Zinc exchange by endothelial cells in culture

Dennis J. Bobilya, Mary Briske-Anderson, LuAnn K. Johnson, and Philip G. Reeves

United States Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND, USA

Endothelial cells form the lining of blood vessels. Therefore, nutrients must cross this barrier each time they enter or leave the blood. While passing through the endothelium, nutrients may interact with intracellular zinc pools. This study was undertaken to characterize the dimensions of the zinc pools that are associated with endothelial cells. ^{65}Zn was employed as a tracer to follow the pattern of zinc uptake and release by bovine pulmonary aortic endothelial cells that had grown into a confluent monolayer. The total cellular zinc content of the endothelial cells was calculated to be 2.48 nmol zinc/mg protein, based upon specific activity. The $t_{1/2}$ for cellular zinc turnover was 244 min. Mathematical modeling of both the uptake and the release processes demonstrated that there are two pools of zinc associated with each. A slow exchange pool was estimated to contain approximately 1.4 nmol zinc/mg protein during both influx and efflux experiments. A fast exchange pool contained 1.11 nmol zinc/mg protein during zinc efflux; zinc entering the cell was exchanged rapidly with a pool of 0.65 nmol zinc/mg protein. The efflux measurement may contain labeled ligands resulting from the catabolism of zinc biomolecules that are absent from the influx study; it is more likely to be a true estimate of the fast exchange pool.

Keywords: zinc ligands; cellular zinc pools; endothelial cells

Introduction

Endothelial cells form the inner lining of blood vessels; along with the basement membrane, they form the barrier through which nutrients must pass in order to be exchanged between the blood and the surrounding tissues. The mechanism by which zinc passes through this barrier has not been described. This study addresses the possibility that zinc, in passing across the endothelium, might interact with endogenous cellular zinc pools.

Few studies have characterized the intracellular pools of zinc. Erythrocytes reportedly contain 5 μM exchangeable zinc/L packed cells, which is exchanged with a half-time of 7 hours.[1] Zinc uptake by hepatocytes has been described as biphasic; the rapidly exchanging component comprises 440 pmol zinc/mg protein and apparently represents net accumulation of zinc into a labile pool[2]; a slow phase represents exchange with a pool comprised of zinc more stably bound to ligands of relatively high affinity.[3] We undertook the present study to examine the dimensions of the intracellular zinc pools of endothelial cells. As nutrient transport is a principal function of this cell type, the size and nature of the nutrient pools may be unique.

Materials and methods

Cell culture

Bovine pulmonary aortic endothelial cells were obtained from American Type Culture Collection (Rockville, MD, USA)* at passage 16. Experiments were conducted using cells at passages 18–20. Cells were subcultured into T-25 flasks (Corning Lab. Sci. Co., Park Ridge, IL, USA) at a density of 15,000 cells/cm^2 and grown in minimum essential medium (MEM, #320-1095, Gibco Labs., Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco Labs., Grand Island, NY, USA), plus 50 μg/mL gentamicin

Address reprint requests to Dr. Philip G. Reeves, Human Nutrition Research Center, Grand Forks, ND 58202-7166, USA.
Presented in part to the North Dakota Academy of Science, Fargo, ND, USA, April, 1990.
Received December 19, 1990; accepted April 2, 1991.

* 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.

and 5 μg/mL amphotericin B (Sigma Chemical Co., St. Louis, MO, USA). By analysis, the growth medium typically contained 7 μmol/L zinc. Incubation was in a water-jacketed incubator (Forma Scientific, Marietta, OH, USA) at 37° C, 90% relative humidity, and 5% CO_2.

Zinc uptake

At 4 days' post-confluence, the growth medium was removed. The cell monolayer was rinsed with 37° C HEPES buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 7 mmol/L KCl, and 5.6 mmol/L glucose, pH 7.4), to remove any remaining medium and cellular debris. Then, 3 mL of labeling medium were added and the flasks were returned to the incubator for the appropriate lengths of time from 0–1500 min. The labeling medium was pre-equilibrated overnight in the incubator; the medium was outside the incubator only briefly when it was being applied.

