Biochem. J. (1988) 255, 483–491 (Printed in Great Britain)

Kinetic lability of zinc bound to metallothionein in Ehrlich cells

Susan K. KREZOSKI, Juanita VILLALOBOS, C. Frank SHAW, III and David H. PETERING*
Department of Chemistry, University of Wisconsin–Milwaukee, Milwaukee, WI 53201, U.S.A.

Ehrlich ascites-tumour cells normally contain a large concentration of Zn-metallothionein. When cells are placed in culture media, containing or pretreated with the metal-ion-chelating resin Chelex-100, they stop growing, remain viable and lose zinc specifically from the metallothionein (MT) pool. The kinetics of loss of zinc are first-order and are very rapid, having a rate constant of greater than or equal to 0.6 h^{-1}. MT protein labelled with ^{35}S is biodegraded with a rate constant of 0.07–0.014 h^{-1} in control cells, 0.08 h^{-1} in cells exposed to the zinc-deficient medium and 0.12–0.18 h^{-1} in cells treated directly with Chelex. Over the 6 h period in which zinc is totally lost from Zn-MT there is relatively little decrease in MT-like protein as measured by cadmium-binding to the 10000-M_r protein fraction. Other pools of zinc and ^{35}S-labelled protein turn over more slowly. There is no loss of zinc from rat liver Zn-MT that is dialysed against Chelex to model the possible reaction of the resin with Ehrlich-cell Zn-MT. However, Chelex does compete slowly for MT-bound zinc when resin and MT are directly mixed. Analysis of the known and possible pathways of zinc metabolism in cells in relationship to these rate constants shows that biodegradation of MT protein cannot account for the rate of loss of zinc from Zn-MT.

INTRODUCTION

Three decades ago Margoshes & Vallee (1957) discovered a metal-binding protein that they called 'metallothionein'. The protein (MT) binds a variety of metals, including zinc, copper and cadmium. Its particular metal content is a function of the history of the tissue or cell from which it is derived. Numerous studies have shown that elevated metal exposure and non-specific stress to the organism induce the synthesis of MT (Webb & Cain, 1982). Fetal and regenerating liver, adult kidney and pancreas contain an abundance of the protein (Ohtake *et al.*, 1978; Onosaka & Cherian, 1982; Petering *et al.*, 1984). Other studies have shown that a number of tumours contain MT-like proteins or their mRNA (Koch *et al.*, 1980; Mayo & Palmiter, 1981; Fenton & Burke, 1984).

Until 1980 the prevailing view of the function of MT was that it acts within cells as a passive unreactive store of metal that becomes available only as the protein is biodegraded (Webb & Cain, 1982). At that time chemical studies began to show that the zinc–thiolate sites in MT were reactive in ligand-exchange reactions and in reactions involving the metal-co-ordinated thiol groups (Udom & Brady, 1980; Li *et al.*, 1980, 1981; Petering *et al.*, 1982). In particular, it was demonstrated that small ligands such as EDTA, apo-(carbonic anhydrase) and some other apo-Zn-proteins can remove zinc from Zn-MT. In fact, apo-(carbonic anhydrase) is reconstituted with zinc from Zn-MT at 25 °C rapidly as with $ZnCl_2$ (Li *et al.*, 1980). It was therefore suggested that Zn-MT may actively donate zinc to other zinc-requiring structures in cells by ligand-exchange reactions, which do not require rate-limiting biodegradation of MT protein to release zinc from its metal-thiolate clusters.

To enquire about the validity of this hypothesis, a tractable model to study had to be found. It has previously been shown that Ehrlich cells normally contain readily measurable amounts of Zn-MT (Koch *et al.*, 1980; Kraker & Petering, 1983; Kraker *et al.*, 1988). Under zinc-deficient conditions the Ehrlich ascites tumour in mice loses zinc preferentially from the MT pool (Kraker & Petering, 1983; Kraker *et al.*, 1988). The present paper describes kinetic studies of zinc metabolism in Ehrlich cells under zinc-deficient conditions in culture that are consistent with the hypothesis that zinc bound to MT is a labile source of zinc and actively participates in ligand-substitution reactions in cells.

MATERIALS AND METHODS

Materials

Most of the materials used were purchased from standard supply houses; all chemicals were reagent grade. The following are specially noted. Chelex-100 beads (120 mesh) were purchased from Bio-Rad Laboratories, Superose 12 column and Sephadex G-75 were from Pharmacia, Spectropor dialysis tubing was from Spectrum Industries, and [^{3}H]thymidine was from New England Nuclear. The cell-culture materials were purchased from GIBCO, except the fetal-calf serum, which was supplied by Flow Laboratories.

Pre-equilibration of Chelex beads

Chelex is comprised of iminodiacetic acid groups bound to an insoluble polystyrene resin bead. Following the procedure of Messer *et al.* (1982), the beads were equilibrated overnight with the major salts present in Eagle's medium. Without this pretreatment the presence of the Chelex in the Eagle's medium might generally alter its cation composition. After incubation, 10 g of beads was placed in 25 ml of fresh medium, and sterilized. Upon sterilization a small number of beads had collapsed.

Abbreviations used: MT, metallothionein; MEM, Eagle's minimal essential medium containing Earle's salts.

* To whom correspondence should be addressed.

These absorbed Phenol Red present in Eagle's medium. This did not affect the ability of the beads to chelate zinc. Chelex was added to the medium in a small amount of the sterile equilibrium buffer, typically 2.5 times the bead volume. In the work described below, 'depleted medium' is medium that had been incubated with pretreated Chelex beads of 2–24 h and had been separated from the beads, which rapidly sediment out of non-stirred suspensions. Zinc and other trace metals were assayed by using flame atomic-absorption techniques on an Instrumentation Laboratories model 357 AA/AE spectrophotometer.

Treatment of cells in culture with Chelex or Chelex-treated medium

Ehrlich tumour cells were maintained in spinner culture as previously described (Krishnamurti *et al.*, 1980). To treat the cells with Chelex, cells were resuspended in fresh Eagle's medium containing Earle's salts (MEM) plus 2.5% (v/v) fetal-calf serum; 10 g of Chelex/100 ml of medium was added. After incubation, the spinner bottle was removed from the incubator and the beads were allowed to settle for 2–3 min. The majority of the beads sank to the bottom, while the cells were still suspended. After the medium above the beads had been decanted and residual beads allowed to settle again, the cell suspension was centrifuged to obtain a cell pellet for subsequent use. In other experiments cells were incubated in the absence of resin in medium pretreated with 10 g of Chelex/100 ml. Both procedures limit extracellular zinc and produce similar results in the experiments described below.

