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On the sensitivity of metallothioneins to oxidation during isolation

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It is demonstrated that the distribution of metals among the Sephadex G-75 fractions of rat liver and horse kidney supernatant is altered by exposure to oxidizing conditions. In particular, the metals bound to metallothionein are displaced into high-molecular-weight fractions and, to a lesser extent, into the low-molecular-weight forms, under aerobic conditions. In this process, metallothionein zinc is much more labile than cadmium. An appreciable proportion of the thionein is also found in the high-molecular-weight fractions and can be recovered along with the metals by treatment with mercaptoethanol. This result shows that the distributions obtained aerobically with large cadmium content in the high-molecular-weight fractions are an artefact due to metallothionein oxidation and suggests that 'spillage' of metals such as cadmium may be due in large part to oxidative processes rather than saturation effects. Evidence is presented that disulphide-bond formation occurs as thionein becomes bound in the high-molecular-weight region and that chemical reduction is necessary to restore its normal elution behaviour. Mercaptoethanol added to the homogenates maintains the reducing conditions normally found in the cellular milieu and prevents the oxidation of the metallothionein redistribution of the metals during isolation. Under these conditions the rat liver metallothionein isolated from animals exposed to chronic low concentrations of cadmium in drinking water contains appreciable quantities of copper as well as zinc and cadmium. Similarly, the metallothionein fraction isolated under reducing conditions contains much of the zinc that is present in horse kidney supernatants. Metallothionein can also be extracted from a 40000**g** pellet after sonication of the pellet. Thus careful analytical studies of the sites of cadmium deposition in rat liver indicate that greater than 95% is bound to metallothionein.

The study of metallothionein has been prompted by the concern about human exposure to toxic heavy metals such as cadmium and the finding years ago by Vallee and co-workers that virtually all of the cadmium in the cytosol of horse kidney is bound to a low-molecular-weight protein fraction called metallothionein (Margoshes & Vallee, 1957; Kägi & Vallee, 1960, 1961). Although many subsequent studies confirmed their findings and showed that cadmium induces the synthesis of thionein protein, progress has been slow in understanding the role of metallothionein in cadmium toxicity (Nordberg *et al.*, 1972; Webb, 1972; Oh *et al.*, 1978*a*; Anderson *et al.*, 1978). Major difficulties are the peculiar structure and probable function of metallothionein, which have resisted analysis. Thus it is likely that thionein is basically a metal-binding protein. The recent evidence that the protein is a fundamental component of zinc metabolism has complicated the problem (Bremner & Davies, 1974; Richards & Cousins, 1976; Oh *et al.*, 1978*b*; Sobocinski *et al.*, 1978; Petering *et al.*, 1978). However, because zinc- and cadmium-thionein are not easily observed by spectroscopic techniques, the investigation of the metal-binding properties of the protein has hardly proceeded beyond Vallee's original work (Kägi & Vallee, 1961).

A second important problem is that studies of cadmium localization have been focused on metallothionein, with little attention given to cadmium possibly bound to other cellular constituents (Shaikh & Smith, 1977; El-Gazaar *et al.*, 1978). Finally, many studies have relied on large short-term acute

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doses of cadmium delivered to animals to induce metallothionein (Weser *et al.*, 1973; Winge *et al.*, 1975; Probst *et al.*, 1977; Anderson *et al.*, 1978). Although the incidence of acute cadmium toxicity is rare, low-dose prolonged exposure of animals produces hypertension and other adverse effects (Miller *et al.*, 1974; Perry, 1976).

The metal ratios of thioneins reported from various laboratories have varied greatly, as documented in the Discussion section. Thioneins are isolated primarily from mammalian livers and kidneys, tissues that are rich in thiol groups, indicative of a reducing environment. During isolation of thioneins, the preparations are exposed to aerobic conditions during homogenization, centrifugation and chromatography. A careful examination of the effects of oxidizing conditions on thioneins has not been reported previously.

The present study utilizes rats given low doses of cadmium in their drinking water for extended periods. The focus of the work is the characterization of the metal content of metallothionein from these rats and comparison of this protein with horse renal metallothionein when they are isolated under reducing conditions that mimic the cellular milieu.

Materials and methods

Exposure of animals to cadmium

Sprague–Dawley weanling rats with an average body weight of 50g were fed commercial diet (Purina Rodent Chow) *ad libitum*. Groups of male or female rats had $CdCl_2$ added to their drinking water at 17 or 34.4μg of Cd/ml respectively. Rats were killed at intervals up to 146 days, livers were excised, immediately frozen and kept at less than −4°C until use.

Preparation of rat liver supernatants

The present method is a modification of the procedures of Winge & Rajagopalan (1972). Frozen livers (about 200g) were placed in a Waring blender and diluted with 5mM-Tris/HCl buffer, pH7.8, to yield a 20% (w/v) dilution and then homogenized for 2min at high speed. In some experiments, 2-mercaptoethanol (Aldrich Chemical Co.) was added before homogenization to give a final concentration of 5–10mM. In early preparations the crude homogenate was centrifuged at 105000g for 1.5h in a Beckman L5-75 ultracentrifuge (Ti75 rotor; 40000rev./min; 0–4°C). A 40000g supernatant was employed in later preparations because it was found that the heat treatment produced comparable removal of microsomal substances. The supernatant fraction was collected and rapidly heated to 60°C in a 100°C water bath, held at that temperature for 1min, then quickly cooled to 0–4°C in an ice bath.