The labeling medium was comprised of 14% FBS in MEM. The FBS had been dialyzed within a molecular sieve bag (1000 MW, Spectra/Por 6, Spectrum Medical Industries, Los Angeles, CA, USA) for 3 days each (1:40, 3 changes) against 10 mmol/L EDTA, 100 mmol/L NaBr, and 150 mmol/L NaCl.[4] The EDTA removed protein-bound zinc, the NaBr facilitated subsequent removal of the EDTA, and the NaCl removed the NaBr and reestablished physiological osmolarity in the serum. Then, 6 μmol/L zinc ($ZnCl_2$) and 250 nCi ^{65}Zn/mL (Dupont NEN, Boston, MA, USA) were added to the medium. Upon analysis, the labeling medium contained 7.2 μmol/L zinc.

Following the timed incubations, the labeling medium was removed and the cell monolayer was bathed for 8 seconds in cold (6° C) HEPES/EDTA buffer (10 mmol/L EDTA, 10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4). The cells were then rinsed twice with cold HEPES buffer. Finally, 2.0 mL of 0.2 N NaOH with 0.2% sodium dodecylsulfate were added to the flasks to denature the cells. The amount of ^{65}Zn in the cell digest was determined by a gamma scintillation detector (Gamma 5500, Beckman Industries, Fullerton, CA, USA).

Zinc release

Cells were subcultured into and grown for 9 days (until 4 days' post-confluence) in medium containing 250 nCi ^{65}Zn/mL. Then, the cell monolayers were treated as in the uptake study. The composition of the media and buffers were the same, except that the medium used in the timed incubations was not radioactive and contained 6.3 μmol/L zinc, by analysis.

Chemical analyses

Zinc was analyzed by aspirating the media directly into a flame atomic absorption spectrophotometer (Perkin Elmer 503, Norwalk, CT, USA). Analysis of the National Bureau of Standards bovine liver reference sample following complete digestion of organic matter gave 122.6 ± 4.9 μg/g (n = 14) as compared to a certified value of 123 ± 7. Analysis of the same sample 14 times resulted in a coefficient of variation equal to 0.4%. Protein was determined by the bicinchoninic acid method.[5]

Data analyses

Rectangular hyperbola, single exponential, and two-component exponential models were fit to the data by using nonlinear regression techniques (SAS, SAS Institute Inc., Cary, NC, USA). The mean square error (MSE) from each model and the plot of the residuals (residual = predicted value − actual value) versus time were examined to determine which model provided the best fit. Compared to the single exponential and rectangular hyperbola, the two-component exponential model significantly reduced the MSE. Adding the second exponential term to the model also removed a nonlinear trend that was apparent in the plot of the residuals from the single exponential model versus time. Thus, a two-component exponential model was used to calculate the rate constants for the uptake and efflux data. The rate constants, k_1 and k_2, are defined as:

$$k_i = 0.693 / T_{1/2} \quad (1)$$

where,

k_i = rate constant min^{-1}

$T_{1/2}$ = half-life of ^{65}Zn turnover.

Because two-component exponential models are nonlinear in their parameters, exact standard error estimates (SEE) can not be calculated; however, asymptotic SEE are reported for each of the parameters estimated.

Previous reports have calculated rate constants for the accumulation and release of calcium and muscle tension in heart by plotting the natural logarithm of the fraction of the final and initial values, respectively, versus time.[6] The rate constant for the slow component was calculated by fitting a regression to the linear portion of the line. The effect of this component was then subtracted from the total zinc values, and a new curve was obtained that was defined as the fast component. This process is known as peeling. To facilitate comparisons to these previous reports, the models that we generated are displayed graphically as if they were computed by using the technique of peeling rather than by directly fitting a nonlinear model.