Cell counting

Cells were counted at the beginning and end of experiments by using a haemocytometer and microscope. During this procedure cells were also treated with 0.04% Trypan Blue solution (1 drop/ml) to determine by vital dye exclusion how many cells had lost membrane integrity. The ratio of blue cells to total cells is expressed as percentage of cells permeable to dye.

Column chromatography

To examine the profiles of metals in cell supernatant, 10^8 cells pelleted after various treatments were mixed with 0.5–0.75 ml of cold water and 5–7.5 μl of 10% (v/v) 2-mercaptoethanol. The cells were then sonicated and then centrifuged at 48000 *g* for 20 min, and the supernatant (cytosol) was applied to a 1.5 cm × 30 cm Sephadex G-75 chromatography column in 5 mM-Tris/HCl buffer, pH 7.8 (Koch *et al.*, 1980; Minkel *et al.*, 1980).

[^{3}H]Thymidine uptake and incorporation by cells

The rate of DNA synthesis was assessed by monitoring the ability of the cells to incorporate extracellular [^{3}H]thymidine into their DNA (Saryan *et al.*, 1979).

Cadmium binding to apo-MT-like protein

To test whether an apo-(Cd-binding protein) similar to MT was present in zinc-depleted cells, cell cytosol was prepared as described above. Then 4.25 nmol of $CdCl_2$/10^7 cells was added directly to the supernatant. The solution was kept for 15 min at room temperature and then applied to a Sephadex G-75 gel-filtration column for analysis.

Kinetics of zinc loss from cells

Time-dependent measurements of MT zinc and high-M_r zinc were made after the addition of Chelex or Chelex-treated medium to cells in spinner culture as described above. Cells (0.7×10^8–1.5×10^8) grown in 100 ml of medium were exposed to 1 or 10 g of Chelex at time zero. Alternatively cells were added at time zero to media pretreated with 10 g of Chelex/100 ml of medium. Periodically the cells were removed from culture for chromatographic analysis. The resulting high-M_r metal and MT metal profiles were integrated and the zinc content was standardized by dividing by the number of cells used. The logarithm of this value was plotted versus time and the slope of the curve was used to determine the first-order rate constant and the $t_{\frac{1}{2}}$ for loss of zinc from cytoplasmic pools of metal.

Kinetics of loss of ^{35}S-labelled protein

The method used for determining the loss of ^{35}S-labelled cysteine from MT and other proteins during biodegradation is a modification of the procedure of Patierno *et al.* (1983). Proteins in cultured cells were labelled with 10 μCi of [^{35}S]cysteine/100 ml for a period of 24 h. These cells were pelleted, then washed three times with fresh MEM, and 8×10^7 cells were resuspended in 100 ml of fresh MEM plus 2.5% (v/v) fetal-calf serum. In some experiments a 10-fold excess of unlabelled cystine was added to the culture medium during the measurement of ^{35}S turnover to dilute out labelled cysteine, which might be re-incorporated into MT and change the apparent rate of loss of radioactivity from the protein. In the Chelex experiment 1 or 10 g of Chelex was added per 100 ml of medium at this time. Samples were removed at 0, 2, 4, 16 and 24 h. Cells were counted, collected by centrifugation, resuspended in 500 μl of phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4), placed in cryotubes and frozen in liquid N_2. The frozen cells were subsequently thawed, and the lysed cells were acidified with cold trichloroacetic acid to a final concentration of 2.2% (wt./vol. of suspension.) The precipitate containing the cell proteins, except apo-MT, and nucleic acids was separated by centrifugation and the supernatant was removed. Then, in order to separate labelled apo-MT, from low-M_r ^{35}S, 90 μl of the supernatant plus 10 μl of 0.1 M-dithiothreitol were chromatographed over a Sephadex G-10 minicolumn containing 1.5 ml of beads equilibrated in phosphate-buffered saline. The pellet was also measured for its isotope content after resolubilization with KOH. Finally, fractions from each column were counted for radioactivity in a liquid-scintillation counter and the results for the high-M_r region of the profile are expressed as c.p.m. per 8×10^7 cells.

Further examination of the distribution of ^{35}S and metals in the soluble fraction of cell supernatant treated with trichloroacetic acid utilized h.p.l.c. The h.p.l.c. studies were carried out with a Waters dual pump system and gradient programmer. First, a Superose 12 column was employed to separate ^{35}S-labelled species according to molecular size. Then ^{35}S-containing fractions were chromatographed over a Bio-Gel TSK-DEAE-5-PW 75 mm × 7.5 mm column with a 10–250 mM-Tris gradient at pH 7.2 to fractionate them on the basis of charge.

Table 1. Chelex treatment of medium

In these experiments 10 g of Chelex was incubated at 4 °C with 100 ml of solution. For the experiment with fetal-calf serum a 20% (v/v) solution of fetal-calf serum was used, and the values given are expressed for 100% fetal-calf serum. In the experiment with MEM plus 2.5% fetal-calf serum the copper content of 0.30 μg/ml did not change.

	Fetal-calf serum		MEM + 2.5% fetal-calf serum	
Time	Zn (μg/ml)	Fe (μg/ml)	Zn (μg/ml)	Fe (μg/ml)
0	2.95	3.00	0.14	0.20
10 min	1.10	2.35	0.11	0.13
30 min	0.75	2.25	0.09	0.14
1 h	0.45	2.35	0.08	0.14
20 h	0.25	2.55	0.06	0.20

Reactions of Zn-MT with Chelex

Rat liver Zn-MT-I, used because relatively large quantities of MT were needed, was placed in the buffer used to pre-incubate the Chelex resin along with 10 mM-2-mercaptoethanol. A 2 ml portion of 37 μM-zinc as Zn-MT-I in Spectropor tubing (6000-M_r cut-off) was disalysed at room temperature against 300 ml of the buffer, to which 10 g of Chelex was added after 17 h. This provided an initial period of time during which metal losses unrelated to the presence of Chelex could be detected. At various times small samples of the solution inside the tubing were taken for zinc analysis by flame atomic-absorption spectrophotometry.