The heat-treated sample was centrifuged at 40000g for 20min. The resulting supernatant was dialysed against 4 litres of distilled water for 4–12h with one change of water. Dialysis bags were prewashed four to five times with doubly distilled water, shown by analysis to contain no Cd, Zn or Cu, before use, and were sealed with plastic clips. Plastic gloves were used in these steps to avoid transfer of zinc and copper from researchers' fingers to the bag and then to the solutions. This dialysed heat-treated supernatant was divided into aliquots (40–50ml) containing approx. 100μg of Cd per sample and freeze-dried. Freeze-dried samples were stored at −4°C until used. Preparation of a soluble supernatant from the pellet derived from the first centrifugation of the crude homogenate was carried out in the same way except that homogenization was carried out in a Branson 200 Sonifier (output 7; 90% continuous cycle; 15min; ~50W output). The protein content of the various preparative steps was determined by the biuret method, with albumin and lysozyme as standards (Layne, 1957). Metal concentrations were determined by atomic-absorption spectroscopy using a Perkin–Elmer 360 spectrophotometer after digestion of crude fractions with 1.0ml of HNO_3 (70%) at 70–80°C for several hours and appropriate dilution with distilled water. Calibration standards for Zn, Cd and Cu were obtained from Fisher Scientific Co.

Horse-kidney-metallothionein preparation and oxidation

The cortex from fresh horse kidneys was homogenized in 2vol. of 0.25M-sucrose/0.02M-Tris/HCl buffer, pH8.6, for 60s in a Waring blender. The homogenate was centrifuged at 23500g for 40min in a GSA rotor on a Sorvall RC5-B Superspeed centrifuge. The supernatant was re-centrifuged at 45000g in an SS-34 rotor. The resulting supernatant was heat-treated by bringing it rapidly to 60°C, holding 60°C for 1min and then cooling in an ice bath. The precipitated proteins were removed by centrifugation at 46000g. The final supernatant was either freeze-dried and stored for further use, or, for the oxidation study, left exposed to air in a covered beaker in a closed refrigerator at 4°C for 15 days. During only one of these trials did bacterial contamination occur, and that material was discarded. At approx. 24h intervals, samples were removed and fractionated on Sephadex G-75. The high-molecular-weight fractions from days 13 and 14 were kept, treated with mercaptoethanol, and then rechromatographed to see if thionein had been oxidized during the incubation.

In a typical preparation, the metal contents of the supernatants were Cd, 1.29, Zn, 4.81 and Cu, 1.92μg/ml, before heat treatment and 1.35, 4.00 and 1.92μg/ml after heat treatment, indicating that substantial metal losses did not occur during this

step. Furthermore, preparations omitting the heat step retained their sensitivity to oxidation.

Sephadex chromatography

Samples prepared, freeze-dried and stored at −4°C as described above were resuspended in 10ml of 5mM-Tris/HCl, pH 7.8 (with or without addition of 10mM-mercaptoethanol and incubation for 24h at 5°C). Some were applied directly to a column (2.5cm × 90cm; Glenco Scientific) containing Sephadex G-75–120 and eluted with 5mM-Tris HCl, pH 7.8. The Sephadex column was calibrated for molecular-weight determinations with the following standards: Blue Dextran (mol.wt. 2000000); haemoglobin (69000); carbonic anhydrase (31000); myoglobin (16700); cytochrome *c* (12750); and $K_3Fe(CN)_6$ (329). Fractions of 9.0ml were collected at a flow rate of approx. 20ml/h. Absorbances of each fraction were determined at 250nm and 280nm on a Beckman Acta V, Cary 16 or Cary 17D u.v.–visible spectrophotometer. Metal analyses for Cd, Zn and Cu were determined directly on each fraction by atomic-absorption spectroscopy. In some cases the thiol content of each fraction was determined by adding 100μl of fraction to 1.9ml of 0.2mM-5,5′-dithiobis-(2-nitrobenzoic acid) in 10mm-KH_2PO_4, pH 7.8, and measuring ΔA_{412} ($\varepsilon = 13600\,M^{-1}\cdot cm^{-1}$) on a Cary 16 spectrophotometer (Ellman, 1959). The protein content of each fraction was measured by the biuret method (Layne, 1957).

DEAE-Sephadex chromatography

The cadmium-containing peak from Sephadex G-75 chromatography corresponding to approx. mol.wt. 10000–12000 (fractions 35–45) was desalted by dialysis, concentrated by freeze-drying, resuspended in 5mM-Tris/HCl, pH 7.8, and applied to a column (1.5cm × 40cm; Glenco Scientific) packed with DEAE-Sephadex A-25. Alternatively, the Sephadex G-75 fractions containing cadmium were applied directly to the column. The sample and column were washed with approx. 100ml of 5mM-Tris/HCl, pH 7.8. The sample was then eluted with a linear gradient of 200–250mM-Tris/HCl, pH 7.8. Fractions of 4.0ml were collected at a flow rate of about 20ml/h and were analysed for protein, metal and thiol content as described above. The gradient was monitored by measuring conductivity on a Yellow Springs Instrument YSI-model conductivity meter calibrated against standard Tris solutions.

Other techniques

Both disc-gel electrophoresis (Davis, 1964) and the enzymic assay for superoxide dismutase activity (Fridovich, 1974) were carried out by the standard methods cited.