Results

Figure 1 is a graphic representation of the movement of ^{65}Zn into the cell; the amount of ^{65}ZN associated with the cells is plotted against the time that the cells were incubated in the labeling medium. These data are from one experiment, but they are similar to those we observed in other studies. Over time, the net influx of ^{65}ZN declined as the system approached equilibrium, which would be when the amount of ^{65}Zn entering the

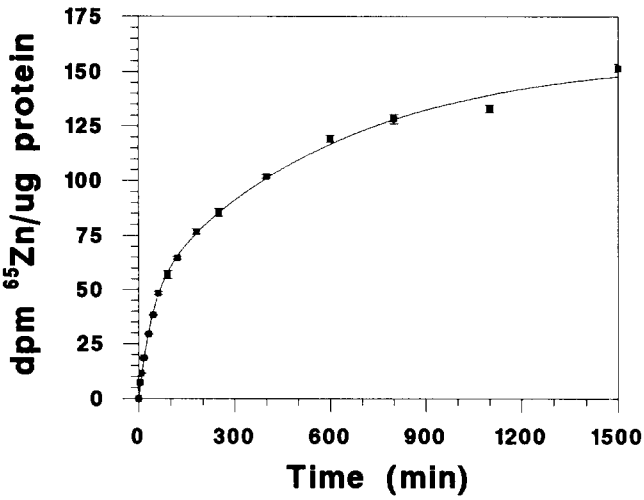

Figure 1 Accumulation of ^{65}Zn by endothelial cells incubated in culturing medium containing 250 nCi ^{65}Zn/mL. Data points are the means of four replicate flasks in one independent trial and include standard error bars.

cells from the medium equals that exiting. ^{65}Zn continued to accumulate, albeit more slowly, even after 25 hr. By dividing the asymptote of the uptake curve by the specific activity of the medium, the cellular zinc content was determined to be 2.07 nmol zinc/mg protein.

^{65}Zn uptake over time was best described by a double exponential model of the form: dpm ^{65}Zn associated with the endothelial cells at time t = $(\alpha + \beta) - \alpha e^{-k_1 t} - \beta e^{-k_2 t}$, where $\alpha e^{-k_1 t}$ and $\beta e^{-k_2 t}$ represent two components (or pools) of exchangeable zinc. The fast component is mathematically defined as $\alpha e^{-k_1 t}$, where α = 49.3 ± 3.6 (SEE) dpm $^{65}Zn/\mu g$ protein and k_1 = 0.0234 ± 0.0026 min^{-1}. The slow component is defined by $\beta e^{-k_2 t}$, where β = 107.9 ± 3.0 dpm $^{65}Zn/\mu g$ protein and k_2 = 0.00164 ± 0.00018 min^{-1}. The existence of the two components is demonstrated graphically in *Figure 2* by plotting the natural logarithm of the percent of ^{65}Zn which remains to be exchanged against time. The fast exchange component accounts for 31.4% of the zinc in the cell (0.65 nmol zinc/mg protein) and has a turnover half-life of 30 min. The slow exchange component accounts for 68.6% of the zinc in the cell (1.42 nmol zinc/mg protein) and has a turnover half-life of 423 min.

Figure 3 depicts the efflux component of zinc exchange as measured by the net movement of endogenous ^{65}Zn out of the cells. Once again, these data are from one experiment, but they are similar to those we observed in other studies. The rate of exchange declines with time as it did during zinc influx. Once again, this decline continued even after 25 hr, indicating that there are zinc pools within the cell that are available for exchange only very slowly. Half of the ^{65}Zn remained at 244 min. By dividing the initial value of the efflux curve by the specific activity of the culturing medium, the cells were estimated to have a zinc content of 2.48 nmol zinc/mg protein.