In a second configuration, 8.8 μM-zinc as rat liver Zn-MT-II and 10 g of Chelex/100 ml were mixed directly under anaerobic conditions in a buffer of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-KCl at room temperature. After 26 h Chelex was removed and supernatant zinc, taken to be MT-bound zinc, was measured. For the control 8.8 μM-zinc as Zn-MT-II was incubated alone anaerobically for 26 h and then exposed briefly to 10 g of Chelex/100 ml. The zinc concentration of the resultant supernatant was determined as a measure of zinc bound to MT. The use of anaerobic conditions precluded thiol-group oxidation as a reason for dissociation of zinc from Zn-MT.

RESULTS

Effects of Chelex-100 on the transition-metal content of cell-culture medium

The Materials and methods section describes a method by which the culture medium for Ehrlich cells (MEM plus 2.5% fetal-calf serum) is selectively depleted of zinc relative to copper or iron. Table 1 shows the time dependence of transfer of zinc, copper and iron from fetal-calf serum or the complete culture medium to the chelating resin. Zinc is efficiently removed. The concentration of iron is only slightly decreased, and that of copper is unchanged.

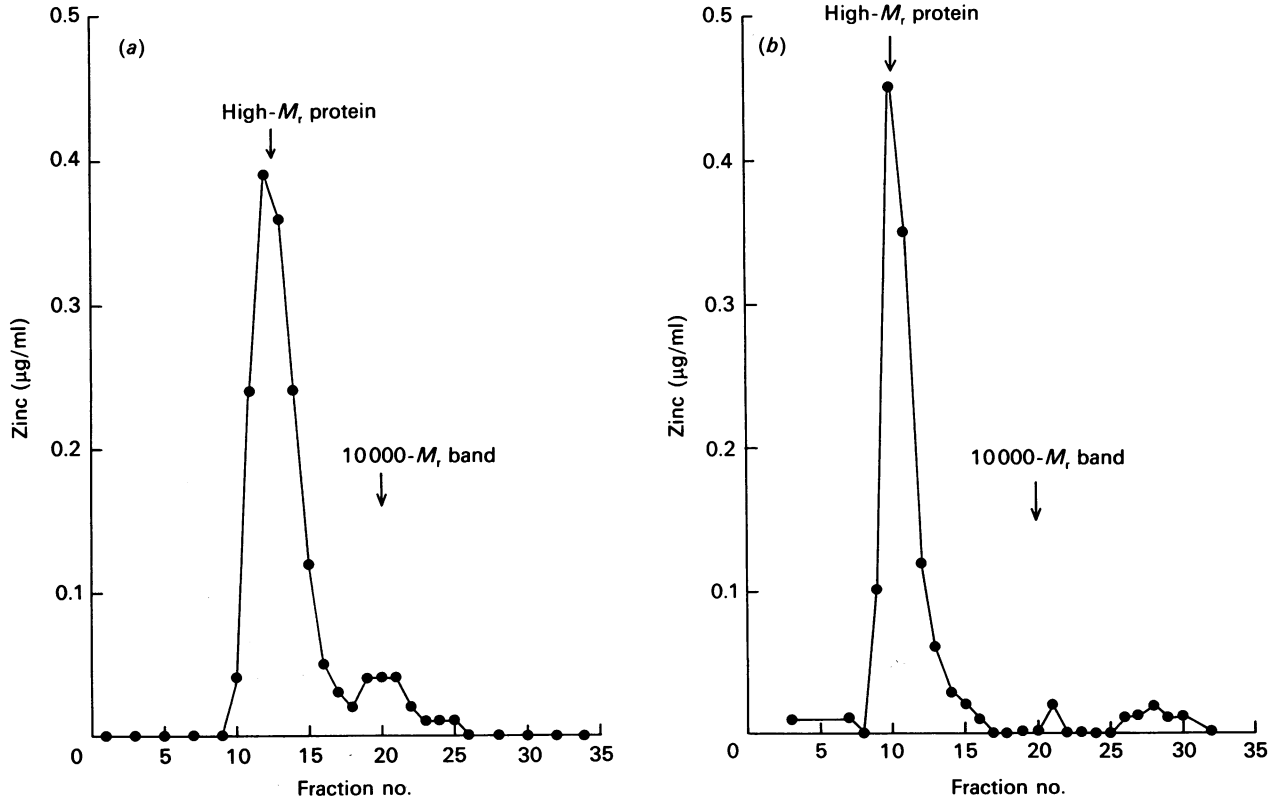


Fig. 1. Sephadex G-75 profiles of zinc in Ehrlich-cell supernatant

For experimental details see the Materials and methods section. (*a*) Control cells; (*b*) cells 4 h after exposure to Chelex. First peak, high-M_r protein; second peak, 10000-M_r band, MT.

The total capacity of the resin to bind zinc has also been experimentally determined by titrating it with $ZnCl_2$. It was determined that 0.21 mg-atom of zinc/g of resin is bound. For 10 g of resin/100 ml, this corresponds to 2.1 mg-atoms of binding capacity/100 ml of medium. One can take this value as the approximate maximal capacity of the resin to bind other metals as well. It is much larger than the concentration of magnesium in the medium (approx. 0.8 mM). Although iminodiacetate has less affinity for Mg^{2+} than for Zn^{2+} at pH 7, the pre-equilibration of the Chelex with buffer and salts including $MgSO_4$ is needed to ensure that the magnesium content of the actual culture medium is not disturbed (Sillén & Martell, 1964).

Effects of Chelex-100-treated media on Ehrlich cells

Ehrlich cells grown in mice or in culture contain a prominent Zn-binding protein that we have identified as MT (Kraker & Petering, 1983; Kraker *et al.*, 1988). It binds about 10% of the cytosolic zinc under the conditions of the present experiments (Fig. 1*a*). When these cells are added to culture medium either containing Chelex-100 resin or pretreated with the resin as described in the Materials and methods section there is a rapid and specific loss of zinc from Zn-MT, as shown in Fig. 1(*b*). In a number of experiments the MT band is largely depleted within about 4 h. In addition, during this 24 h period the culture medium, pretreated with Chelex and then incubated separately with cells, is further depleted of residual zinc by the cells.