Results

Effect of preparative conditions on the isolation of rat liver metallothionein

The distribution of cadmium, zinc, and copper in fractions of liver cytosol was found to be related to the length of exposure of the sample to oxidizing conditions and was readily reversed by incubation with the reducing agent 2-mercaptoethanol. Fig. 1(*a*) shows the G-75 Sephadex profile of a heat-treated rat liver supernatant prepared entirely in the presence of 2-mercaptoethanol. Cadmium was almost entirely localized in band III, corresponding to 10000 mol.wt., and was identified as the metallothionein fraction. Zinc was located in three bands: I, II and III. Copper was found largely in bands II and III. The g-atom ratio Zn/Cu/Cd in the metallothionein band was 2.5:1:2. The second band, containing equal g-atom amounts of zinc and copper, contained proteins with mol.wts. of about 30000. The entire superoxide dismutase activity of the profile co-chromatographed with the zinc and copper in this band (D. T. Minkel, unpublished work). Since the liver superoxide dismutase contains equal numbers of zinc and copper atoms, the metal content of this peak is attributed to the presence of this enzyme (Fridovich, 1975). There was little if any metal in the low-molecular-weight band-IV material.

When the heat-treated supernatant prepared in the absence of mercaptoethanol was stored at 4°C for 3 days before Sephadex chromatography, the chromatographic profile of Fig. 1(*b*) was obtained. Band-III material is completely devoid of metals. The zinc, copper and cadmium have shifted into bands I, II, and IV. That this metal redistribution is related to thiol oxidation in metallothionein is shown in Fig. 1(*c*) in which the heat-treated supernatant of Fig. 1(*b*) was incubated with 0.1M-mercaptoethanol at 4°C for 24h before column chromatography. Fig. 1(*c*) shows that much of the zinc, copper and cadmium have returned to the metallothionein band. Some cadmium remaining in band I and copper in band IV distinguish this elution profile from Fig. 1(*a*). It will be noted that only metals initially in metallothionein redistribute under the oxidizing or reducing conditions.

To examine this reversible effect further, heat-treated supernatant was dialysed for 20h before Sephadex chromatography. Fig. 2(*a*) illustrates the metal distribution of the preparation. It is almost entirely oxidized. Peak I (fractions 18–20) was freeze-dried and rechromatographed on Sephadex G-75; it was located entirely in fractions 17–20. Thus this high-molecular-weight mixture is stable in its binding of zinc and cadmium. Again, these fractions were concentrated, resuspended in 0.01M-potassium phosphate buffer containing 0.1M-mercaptoethanol and incubated for 72h at 4°C. This material

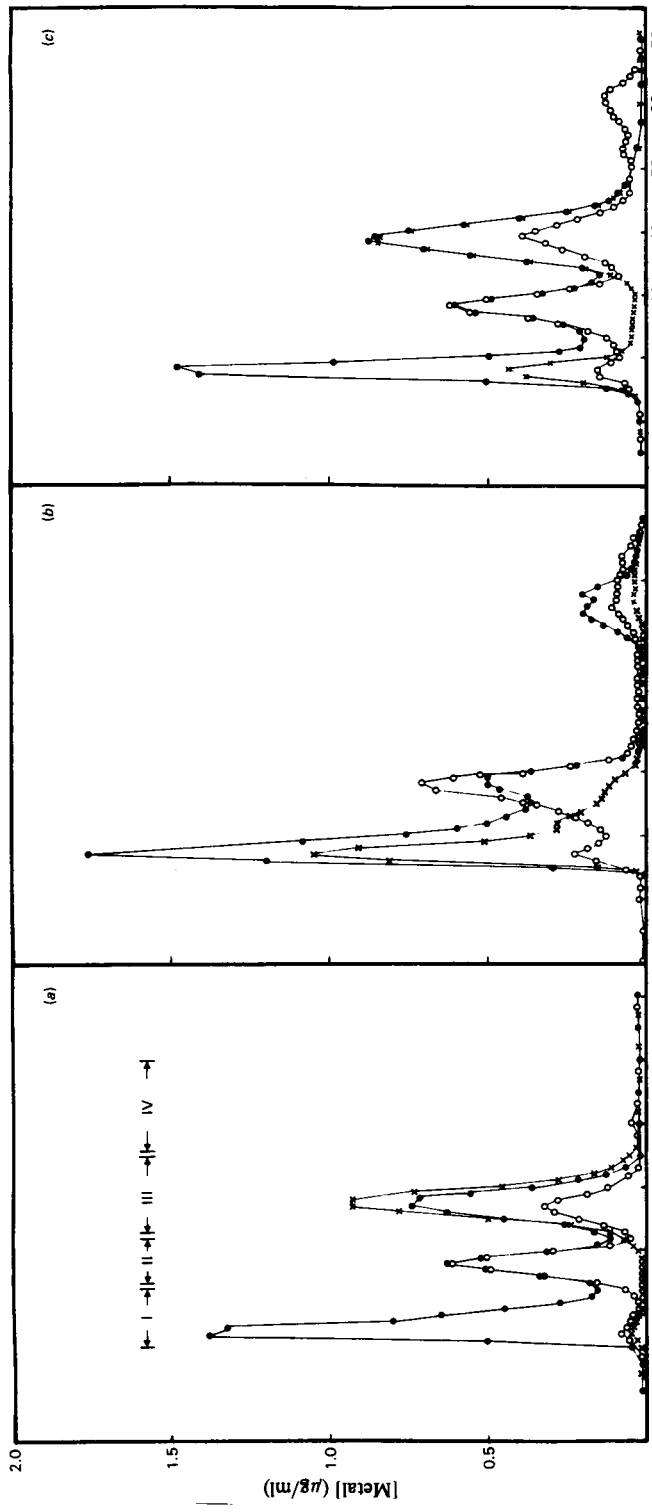

Fig. 1. *Sephadex G-75 profile of reduced and oxidized samples of rat liver supernatants*