^{65}Zn efflux over time also was best fit by a double exponential model of the form: dpm ^{65}Zn associated with the endothelial cells at time $t = \alpha e^{-k_1 t} + \beta e^{-k_2 t}$, where $\alpha e^{-k_1 t}$ and $\beta e^{-k_2 t}$ represent two components (or pools) of exchangeable zinc. The fast component is defined mathematically as $\alpha e^{-k_1 t}$, where α = 94.4 ± 3.8 dpm $^{65}Zn/\mu g$ protein and k_1 = 0.0116 ± 0.0009 min^{-1}. The slow component is defined as $\beta e^{-k_2 t}$, where β = 116.5 ± 3.9 dpm $^{65}Zn/\mu g$ protein and k_2 = 0.000626 ± 0.000044 min^{-1}. The two components of zinc efflux are demonstrated graphically in *Figure 4* by plotting the natural logarithm of the percent of ^{65}Zn that has been exchanged against time. The rapidly exchanged pool accounted for 44.8% of cellular zinc (1.11 nmol zinc/mg protein) and had a turnover half-life of 60 min. The slow component comprised 55.2% of the total (1.37 nmol zinc/mg protein) and has a turnover half-life of 1100 min.

Discussion

The total cellular zinc content of endothelial cells was estimated, based on the specific activity of the culturing medium. This calculation assumes that equilibrium exists between the cells and the medium. This is a valid assumption because the concentrations of zinc in the experimental media used for the timed incubations were similar to that in the growth media; the cells were not changing their zinc concentration. Zinc exchange was occurring, rather than net accumulation or release of zinc over time. Because the labeling period for the measure of efflux was longest (9 days), its value of

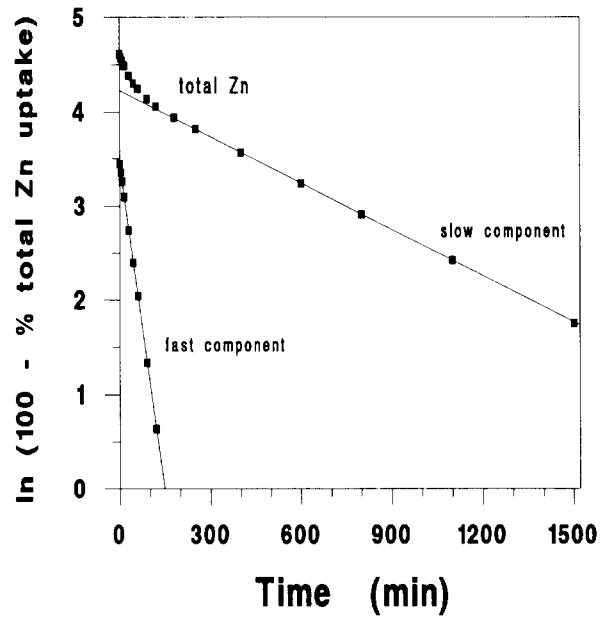

Figure 2 Two components of zinc exchange during ^{65}Zn uptake by endothelial cells are demonstrated by plotting the natural logarithm of the fraction of ^{65}Zn remaining to be exchanged against time. The linear tail of the total zinc curve represents the slower component. Subtracting the influence of the slow pool from the total generated another line that describes a faster component.

