arrived at this conclusion in part because an increase in the histidine concentration in medium containing serum had two consequences: (i) there was a redistribution of Zn from the serum proteins to LMWL (gel filtration studies) and (ii) the transport rate increased (kinetics studies). The free Zn ion concentration could only have decreased in the presence of additional Zn ligands. Thus, the free Zn ion concentration was not a relevant factor, as has been suggested by Ackland and McArdle (13)—at least not from medium containing serum. The LMWL fraction contained histidine and other amino acids which presumably formed complexes with Zn, such as His·Zn, His_2·Zn, and His·Zn·Cys. These data suggest that LMWL-bound Zn is transported more rapidly than protein-bound Zn.

One might also interpret these results as evidence that histidine was acting as a cotransporter that assisted physically in the transport of Zn. This is unlikely, however, because Zn transport occurred in histidine-free, serum-free medium at a significantly higher rate than from medium containing serum and histidine. Adding histidine to serum-free medium decreased the transport rate. The results imply that Zn bound to histidine was more available for transport than Zn bound to serum proteins, but less available than Zn bound to the other ligands in MEM. Similar results were observed when picolinic acid was added to the medium, demonstrating that this phenomenon is not specific for histidine. Cysteine and citrate were relatively ineffective under the conditions employed, possibly because their affinities for Zn are lower than those of the other ligands and a significant change in the amount of Zn bound to them did not occur. The gel filtration experiment confirms this hypothesis because the addition of 500 μM cysteine or citric acid did not cause a redistribution of Zn. This is contradictory to the prediction of Giroux and Henkin (2), which suggests that increasing the cysteine concentration would shift more Zn away from albumin than a similar increase in histidine; their prediction was based on a computer model using the estimated binding constants for Zn associated with various ligands in human serum. However, our results agree with those of Prasad and Oberleas (22), who found that the addition of histidine, glutamine, threonine, cysteine, or lysine caused a shift of Zn from proteins to ultrafilterable ligands of human serum; this effect was most pronounced when the Zn concentration exceeded the albumin concentration.

Why was Zn associated with LMWL transported more rapidly than Zn bound to serum proteins? Apparently, LMWL were more likely to release their Zn to the transporter than the HMWL. The LMWL had a lower affinity for zinc; they only bound a significant fraction of the total Zn when their concentrations were elevated. Zn bound to LMWL may also have been better positioned physically for transferral to the transporter than Zn in the binding site of a large protein like albumin. The ability to influence Zn transport appears to be related more to the affinity of the ligand for Zn than to some other unique property because similar responses were observed with such diverse molecules as histidine and picolinic acid. The LMWL concentrations employed in our studies exceed those observed under normal physiological situations, and were chosen to identify effects that were detectable with our methods. Nevertheless, a similar pattern would be expected to occur in response to changes in physiological concentrations of these ligands.

Our chromatographs also identified a ^{65}Zn fraction associated with ligands of intermediate molecular weight. The identity of these ligands was not established, but there was no detectable absorbance at 215 or 280 nm. The experimental addition of the ligands did not significantly affect the ^{65}Zn composition of this IMWL fraction.

Albumin is believed to be the most important Zn ligand of the HMWL fraction (2). Therefore, we examined the influence of albumin concentration on the Zn transport rate. The results indicate that the relative amounts of albumin and Zn in the medium were very important to the rate of Zn transport. When we increased the albumin concentration relative to the Zn concentration, the transport rate declined until a ratio of approximately 4:1 (albumin to zinc) was reached.

The rate changes occurred in a stepwise manner reminiscent of a titration curve—the type that describes the number of metal ions bound by a metalloprotein with multiple binding sites of differing affinity at increasing concentrations of the metal. The plateaus in transport rate occurred when the albumin to Zn ratio approached 1:1, 2:1, and 4:1. Similar data were presented by van den Berg and van den Hamer (23), but were not discussed. The albumin to Zn ratio is greater than 4:1 in serum of healthy people. These data imply that the successive association of up to four albumin molecules per Zn atom contribute allosterically to the controlled transport of Zn. This might be accomplished by regulating the formation of the Zn-transporter complex. The effect is not likely to be the result of a change in the concentration of free Zn, which is negligible. Also, if the effect was caused solely by a change in free Zn concentration, it would not explain the stepwise shape of the results.

Previous investigators have also observed a decrease in the Zn transport rate when the albumin concentration was increased (11–14). This is commonly reported as an inhibitory effect of albumin on Zn transport. However, we consider this effect to be a moderating influence, and that the "normal" rate of Zn transport is the lower one observed when the albumin to Zn ratio exceeds 4:1—as it would in blood. The elevated transport rates observed when this ratio is lower than 4:1 apparently are aberrations—perhaps even pathological.

These results demonstrate the potential consequences when the relative concentrations of Zn ligands change in extracellular fluid. The physiological significance of this in the redistribution of Zn between organs, cells, and organelles remains to be elucidated. The synthesis, transport, and catabolism of various Zn ligands may be a significant factor in regulating the distribution and localization of Zn in normal, as well as pathological, conditions. These observations also help explain why different, and often conflicting, results regarding Zn transport have been reported previously by different laboratories; they did not control the relative ligand concentrations. This was demonstrated most clearly by our observation that histidine increased Zn transport in the presence of albumin, but decreased it from serum-free medium. The relative ratios of Zn and Zn ligands in experimental research should be maintained as near to physiological levels as possible in order to enable reliable interpretations.

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