(*a*) Sample containing mercaptoethanol throughout the preparation. (*b*) Supernatant after heat treatment prepared in the absence of mercaptoethanol and incubated for 3 days at 4°C before freeze-drying and later chromatography. (*c*) Sample (*b*) incubated with 0.1 M-mercaptoethanol for 24 h at 4°C before chromatography. ×, Cd; ●, Zn; ○, Cu. Samples were prepared from liver of female rats given 34.4 μg of Cd/ml in water for 60 days.

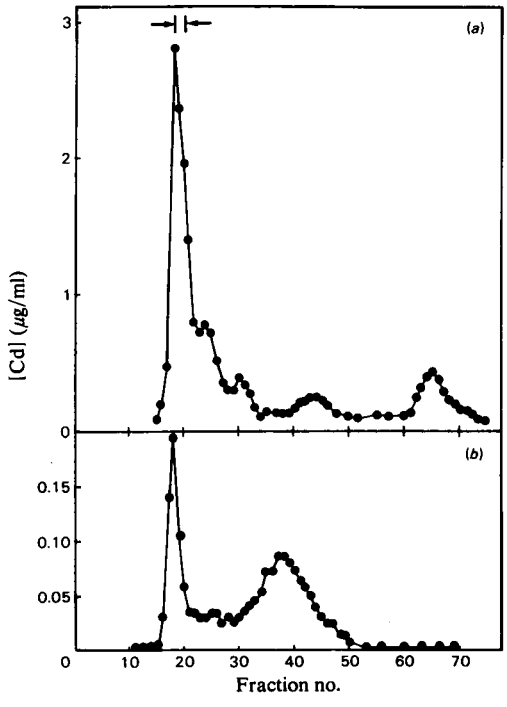

Fig. 2. *Sephadex G-75 profile of oxidized rat liver supernatant and reduced fraction I*
(*a*) Sample prepared without mercaptoethanol and dialysed 20h after heat step before Sephadex G-75 chromatography. (*b*) Fractions 18–20 from (*a*) (indicated by →||←) were first rechromatographed on Sephadex G-75 and then incubated for 72h with mercaptoethanol before final chromatography on Sephadex G-75. Samples were from liver of female rats given 34.4μg of Cd/ml in water for 60 days.

was chromatographed a third time. The results of Fig. 2(*b*) show that a majority of the cadmium (60%) shifts into the metallothionein band. (This shift of cadmium accounts for the decreased scale in Fig. 2*b* compared with Fig. 2*a*.) No metal was detected in band IV. Some cadmium remains behind in the original sharp peak of protein in fractions 15–19. This is also observed in Fig. 1(*c*). Clearly, thionein protein is released by mercaptoethanol to re-form the metal–mercaptide protein complexes initially present.

As a result of experiments such as these, it was determined that lengthy dialysis before chromatography was causing redistribution of metals associated with metallothionein. Hence dialysis time was limited to 3–4h. Table 1 compares the cadmium and zinc contents of bands I–IV for samples dialysed in the presence and absence of mercaptoethanol. Under oxidizing conditions, 86% of the cadmium is bound to metallothionein, with only minor amounts observed in bands I, II and IV. When mercaptoethanol is included in the dialysis, the percentage rises to 94% More significantly, however, the amount of zinc in this band increases sharply from 37μg to 65μg at the expense of both high- and low-molecular-weight regions of the elution profile. There was little effect of the conditions on copper distribution. With mercaptoethanol present, the zinc/copper/cadmium ratio is 3.2:1:2.6. In its absence, the metal ratio becomes 2.2:1:3.0. Thus the metal ratio is altered subtly even under conditions in which cadmium is stable in the metallothionein band. Clearly, it is zinc-binding sites that seem most sensitive to oxidation, and it is the shift in zinc among constituents of the supernatant that lead to the

Table 1. *Metal contents of rat liver Sephadex G-75 elution bands I–IV*
Bands I–IV were as defined in Figure 1. Livers were from female rats given 34μg of Cd/ml in drinking water for 146 days. Supernatants were isolated with and without 2-mercaptoethanol present.

	Metal content					
	Cd		Zn		Cu	
Band	(μg)	(%)	(μg)	(%)	(μg)	(%)
(*A*) Mercaptoethanol absent						
I	4.19	4	67.27	46	3.78	8
II	4.91	5	31.27	21	24.08	53
III	87.40	86	37.22	25	16.38	36
IV	4.57	4	11.79	8	1.30	3
Total (μg)	101.07	100	147.55	100	45.54	100
(*B*) Mercaptoethanol present						
I	2.86	3	57.60	38	5.89	10
II	1.75	2	25.47	17	25.11	45
III	86.00	94	64.71	42	19.66	35
IV	0.90	1	5.76	4	5.72	10
Total (μg)	91.51	100	153.54	101	56.38	100

variation in metal ratios of various metallothionein preparations.