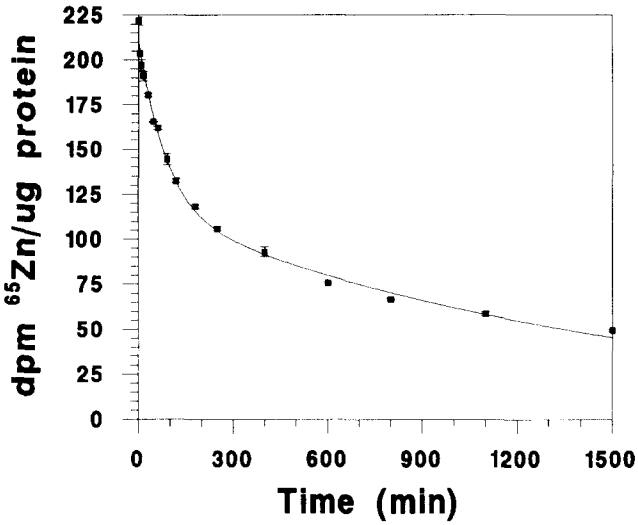

Figure 3 Retention of ^{65}Zn by endogenously labeled endothelial cells incubated in unlabeled culturing medium. Data ponts are the means of four replicate flasks in one independent trial and include standard error bars.

2.48 nmol zinc/mg protein is most accurate. (This is in agreement with unpublished values obtained in our laboratory by direct analysis.) Hepatocytes have a similar zinc concentration.[3] One mg protein corresponds to 3.8 million confluent endothelial cells. Therefore, each endothelial cell contains approximately 400 million zinc atoms. Erythrocytes reportedly contain only 10 million atoms of zinc/cell.[7] The latter is not surprising, because a major physiological function of zinc is to catalyze reactions, and red blood cells have little active metabolism.[8]

Zinc associated with endothelial cells is readily exchanged with zinc in the culturing medium. The efflux study permitted an estimation of the half-time for total cellular zinc turnover, which was 244 min. For comparison, this period is 7 hr in erythrocytes[1] and 15 hr in hepatocytes.[3]

The observation that ^{65}Zn continued to accumulate in the cells after 25 hr leaves open the possibility that there are zinc pools within the cell, not resolved by our experiments, from which zinc is exchanged only very slowly. It may take a long time for the extracellular tracer to infiltrate some of these pools. Likewise, there are forms of cellular zinc that are released only very slowly, as evidenced by the continuous decline in ^{65}Zn during its efflux.

Two distinct pools of exchangeable zinc were described by the mathematical models of zinc uptake and release by the endothelial cells. The size of the slow exchange pool was estimated to be 1.42 nmol zinc/mg protein during influx and 1.37 nmol zinc/mg protein during efflux. The rate constant for exchange by the slow pool was estimated to be 0.00164 min^{-1} during influx and 0.00063 min^{-1} during efflux. Thus, the size of the slow pool was found to be similar during influx and efflux, but the rate constant was 2.6 times faster for influx than efflux. This implies that the slow exchange pool has a relatively high affinity for zinc.

The intracellular free zinc ion concentration is believed to be very low[9,10]; most zinc is complexed to ligands. The pool of slowly exchanged zinc might consist of zinc bound tightly to metalloproteins. Some proteins bind zinc so tightly that it is exchanged primarily during their turnover. Zinc enters the binding site as it forms during protein synthesis and is released during catabolism. In this case, the turnover rate of zinc would approach that of the protein. Such proteins might include metalloenzymes[11] and structural molecules such as chromosomal[10] and biomembrane[12] proteins.

The size of the fast exchange pool was estimated to be 0.65 nmol zinc/mg protein during influx and 1.11 nmol zinc/mg protein during efflux. The rate constants were 0.0116 min^{-1} and 0.0234 min^{-1} for influx and efflux, respectively. The efflux component was nearly twice as large as the influx component, and the efflux rate constant was twice as fast as for influx. Therefore, certain ligands are capable of rapidly releasing zinc, but are relatively incapable of binding new zinc. Perhaps these ligands are the oligopeptide intermediates of zinc metalloprotein degradation. Such ^{65}Zn labeled components could not yet have arisen during the influx experiment. As the cell's zinc metalloproteins are catabolized, some oligopeptide segments might tenaciously retain their bound zinc. Catabolism of these ligands, resulting in their subsequent release of zinc, would proceed rapidly. This suggests that the efflux experiment was a more accurate estimate of true pool size.