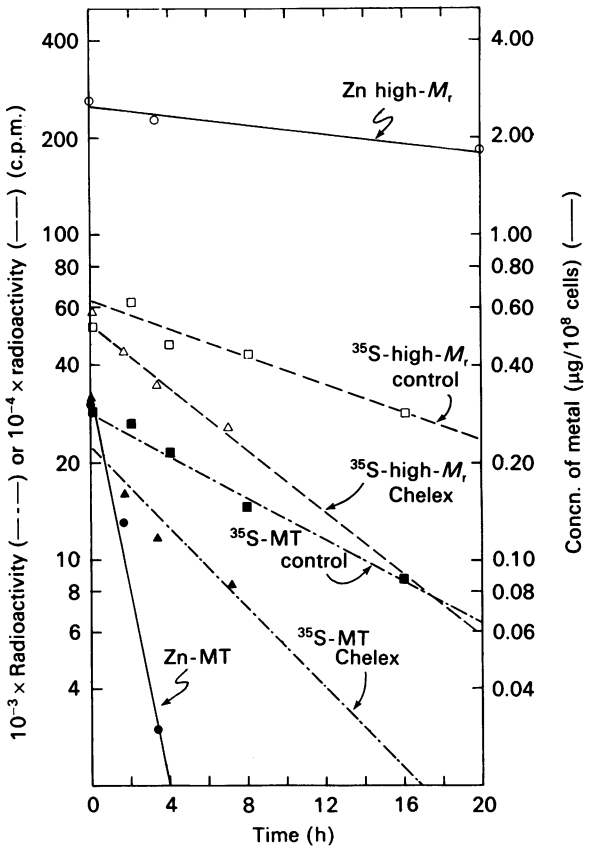


Fig. 2. Rates of loss of zinc and ^{35}S label from proteins in Ehrlich cells

For experimental details see the Materials and methods section. ○, Zinc in high-M_r protein; ●, zinc in MT; △, ^{35}S in non-MT high-M_r protein of Chelex-treated cells; ▲, ^{35}S in MT protein of Chelex-treated cells; □, ^{35}S in non-MT high-M_r protein of untreated cells; ■, ^{35}S in MT protein of untreated cells. The concentration of Chelex in the cell suspension was 10 g/100 ml.

Table 2. First-order rate constants (k_d) for zinc and ^{35}S loss from cytosolic pools

	k_d (h^{-1})	
	MT	High-M_r non-MT protein
Zn loss		
10 g of Chelex	0.69	0.016
	0.58	
	0.63	
Zn-depleted medium + cystine	1.0	
^{35}S loss		
Control	0.072	0.044
	0.072	0.049
1 g of Chelex*	0.12	0.051
	0.12	0.044
10 g of Chelex	0.16	0.081
	0.17	0.076
	0.18	0.076
Control + cystine	0.12	0.047
	0.14	0.048
10 g of Chelex + cystine	0.15	0.049
Zn-depleted medium + cystine	0.082	0.032

* This amount of Chelex does not halt cell proliferation or remove zinc from Zn-MT.

Over a period of 24 h, during which control cells double in number, there is little proliferation among the Chelex-treated cells. By 24 h thymidine incorporation into DNA is greatly depressed. However, the inhibition of thymidine incorporation does not occur until after 3 h, when zinc in MT has been mostly lost. At 3 h the cells are viable in the treated culture medium, as indicated by the exclusion of Trypan Blue dye by greater than 90% of the cells and by the ability of these cells to proliferate again at normal rates when they are transferred to the complete medium.

Kinetics of loss of zinc from the Zn-MT pool

The kinetics of loss of zinc from both Zn-MT and the high-M_r band of zinc, noted in Fig. 1, were determined by using Sephadex G-75 chromatography. Ehrlich cells were placed in the presence of Chelex (10 g/100 ml of medium) or Chelex-treated medium and then removed at intervals for analysis. Fig. 2 presents typical results in the form of semi-logarithmic first-order decay plots. The rate of loss of zinc from Zn-MT is very rapid with a rate constant of 0.69 h^{-1} ($t_{\frac{1}{2}} = 1$ h). In repetitions of this experiment with cells treated with Chelex or incubated in the zinc-depleted medium, rate constants averaged 0.74 h^{-1} (Table 2). In contrast, the decrease in high-M_r zinc is very slow, exhibiting a rate constant of approx. 0.016 h^{-1} ($t_{\frac{1}{2}} = 42$ h), assuming a homogeneous first-order decay of all of the high-M_r zinc. Cells were also exposed to 1 g of Chelex/100 ml of medium. At this

concentration cells do not stop growing and do not lose zinc from Zn-MT.

Reaction of Zn-MT with Chelex

To test whether the loss of zinc from Zn-MT might represent a dissociative process driven thermodynamically by the presence of the chelating agent outside the cell, Zn-MT (37 μM-zinc) was dialysed anaerobically at room temperature against 3.3 g of Chelex/100 ml as described in the Materials and methods section. No transfer of zinc from Zn-MT to Chelex occurred over a 72 h period. Similarly, when 8.8 μM-zinc as Zn-MT was mixed anaerobically with 10 g of Chelex/100 ml, only 32% of the zinc was transferred to the resin over 26 h, indicating that ligand exchange is quite slow but favourable when ligand and Zn-MT are in direct contact.

Labelling of cells with [^{35}S]cysteine

The turnover of MT and high-M_r protein in Ehrlich cells was assessed by a method commonly used in other studies of the biodegradation rates of various MT proteins (see the Materials and methods section). Cells were first labelled with [^{35}S]cysteine over a 24 h period, washed free of extracellular label and then examined for loss of ^{35}S from proteins in the cell supernatant over time with or without the addition of unlabelled cystine to dilute out [^{35}S]cysteine, which otherwise might be reincorporated into MT. This labelling procedure differs from the pulse exposure of animals or cells, where new MT protein is rapidly induced by exposure to an acute dose of a toxic metal. In the Ehrlich-cell system the protein is already present in a steady state of biosynthesis and degradation, and induction techniques are unnecessary.

Fig. 3 shows the Sephadex G-75 profile of ^{35}S in Ehrlich-cell supernatant. The profile resembles closely that obtained when the concentration of cysteine in each chromatographic fraction is analysed chemically by using Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoate) (Ellman, 1959; Kraker *et al.*, 1985). Although there is ^{35}S or thiol in the MT fraction it does not stand out as a clear separate peak. One can also see a large band of low-M_r ^{35}S, which may be free cysteine or label incorporated into GSH.