Given the sensitivity of metallothionein to oxidizing conditions during its isolation, a preparation of metallothionein I and II from rat liver was carefully monitored for metal distribution by using 5 mM-mercaptoethanol throughout the procedure up to the Sephadex-G-75-chromatographic step. Although the cadmium content decreases during purification of metallothionein, as tabulated in Table 2, the decrease is primarily due to loss in total volume. The concentration of cadmium in the supernatant remains approximately constant; 15% of the cadmium was lost to the pellet when the crude homogenate is centrifuged at 40000g. This finding mirrors the results of others, who have found most of the cadmium in rat liver supernatant, but also a significant percentage in the particulate fraction (El-Gazaar *et al.*, 1978; Shaikh & Smith, 1977).

The metal and thiol distributions among bands I–IV of the Sephadex G-75 elution profile are shown in Table 2. Significant percentages of the total copper and zinc in the treated supernatant are bound in the metallothionein peak. Bands I–III contain thiol groups reactive with 5,5′-dithiobis-(2-nitrobenzoic acid). However, since free thiol groups are usually necessary to react with the aromatic disulphide 5,5′-dithiobis-(2-nitrobenzoic acid), it was surprising to observe substantial amounts of free thiol in band III, where thiol groups were thought to be firmly bound to metal. However, a second-order biphasic reaction of metallothionein with 5,5′-dithiobis-(2-nitrobenzoic acid) has been found (Li *et al.*, 1980). Disc-gel electrophoresis of the metallothionein fraction showed two major bands and several minor protein components that can be resolved by DEAE-Sephadex A-25 chromatography.

Band III from the G-75-Sephadex-chromatographic elution was loaded on to a column of DEAE-Sephadex A-25 and eluted with 5–250 mM-Tris/HCl gradient, pH 7.8. Two major cadmium-containing bands, corresponding to metallothioneins I and II, were eluted at 0.06 M- and 0.125 M-Tris respectively. A much smaller band (fraction A) containing cadmium was eluted between the two major peaks at 0.10 M-Tris. These protein

Table 2. *Purification of rat liver metallothionein*

The livers used were from female rats given 34.4 μg of Cd^{2+}/ml in their drinking water for 60 days.

Step	Cd (μg/ml)	Protein (mg/ml)	Volume [ml (%)]	Total Cd (%)	Total protein (%)	Cd (μg/mg of protein)
(A) Rat liver						
1. Crude homogenate	1.27	40.0	536 (100)	100	100	0.032
2. Centrifuged supernatant	1.29	31.9	447 (83)	85	67	0.040
3. Heat-treated supernatant	1.35	9.7	374 (70)	74	17	0.139
(B) Pellet from crude homogenate						
1. Crude homogenate	0.33	26.1	309 (100)	100	100	0.013
2. Centrifuged supernatant	0.36	20.2	271 (88)	96	68	0.018
3. Heat-treated supernatant	0.31	2.7	235 (76)	72	8	0.113

(C) Sephadex G-75 profile for supernatant (A)3 above

Band	Cd [μg(%)]	Zn [μg(%)]	Cu [μg(%)]	Protein [mg(%)]	Thiol (μmol)
I	2.2 (2)	62.4 (40)	6.3 (12)	173 (56)	5.67 (43)
II	2.3 (2)	26.5 (17)	25.4 (48)	97 (32)	2.07 (16)
III	114.4 (96)	62.2 (40)	21.5 (40)	37 (12)	5.46 (41)
IV	0.0 (0)	3.9 (3)	0.0 (0)	— —	— —
Total	118.9 (100)	155.0 (100)	53.2 (100)	307 (100)	13.2 (100)

(D) DEAE-Sephadex A-25 fraction of (C)b and III above

	Cd [μg(%)]	Zn [μg(%)]	Cu [μg(%)]	Protein [mg(%)]	Thiol (μmol)
Loading	7.6 (10)	3.1 (7)			0.15 (4)
Metallothionein 1	33.9 (47)	21.8 (46)	11.3		1.94 (47)
Band A	4.7 (7)	1.7 (4)	—		0.14 (3)
Metallothionein 2	25.6 (36)	20.3 (43)	6.8		1.88 (46)
Total recovered	71.8 (100)	46.2 (100)	17.4		4.12 (100)
Total applied	73.2	49.1	16.7		3.49

components contain 90% of the applied cadmium, as indicated in Table 2. A small amount of cadmium is washed through the column in the initial loading procedure and may represent oxidized metallothionein. Generally, cadmium, zinc, copper and thiols co-chromatograph, giving metal proportions for zinc/copper/cadmium of 1.9 : 1 : 1.7 and 3 : 1 : 2.2 for metallothioneins I and II, respectively and thiol-to-metal ratios of 2.4 and 2.9. In each protein the zinc concentration is greater than that of cadmium, and there is a significant amount of copper, which is bound firmly to each metallothionein even though mercaptoethanol is not used in the chromatographic procedures.

Table 2 also shows data for the purification of cadmium-binding constituents of the pellet after vigorous sonication in the presence of mercaptoethanol. The progression of the steps yields results qualitatively similar to those seen with the initial purification of the supernatant fraction. On chromatography of the heat-treated material on Sephadex G-75, the elution profile of Fig. 3 is observed. Most of the cadmium (85%) is bound in band III, corresponding in molecular weight to metallothionein. Zinc is distributed much as in Fig. 1 among bands I–III. Besides its presence in band II-containing superoxide dismutase, the majority of the copper is located in the metallothionein-like species. The peak fraction of that material has a zinc/copper/cadmium ratio of 1 : 2.3 : 1 with copper present in highest amount.