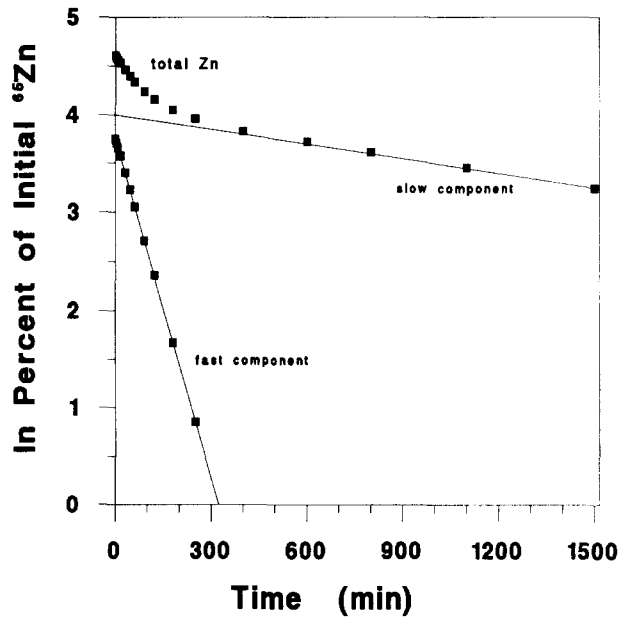

Figure 4 Two components of zinc exchange during ^{65}Zn efflux by endothelial cells are demonstrated by plotting the natural logarithm of the fraction of ^{65}Zn that remains to be exchanged against time. The linear tail of the total zinc curve represents the slower component. Subtracting the influence of the slow pool from the total generated another line that describes a faster component.

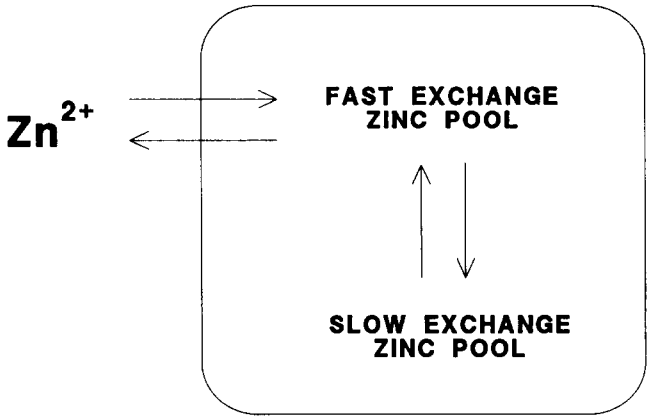

Figure 5 The kinetics of zinc exchange by endothelial cells describes two zinc pools. One pool has relatively high affinity and exchanges zinc slowly—the slow exchange pool. It draws its zinc from a labile pool that is capable of rapid exchange with the exterior—the fast exchange pool. Concomitantly, zinc leaving the slow pool reenters the fast pool.

Zinc in the fast exchange pool might be associated with cytosolic ligands that form relatively weak complexes, such as amino acids, oligopeptides, and citrate. Metallothionein is a protein of intermediate affinity that might also be a component of this pool, because it exchanges zinc more rapidly than the protein is turned over.[13,14] Such ligands readily bind zinc, thereby preventing an accumulation of free zinc ions within the cell. However, they readily pass their zinc to other ligands with a higher affinity such as newly synthesized apoenzymes. It is reasonable to suggest that zinc bound to these low affinity ligands is also readily exchangeable with extracellular zinc. Thus, the fast exchange component may function as an intermediate pool in a state of dynamic flux between the critical zinc functions of the cell and the extracellular environment.