Validity of the measurement of ^{35}S-labelled MT

To analyse the cell supernatant for high-M_r protein and MT protein labelled with ^{35}S, the method of Patierno *et al.* (1983) was employed. Although these workers showed that MT was unique among proteins in cytosol exposed to $^{203}HgCl_2$ in that it was not precipitated by 2.2% trichloroacetic acid, it was important for the present study to establish that this binding also applied to Ehrlich-cell MT labelled with ^{35}S. When trichloroacetic acid was added to Ehrlich-cell supernatant, the pH fell to 1.5. The apo-MT is fully dissociated from zinc or copper at this pH (Kägi & Vallee, 1961). Furthermore, SDS/polyacrylamide-gel electrophoresis of the Ehrlich-cell supernatant indicated that proteins normally seen in untreated control samples were absent and had been precipitated by the acid treatment. To determine whether apo-MT is still in the acidified supernatant and not contaminated by other ^{35}S-labelled species, samples were taken from Ehrlich cells that had been incubated for 12 h in the culture medium containing [^{35}S]cysteine. After trichloroacetic acid precipitation, the remaining

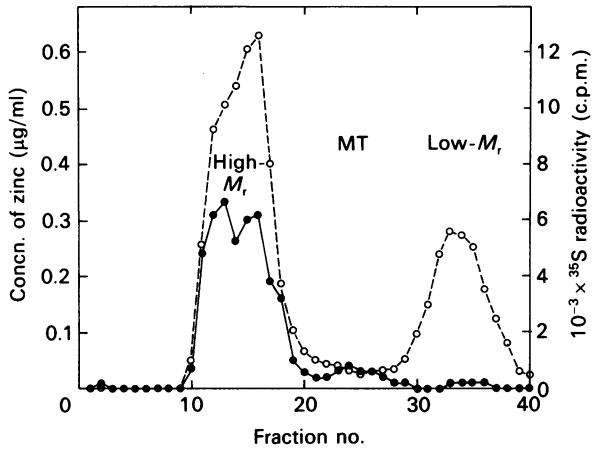


Fig. 3. Sephadex G-75 profile of zinc and ^{35}S in Ehrlich-cell supernatant

For experimental details see the Materials and methods section. ●, Zinc; ○, ^{35}S.

supernatant was removed, neutralized and chromatographed over Superose 12. Because the pH was elevated to 7 before chromatography, one may expect that holo-Zn-MT will be present during the fractionation procedure. According to Fig. 4(*a*), coincident bands of radioactivity and zinc are observed. These bands are co-eluted with authentic rat liver Zn-MT-I or -II taken through the same procedure. The Superose band of ^{35}S was placed directly on the TSK-DEAE-5-PW column and chromatographed with the salt gradient described for Fig. 4(*b*). The radiolabel and zinc are eluted in only two bands, having conductivities of 4 and 6 mS. These are the conductivities at which native rat liver and mouse Ehrlich ascites-tumour Zn-MT-I and -II are eluted from DEAE-cellulose columns (Kraker *et al.*, 1988). Rat liver Zn-MT-I and -II recovered from the Superose 12 column above are also eluted at these conductivities when chromatographed over the TSK-DEAE-5-PW column. To test the validity of the Patierno *et al.* (1983) procedure further, it was shown that the final minichromatography of the supernatant present after trichloroacetic acid treatment separated the ^{35}S-labelled protein from unincorporated [^{35}S]cysteine and other low-M_r species.

Kinetics of loss of ^{35}S from supernatant pools

The losses of ^{35}S from MT and non-MT proteins are first-order rate processes (Fig. 2). Table 2 summarizes the results of a number of kinetic runs under different conditions. In all experiments MT is degraded 2–3-fold faster than other proteins with a rate constant in the range 0.07–0.18 h^{-1}, depending on the particular conditions of the kinetic run. Importantly, for this study the rate constant for the loss of zinc from Zn-MT is 4–12-fold larger than the corresponding k_d for MT biodegradation. Hence under conditions of zinc-depletion redistribution of zinc from Zn-MT is not limited by the biodegradation of the protein leading to destruction of the metal cluster.

Considering the individual results for each experiment, the derived rate constants are quite reproducible for each experiment. In normal Ehrlich cells the biodegradation rate constants for MT and non-MT protein are 0.072 h^{-1} and 0.046 h^{-1} respectively. The presence of cys-

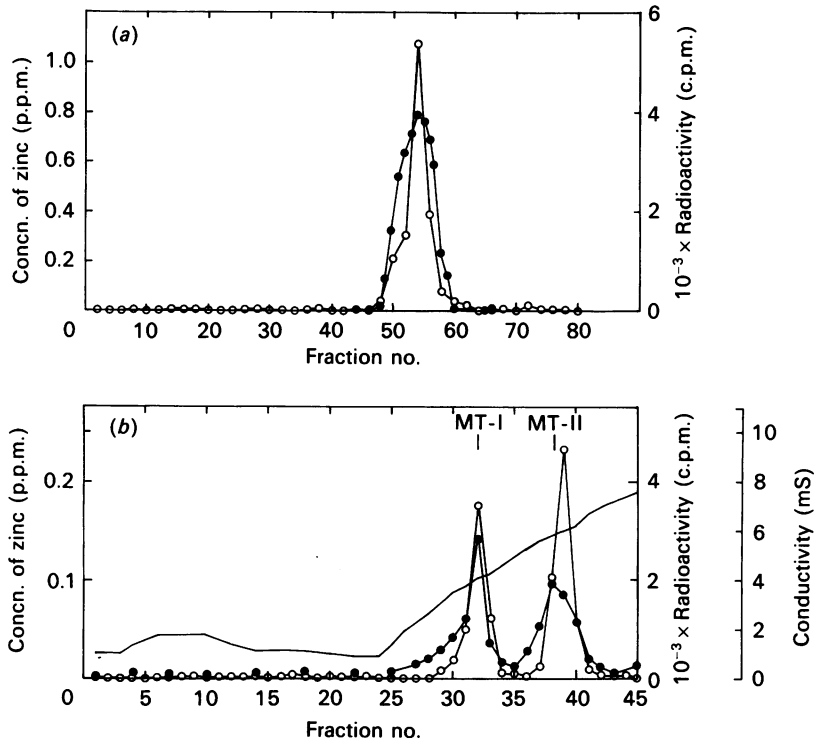


Fig. 4. Chromatography of supernatant from Ehrlich-cell cytosol after trichloroacetic acid treatment

For experimental details see the Materials and methods section. ●, Zinc; ○, ^{35}S. (*a*) Supernatant adjusted to pH 7, chromatographed over Superose 12 in 10 mM-Tris-buffer, pH 7.2, at room temperature; (*b*) zinc or ^{35}S-labelled band from Superose 12 chromatography, passed over TSK-DEAE-5-PW with a 10–250 mM-Tris gradient at pH 7.2. Authentic samples of rat liver MT-I or -II taken through the same procedure are eluted at the same positions on the Superose and TSK-DEAE-5-PW columns as the ^{35}S-labelled material.

tine in the medium raises the constant for MT to 0.13 h^{-1} but does not alter the kinetics of apparent turnover of non-MT protein. However, because cystine does not enhance rate constants in the Chelex-treated sample, it is not possible to attribute its effects on some measurements to a specific mechanism such as the inhibition of reincorporation of ^{35}S into newly synthetized MT during the experiment.