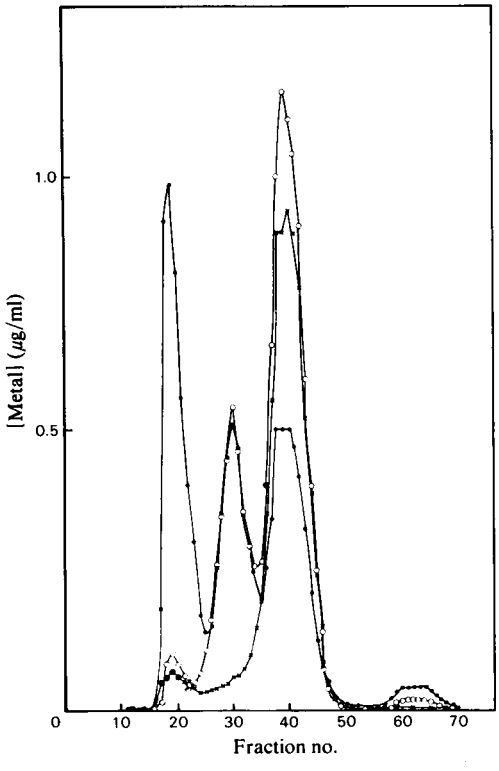

Fig. 3. *Sephadex G-75 profile of supernatant from rat liver pellet*

The supernatant is the same as that described in Table 2(B)3. ×, Cd; ●, Zn; ○, Cu.

Oxidation of horse kidney metallothionein

Because the facile thiol oxidation of the metallothionein from these rat livers had not been observed previously in preparations of metallothionein from a number of sources, the effect of aerobic oxidizing conditions on horse kidney metallothionein was investigated. The method used to prepare rat liver metallothionein was adapted to make a non-heat-treated supernatant from fresh horse kidney. Mercaptoethanol was omitted so that possible oxidation might be detected. The behaviour of zinc, copper, and cadmium in profiles from Sephadex G-75 chromatography of the supernatant exposed to oxidizing conditions was analysed over a period of 14 days. Figs. 4 and 5 with sample chromatographic runs and detailed kinetics of the metal redistributions summarize the rather surprising results. Indeed, as previously observed, the shift of cadmium from metallothionein into the high-molecular-weight fraction of the profiles is slow and unlikely to be observed during normal preparations of the protein. However, zinc shifts more rapidly out of this protein into the high-molecular-weight-protein band. In fact, in the first profile, run within hours of obtaining the fresh kidneys, a remarkable percentage of the total supernatant zinc is found in the metallothionein fraction (60%) along with a zinc/copper/cadmium g-atom proportion of 1 : 0.22 : 1.5. However, in the absence of mercaptoethanol, this is unstable, and later profiles reveal the redistribution of zinc and cadmium. There is no clear shift in the copper profile over time.

The rechromatography of mercaptoethanol-treated fraction I, which contains redistributed zinc and cadmium, leads to the recovery of a significant amount of zinc and cadmium in the metallothionein band II noted in Fig. 4. Thus, as for the rat liver supernatant, both metals and thionein protein move into the high-molecular-weight fraction during oxidation. An example of this is summarized in Table 3. The high-molecular-weight fraction I from day 13 of another kinetic study similar to the one reported above was incubated with mercaptoethanol and then chromatographed: 67% of the cadmium, 26% of the zinc, and 40% of the copper shift into the metallothionein band. The g-atom ratio of metals in this band was now 2 : 1 : 1.4, reflecting the fact that more

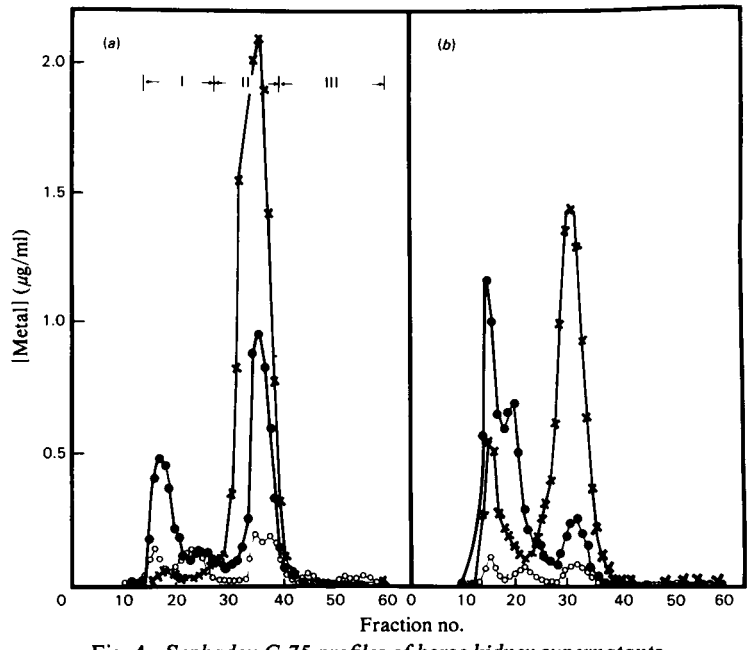

Fig. 4. *Sephadex G-75 profiles of horse kidney supernatants*
(*a*) Day 1 of incubation of heat-treated supernatant at 4°C. (*b*) Day 8 of incubation of supernatant. ●, Zn; ×, Cd; O, Cu.