A model of the zinc pools in endothelial cells grown on a solid surface is proposed in *Figure 5*. Extracellular zinc is exchanged with intracellular zinc that is kinetically defined as two pools. However, the pools may not be spatially separated into specific regions of the cell. The rate limiting step for zinc exchanged by the fast pool might depend upon the affinity of the ligands or might be a function of zinc transport across the cellular membrane. The rate constants for zinc uptake and release by the slow pool are relatively small, so as to make its exchange independent of membrane transport or interaction with the fast pool. Because the slow exchange pool has a greater affinity for zinc than the fast exchange pool, it is probably capable of drawing zinc away from this relatively labile pool. Likewise, zinc released from the slow pool might engage an available ligand of the fast pool rather than, or prior to, exiting the cell. Such a detour would be kinetically undetectable because of the relatively rapid efflux rate from this pool. Thus, zinc may be exchanged between the two intracellular pools. The fast pool serves as an intermediary buffer between the stable intracellular zinc complexes of the slow exchange pool and the extracellular fluid.

Zinc entering a capillary endothelial cell probably joins the fast exchange pool. It might be drafted subsequently into the slow exchange pool of high affinity metalloproteins or might be released back into the extracellular fluid, perhaps in the opposite direction from whence it entered the cell. Therefore, research into the movement of zinc across the endothelial cell barrier between blood and interstitial fluid should focus on the fast exchange pool. The size of this pool was estimated to be 1.11 nmol zinc/mg protein—45% of the cell's zinc.

Acknowledgment

The authors wish to thank Drs. Jack Saari and Scott Pattison for critically reviewing the manuscript.

References

1 Van Wouwe, J.P., Veldhuizen, M., De Goeij, J.J.M., and Van Den Hamer, C.J.A. (1990). In vitro exchangeable erythrocytic zinc. *Biol. Trace Elem. Res.* **25,** 57–69
2 Pattison, S.E. and Cousins, R.J. (1986). Kinetics of zinc uptake and exchange by primary cultures of rat hepatocytes. *Am. J. Physiol.* **250,** E677–E685
3 Cousins, R.J. (1986). Toward a molecular understanding of zinc metabolism. *Clin. Physiol. Biochem.* **4,** 20–30
4 Pilz, R.B., Willis, R.C., and Seegmiller, J.E. (1982). Regulation of human lymphoblast plasma membrane 5'-nucleotidase by zinc. *J. Biol. Chem.* **257,** 13544–13549
5 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150,** 76–85
6 Saari, J.T. and Johnson, J.A. (1971). Decay of calcium content and contractile force in the rabbit heart. *Am. J. Physiol.* **221,** 1572–1575
7 Hinks, L.J., Clayton, B.E., and Lloyd, R.S. (1983). Zinc and copper concentrations in leucocytes and erythrocytes in healthy adults and the effect of oral contraceptives. *J. Clin. Pathol.* **36,** 1016–1021
8 Williams, R.J.P. (1984). Zinc: what is its role in biology? *Endeavour* **8,** 65–70
9 Magneson, G.R., Puvathingal, J.M., and Ray, W.J., Jr. (1987). The concentrations of free Mg^{2+} and free Zn^{2+} in equine blood plasma. *J. Biol. Chem.* **262,** 11140–11148
10 Williams, R.J.P. (1989). In *Zinc in Human Biology* (C. F. Mills, ed.), pp. 15–31, Springer-Verlag, London
11 Vallee, B.L. (1983). In *Zinc Enzymes* (T. G. Spiro, ed.), pp. 1–24, Wiley Press, New York
12 Bettger, W.J. and O'Dell, B.L. (1981). A critical physiological role of zinc in the structure and function of biomembranes. *Life Sci.* **28,** 1425–1438
13 Petering, D.H., Krezoski, S., Villalobos, J., Shaw, C.F., III, and Otvos, J.D. (1987). Cadmium-zinc interactions in the ehrlich cell: metallothionein and other sites. *Experient. Suppl.* **52,** 573–580
14 Thomas, D.G., Dingman, A.D., and Garvey, J.S. (1987). The function of metallothionein in cell metabolism. *Experient. Suppl.* **52,** 539–543