Adding 1 or 10 g of Chelex/100 ml increases the k_d of MT protein, and the latter amount of resin has some effect on the non-MT group of proteins as well. In contrast, when cystine is included in the incubation medium, adding Chelex does not further increase the turnover rates. Finally, when zinc-depleted medium replaced Chelex as a means to cause cellular zinc redistribution, the k_d for MT protein was somewhat smaller than when Chelex was present.

Presence of apo-MT-like protein in Chelex-treated Ehrlich cells

If zinc from Zn-MT is depleted more rapidly than the apoprotein is biodegraded, the cells should retain apo-MT metal-binding sites. To explore this hypothesis, cells undergoing Chelex-treatment were taken at various times, sonicated and their supernatants exposed to cadmium. The resultant fractions were chromatographed over Sephadex G-75 to determine if apo-MT or a MT-like protein was still present after zinc had left the protein. According to this experiment, summarized in Table 3, 7.60 nmol of MT/10^8 cells was present initially,

Table 3. Loss of 10000-M_r Cd-binding in Chelex-treated cells

Cells were treated with 10 g of Chelex/100 ml of medium. The concentration of Cd-binding protein was calculated assuming 7 g-atoms of Cd bind per mol of Cd-binding protein.

Time (h)	Cd-binding protein (nmol/10^8 cells)
0	7.6
6	5.6
12	3.6

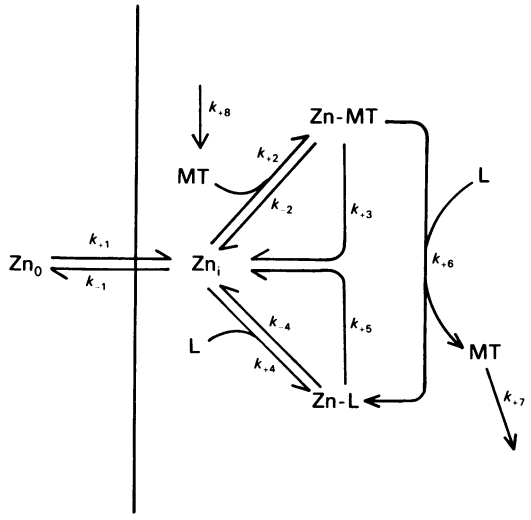
as measured by cadmium binding to the 10000-M_r peak of cadmium in the chromatographic profile and assuming 7 Cd atoms bound per molecule of MT. A significant amount of Cd-binding protein (5.6 nmol/10^8 cells) was present after 6 h even though 50% of the labelled protein has been biodegraded and no zinc remained bound to MT. Although this experiment does not prove that all of the Cd-binding material at 6 h is MT, it does indicate directly that an apo-Cd-binding protein still remains after zinc has been lost from this band. Furthermore, its concentration of binding sites for cadmium remains high despite the turnover of MT protein, consistent with continual synthesis of MT protein in these cells (Kraker *et al.*, 1985). In a second experiment, the concentration of binding protein at 24 h after the addition of Chelex to

the cells was still 20–30 % of the time-zero control value. According to the results in Table 3 the rate constant for net loss of MT and any MT-like binding protein is 0.065 h^{-1}, or less than one-half the rate constant for the loss of label from MT.

DISCUSSION

Chemical studies have shown that Zn-MT is reactive in ligand-substitution reactions, including ones involving apo-Zn-proteins as competing ligands (Li *et al.*, 1980; Udom & Brady, 1980). The question arises, therefore, whether such chemistry may also occur in cells. Scheme 1 summarizes the possible chemistry of Zn-MT in cells in relationship to the binding of zinc by other high-M_r proteins, designated collectively as Zn-L. After its uptake into Ehrlich cells (rate process, k_{+1}), Zn_i can, in principle, bind either to MT or to other sites, L, with rate constants k_{+2} or k_{+4}. There is no evidence that typical Zn-metalloproteins can directly donate zinc to other cellular ligands (Kraker *et al.*, 1988). Thus Zn-L is envisioned to lose zinc by dissociation (k_{-4}) or biodegradation (k_{+5}). Similarly, Zn-MT could lose its metal by such processes (k_{-2} or k_{+3}) but possibly also by direct ligand-substitution reactions, k_{+6}, as has been shown to occur in model studies (Li *et al.*, 1980; Petering *et al.*, 1982). There are no other obvious ways by which zinc can leave Zn-MT.

It has been shown with zinc-induced Zn-MT that metal and protein turn over with identical kinetics (Feldman & Cousins, 1976). This has usually been interpreted to mean that there is rate-limiting protein biodegradation (k_{+3}). However, slow loss of zinc followed by rapid proteolysis of apo-MT would display the same kinetics (Bremner, 1987). None of these studies has examined the fate of the zinc released from Zn-MT in this process. The present investigation addresses the question of whether the cellular metabolism of zinc bound to basal uninduced MT is governed in all cases solely by the biodegradation rate of the protein (k_{+3}) to release zinc or whether the protein-bound zinc can leave MT at higher rates, implying the operation of metal–ligand processes (k_{-2}, k_{+6}) in the redistribution of zinc.



Scheme 1. Model for the metabolism of zinc in Ehrlich cells

Because of evidence that one can control the zinc content in basal Zn-MT by varying nutrient zinc, it was decided to examine the behaviour of Zn-MT in Ehrlich cells in a culture model that limits the availability of extracellular zinc (Petering *et al.*, 1984; Kraker *et al.*, 1988).