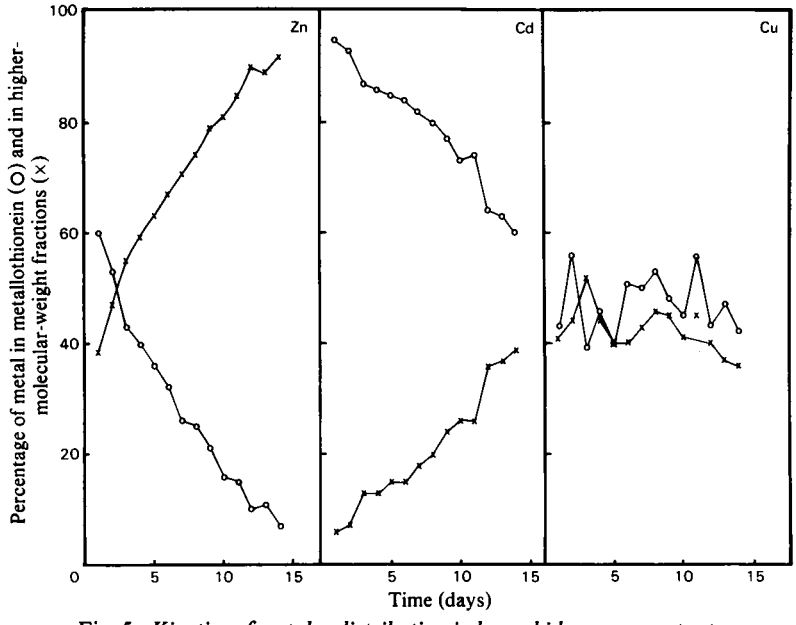

Fig. 5. *Kinetics of metal redistribution in horse kidney supernatant*
The metal content of band I (×) and band II (O) as a percentage of the total metal in the supernatant during 15 days exposure to aerobic conditions is shown.

zinc than cadmium had moved into the high-molecular-weight band during oxidation. Thus the oxidized and re-reduced thionein protein is enriched in zinc relative to the starting metallothionein.

Discussion

The present study was initiated to characterize rat liver metallothionein isolated from animals exposed

Table 3. *Reduction of oxidized horse kidney metallothionein*

Metal contents of high-molecular-weight fractions, peak Ia, before mercaptoethanol treatment (day 13). The day-0 g-atom ratio of metallothionein peak II is Zn/Cu/Cd = 2.2:1:3.5. After mercaptoethanol reduction of these fractions, they were rechromatographed on Sephadex G-75, giving peaks Ib, IIb and IIIb.

		Content (nmol)		
		Cd	Zn	Cu
Peak Ia (−mercaptoethanol)		205	727	241
Peak Ib	peak Ia (+mercaptoethanol)	65	445	116
Peak IIb		136	119	97
Peak IIIb		3	82	28

to low concentrations of cadmium for a number of weeks. As the work began, the report of El-Gazaar *et al.*, (1978) appeared in which this same model of cadmium intoxication yielded data that higher levels of cadmium exposure caused 'spillage' of cadmium into other proteins of liver supernatant and a re-organization of zinc in liver cytosol. In examining this finding, it became clear that shifts of cadmium and zinc into other protein fractions were due to the oxidation of metallothionein, which could largely be prevented by the presence of the reducing agent 2-mercaptoethanol in the preparation. Thus redistribution of cadmium does not occur when mercaptoethanol is used throughout the preparation (Fig. 1*a*). Furthermore, the altered metal profile of an oxidized sample of supernatant can be substantially restored to that of Fig. 1(*a*) by incubation with mercaptoethanol before Sephadex chromatography (Fig. 1*c*). Similarly, the movement of cadmium out of the metallothionein band is prevented by minimizing the exposure to oxidizing conditions during the initial purification steps preceding chromatography (Table 1).

The introduction of 5 mM-2-mercaptoethanol into the preparation was based on the report that liver contained approximately this concentration of thiol groups (Jocelyn, 1972). Thus, averaged over the whole cell, the proteins of liver exist in a reducing environment with respect to the thiol–disulphide redox couple.

In considering the mechanism of this oxidation, rechromatography of high-molecular-weight fractions containing cadmium after treatment with mercaptoethanol showed that the thionein protein had shifted along with metal, for the elution profile showed cadmium reappearing in the metallothionein band (Fig. 2). Hence it is the entire protein, not metal alone, that is becoming bound in higher-molecular-weight forms during oxidation. The observation that the thionein and metals remain in the high-molecular-weight fractions during simple rechromatography but not after chemical treatment with mercaptoethanol indicates that it is polymerized or chemically bound and not just loosely associated with high-molecular-weight proteins. The obvious mechanistic possibilities are that either some thiol groups of metallothionein undergo inter-metallothionein oxidation to produce polymeric species of the protein or that, during thiol oxidation, metallothionein becomes bound to other thiol-containing proteins in band I of the Sephadex elution profile. In either model, 2-mercaptoethanol would reduce the disulphide bonds that had formed and restore the original metal profile. Oxidation of thiol groups that bind the metals might be expected to release some cadmium, zinc, and copper from metallothionein, so that, although both metals and proteins appear in band I, they do not necessarily remain together in the oxidation process. However, this has yet to be determined.