An earlier report described the use of Chelex-100 resin to establish zinc-deficient conditions in culture (Messer *et al.*, 1982). It was not necessary to mimic all of the details of zinc deficiency *in vivo* in the present experiments because they focused only on possible cellular reactions of Zn-MT, not as such on cellular zinc deficiency. However, it was demonstrated, first, that the resin does deplete the medium of zinc in preference to iron and copper (Table 1). Secondly, Ehrlich cells do not grow but remain viable in Chelex-treated medium, as is the case for such cells in zinc-deficient mice (Minkel *et al.*, 1979; Kraker & Petering, 1983; Kraker *et al.*, 1988). However, during the period of redistribution of 90 % of the zinc (3 h) from Zn-MT there is no loss of capacity of cells to carry out DNA synthesis. Indeed, after 24 h in the presence of Chelex more than 90 % of the cells still exclude Trypan Blue. Importantly, transfer of these cells to complete medium rapidly restored cell proliferation. Thirdly, as in zinc-deficient Ehrlich cells from animals, zinc is lost preferentially from Zn-MT during the course of the cell-culture experiments (Figs. 1 and 2 and Kraker *et al.*, 1988).

In sum, cells placed into Chelex-treated medium respond in important ways like cells in zinc-deficient mice, and do not appear to be adversely stressed either during the period of zinc loss from Zn-MT, the key period for the interpretation of the kinetics, or over the full 24 h of the experiment. Therefore the effects of Chelex treatment on Zn-MT occur in intact Ehrlich cells. Whether this is an adequate model for all of the effects of zinc deficiency on the Ehrlich ascites tumour in mice requires further investigation.

Chelex treatment decreases the concentration of extracellular zinc (Zn_0 in Scheme 1), so that internal redistribution of zinc can be observed uncomplicated by continuous uptake of extracellular metal. Because a well-washed resin was used to bind metal ions in the medium, there was no possibility that the observed effects of Chelex on cellular zinc distribution could be due to direct interaction of unbound iminodiacetate with pools of zinc within the cell.

The rate of change of zinc bound to Zn-MT can be expressed in terms of kinetic processes for the full dissociation of zinc from Zn-MT (k_{-2}), biodegradation of Zn-MT (k_{+3}), possible ligand-exchange processes involving Zn-MT (k_{+6}) and the formation of Zn-MT from apo-MT and Zn_i (k_{+2}):

$$\begin{aligned} d[Zn]_{Zn\text{-}MT}/dt &= -k_{obs.}[Zn]_{Zn\text{-}MT} \\ &= -(k_{-2}+k_{+3}+k_{+6}[L])[Zn]_{Zn\text{-}MT} \\ &\quad +k_{+2}[Zn_i][MT] \end{aligned} \quad (1)$$

According to the present studies $k_{obs.} = 0.7\ h^{-1}$. Under normal conditions for Ehrlich cells in culture k_{+3} is 0.07–0.14 h^{-1}, which is about 2–4-fold faster than the turnover rate constant for excess Zn-MT in rat liver and kidney (Feldman & Cousins, 1976; Webb, 1979). The high-M_r cytosolic proteins labelled with ^{35}S are biodegraded more slowly, i.e. with an average rate constant of 0.047 h^{-1}. Under zinc-deficient conditions the rate constant for turnover of MT protein, k_{+3}, remains within

the range 0.08–0.18 h^{-1}, depending on conditions; high-M_r protein is biodegraded with the rate constant of 0.03–0.08 h^{-1}. There is presumptive evidence that apo-MT continues to be synthesized (k_{+8}) as the labelled protein turns over, for total cadmium binding to 10000-M_r protein decreases more slowly than does ^{35}S in this fraction (Table 3). It is also consistent with the slower rate constant for turnover of MT protein than for MT-bound zinc. In turn this suggests that, as MT protein is degraded to release zinc, apo-MT or apo-Cd-binding protein remains present to compete with other apo-Zn-proteins for Zn_i (k_{+2}). Thus it is clear that the process of degradation of the protein cannot account for the rapid loss of zinc from Zn-MT expressed as $k_{obs.}$. Therefore the results provide strong indirect evidence that zinc normally present in the cell as Zn-MT leaves the protein via ligand-substitution chemistry either indirectly (via k_{-2} followed by ligand binding k_{+4}) or directly (via k_{+6}). At present it is not possible to discriminate between the k_{-2} and k_{+6} paths as mechanisms by which metal leaves MT, though the study of the reaction of Zn-MT with Chelex suggests that k_{-2} is unimportant.

It is recognized that the present experiments, like previous studies, have not defined where zinc goes upon leaving Zn-MT (Feldman & Cousins, 1976; Oh *et al.*, 1978; Day *et al.*, 1978). However, since the removal of zinc from Zn-MT by Chelex is much slower than the effects of Chelex on cellular redistribution of zinc and requires direct interaction of zinc with the competing ligand, it is hypothesized that zinc moves from the Zn-MT pool to other sites within the cell and not to Chelex. At the present level of analysis a 10% increase in high-M_r zinc content would not be detectable after it moved from Zn-MT, so that transfer of metal between these pools could not be quantified.

These considerations suggest that the turnover of Zn-MT under zinc-deficient conditions can be understood in terms of eqns. (2) and (3), in which Zn-L represents members of the intracellular Zn-metalloprotein pool:

$$\text{Zn-MT} + \text{L} \xrightarrow{k_{-2}\text{ and }k_{+6}} \text{Zn-L} + \text{MT} \qquad (2)$$

$$\frac{d[Zn]_{Zn\text{-}MT}}{dt} = -k_{-2}[Zn]_{Zn\text{-}MT} - k_{+6}[Zn]_{Zn\text{-}MT}[L] \qquad (3)$$

For first-order kinetics to be observed, either the dissociative path (k_{-2}) must be followed or $[L] \gg [Zn]_{Zn\text{-}MT}$ in eqn. (3). Given that Zn-MT constitutes about 10% of the supernatant zinc, but only a small fraction of Zn-L is being constituted from zinc and apo-L at any time during the cell cycle, the k_{+6} path alone would not display first-order kinetics. However, one also recognizes that during the cell cycle apo-Zn-proteins that require zinc for their function are continually being synthesized. This is occurring at some average rate for cells dispersed throughout the cell cycle, as they are at the beginning of the present experiments. It is likely, then, that [L] remains effectively constant because of protein biosynthesis. This condition would also lead to pseudo-first-order kinetics for the loss of zinc from Zn-MT.

The rate constant observed in Ehrlich cells for the loss of metal from Zn-MT seems attainable on the basis of model ligand-exchange reactions. For example, apo-(carbonic anhydrase) is an acceptor ligand with a rate constant for ligand substitution with Zn-MT of $1.5 \times 10^3\ M^{-1} \cdot s^{-1}$ (Li *et al.*, 1980). The cellular concentration of Zn-MT calculated above is 13 μM. Estimating the steady-state concentration of L due to continual protein biosynthesis as 1 μM (about 1% of the concentration of Zn-L) and equating the carbonic anhydrase rate constant with k_{+6} yields a pseudo-first-order rate constant of about 5.4 h^{-1}. This value is an order of magnitude larger than the empirical first-order constants obtained with Chelex (Table 3).

The turnover rate constant for MT protein in the normal culture medium can be used to define the balancing rate constant for MT synthesis that must exist to maintain the protein in its steady state. Assuming, as is commonly done, that the steady state of MT protein may be described as:

$$\frac{d[MT]}{dt} = 0 = k_{+8} - k_{+3}[MT] \qquad (4)$$

in which k_8 is the zero-order constant for biosynthesis for apo-MT, k_{+3} is the average degradation constant from Table 3 and [MT] is that measured at time zero (Table 3). One calculates that $k_{+8} = 0.1\ h^{-1} \times 0.076$ μmol of MT/10^{10} cells or 7.6 nmol of MT/h per 10^{10} cells. This number is about 40% of the value calculated directly for the net synthesis of Zn-MT in Ehrlich cells treated with the copper complex 3-ethoxy-2-oxobutyraldehyde-bis-(thiosemicarbazonato)copper(II), to displace zinc from Zn-MT (Kraker *et al.*, 1985).

Once one recognizes that the loss of zinc from Zn-MT can result from rate-limiting ligand-substitution processes, one needs to consider that the simultaneous loss of metal and radiolabel from MT observed in other studies (Feldman & Cousins, 1976) could result from a rate-limiting loss of zinc from MT followed by rapid proteolysis of apo-MT. Indeed, different rate-limiting steps may apply, depending on the physiological condition. For example, in a mature slowly dividing tissue such as liver, which takes up extra zinc into MT after injection of the metal into the host, the turnover of this excess Zn-MT may well be rate-limited by MT biodegradation. The synthesis of apo-Zn-proteins to compete for MT-bound zinc is relatively slow and would contribute little to the loss of zinc from induced Zn-MT. In other situations, however, active zinc transfer from Zn-MT to other sites might be important. For example, Zn-MT is induced in regenerating rat liver in a probable host stress response (Ohtake *et al.*, 1978). In this physiological situation Zn-MT turns over with an approximate rate constant of 0.07 h^{-1}, which is about twice as large as that induced in liver Zn-MT by injections of Zn^{2+} (Feldman & Cousins, 1976). Similarly, McCormick *et al.* (1981) found that the concentration of liver MT-Zn decreased more rapidly during the transition from a fasting to a feeding state than in the fed state.

We acknowledge the support of the National Institutes of Health through Grants GM-29583 and ES-04026 and thank Professor J. Otvos for his critical input.

REFERENCES

Bremner, I. (1987) J. Nutr. **117**, 19–29
Day, F. A., Coles, B. J. & Brady, F. O. (1978) Bioinorg. Chem. **8**, 93–105
Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–77
Feldman, S. L. & Cousins, R. J. (1976) Biochem. J. **160**, 583–588

Fenton, M. R. & Burke, J. P. (1984) Fed. Proc. Fed. Am. Soc. Exp. Biol. **43**, 855 (abstr. 3334)
Kägi, J. H. R. & Vallee, B. L. (1961) J. Biol. Chem. **236**, 2435–2442
Koch, J., Wielgus, S., Shankara, B., Saryan, L. A., Shaw, C. F., III & Petering, D. H. (1980) Biochem. J. **189**, 95–104
Kraker, A. & Petering, D. H. (1983) Biol. Trace Elem. Res. **5**, 363–374
Kraker, A., Krezoski, S., Schneider, J., Minkel, D. & Petering, D. H. (1985) J. Biol. Chem. **260**, 13710–13718
Kraker, A., Krakower, G., Shaw, C. F., III & Petering, D. H. (1988) Cancer Res. **48**, 3381–3388
Krishnamurti, C., Saryan, L. A. & Petering, D. H. (1980) Cancer Res. **40**, 4092–4099
Li, T.-Y., Kraker, A. J., Shaw, C. F., III & Petering, D. H. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 6334–6338
Li, T.-Y., Minkel, D. T., Shaw, C. F., III & Petering, D. H. (1981) Biochem. J. **193**, 441–446
Margoshes, M. & Vallee, B. L. (1957) J. Am. Chem. Soc. **79**, 4813–4814
Mayo, K. E. & Palmiter, R. D. (1981) J. Biol. Chem. **256**, 2521–2524
McCormick, C. C., Menard, M. P. & Cousins, R. J. (1981) Am. J. Physiol. **240**, E414–E421
Messer, H. H., Murray, E. J. & Goebel, N. K. (1982) J. Nutr. **112**, 652–657
Minkel, D. T., Dolhun, P. J., Calhoun, B. L., Saryan, L. A. & Petering, D. H. (1979) Cancer Res. **39**, 2451–2456
Minkel, D. T., Poulsen, K., Wielgus, S., Shaw, C. F., III & Petering, D. H. (1980) Biochem. J. **191**, 475–485
Oh, S. H., Deagen, J. T., Whanger, P. D. & Weswig, P. H. (1978) Bioinorg. Chem. **8**, 245–254
Ohtake, H., Hasegawa, K. & Koga, M. (1978) Biochem. J. **183**, 999–1005
Onosaka, S. & Cherian, M. G. (1982) Toxicology **23**, 11–20
Patierno, S. R., Pellis, N. R., Evans, R. M. & Costa, M. (1983) Life Sci. **32**, 1629–1636
Petering, D. H., Krakower, G., Bachowski, G. & Shaw, C. F., III (1982) Fed. Proc. Fed. Am. Soc. Exp. Biol. **41**, 641 (abstr. 2168)
Petering, D. H., Loftgaarden, J., Schneider, J. & Fowler, B. (1984) EHP Environ. Health Persp. **54**, 73–81
Saryan, L. A., Ankel, E., Krishnamurti, C., Petering, D. H. & Elford, H. (1979) J. Med. Chem. **22**, 1218–1221
Sillén, L. G. & Martell, A. E. (1964) Spec. Publ. Chem. Soc. **17**
Udom, A. O. & Brady, F. O. (1980) Biochem. J. **187**, 329–335
Webb, M. (1979) The Chemistry, Biochemistry, and Biology of Cadmium, chapter 6, Elsevier, Amsterdam
Webb, M. & Cain, K. (1982) Biochem. Pharmacol. **31**, 137–142

Received 3 November 1987/8 March 1988; accepted 22 June 1988