The oxidation is a subtle process, and a detailed understanding may show more than one of these mechanisms to be operative. Thus, although the horse kidney metallothionein is considered to be saturated with metal (Kägi & Vallee, 1961; Kägi *et al.*, 1974), oxidation does slowly occur, with zinc shifting much more rapidly than cadmium into higher-molecular-weight fractions (Fig. 5). In fact, zinc moves out of the metallothionein peak more quickly than cadmium with either the horse kidney or rat liver proteins. Copper is also mobile in the rat liver protein, where it is present in large concentration, leaving band III before cadmium. During the oxidation process some of the zinc and copper are liberated as lower-molecular-weight forms that are virtually absent in reduced control supernatants (Fig. 1*a*; Table 2).

It is in the discovery of the oxidative sensitivity of zinc- and copper-bound metallothionein that these results may have general applicability. Although it is easy to recognize the perturbation of the cadmium profile by oxidation, the shifts in zinc and copper may be attributed to altered essential-metal metabolism or be missed entirely because control reduced supernatant samples were not run (El-Gazaar *et al.*, 1978). One would not detect this by metal-to-thiol

ratios, for oxidation is accompanied either by polymerization of this protein or by concomitant loss of metal and thiol. This is most striking in horse kidney, in which our normal preparation leads to the profile similar to that in Fig. 4(*b*), in which there is much zinc in the high-molecular-weight fractions and a smaller amount in the cadmium-metallothionein fractions. Yet rapid handling of the preparation shows that there is normally almost no high-molecular-weight-fraction-associated zinc in horse kidney supernatant and large amounts bound in the metallothionein band III. This result stands in contrast with that for rat liver, in which there is a large amount of stable high-molecular-weight-associated zinc.

It is the oxidative sensitivity of zinc and copper bound to metallothionein during preparations of the protein that may account for a considerable proportion of the wide variation in metal ratios reported for metallothioneins (Anderson *et al.*, 1978; Weser *et al.*, 1973; Kimura *et al.*, 1974; Irons & Smith, 1977; Oh *et al.*, 1978*a*; Tsunoo *et al.*, 1978; Winge *et al.*, 1978). Although differences in conditions of metal exposure and species may also alter these ratios, only when the reductive conditions of cells such as liver are employed in the isolation method will it be possible to know whether particular metal compositions of metallothioneins have significance.

The finding of substantial amounts of copper in rat liver metallothionein isolated under reducing conditions is also noteworthy. Few studies have actually measured the copper content of metallothionein. Until the present report, only the finding of a trace of copper in the horse kidney protein has suggested this is a normal component of the cadmium/zinc-metallothionein (Kägi *et al.*, 1974). These results suggest that in considering the dynamics of metal binding in this protein, zinc and copper must be considered as well as cadmium. More recently a metal-binding protein having a number of properties associated with metallothioneins has been isolated from Ehrlich ascites-tumour cells (Petering *et al.*, 1978; Koch *et al.*, 1980). It binds nearly equal amounts of zinc and copper. It has been shown that copper ion added directly to rat liver supernatants or chelated copper taken into Ehrlich cells binds specifically to either liver metallothionein or the analogous Zn,Cu-binding protein of Ehrlich cells (D. T. Minkel, C. F. Shaw, & D. H. Petering, unpublished work).

The finding illustrated in Fig. 2(*b*) that the cadmium bound to the particulate fraction of the liver homogenate chromatographs on Sephadex G-75 as a metallothionein is an important observation. Thus, assuming that losses of cadmium during preparative steps listed in Table 1 do not reflect selective removal of cadmium bound to other cellular components, virtually all of the cadmium in the liver cell is bound to metallothionein. Hence subtle effects on liver caused by low-level chronic exposure to cadmium are reasonably attributed to some effect of cadmium bound to metallothionein (Miller *et al.*, 1974; Petering, 1978).

Significantly, once out of the environment of the liver supernatant, the copper found in liver metallothionein is bound in stable form despite the close association with the thiol groups in the protein. Thus copper co-chromatographs with cadmium and zinc in the Sephadex G-75 chromatography and during the subsequent DEAE-Sephadex chromatographic run (Table 2). In a previous study, Rupp & Weser, (1974) titrated apo-metallothionein with Cu(I) under anaerobic conditions, but found the product to oxidize rapidly on exposure to air. Similarly, two groups have isolated zinc, copper- and copper-metallothionein under aerobic conditions, which tends to polymerize once exposed to air (Bremner & Marshall, 1974; Bremner & Young, 1976, 1977; Hartmann & Weser, 1977). However, the copper-thionein reported by Rydén & Deutsch (1978), though sensitive to oxidation, does exhibit a degree of stability in air which could not be expected of simple mercaptans in the presence of copper ion.

Thus it appears that the metal–thiol chemistry of metallothioneins must be examined closely in order to gain a better understanding of the detailed reactions that the metal-binding sites of these interesting proteins may undergo.

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References

Anderson, R. D., Winter, W. P., Maher, J. J. & Bernstein, I. A. (1978) *Biochem. J.* **174**, 327–338

Bremner, I. & Davies, N. T. (1974) in *Trace Elements Metabolism in Animals 2* (Hoekstra, W. G., Suttie, J. W., Ganther, H. E. & Mertz, W., eds.), pp. 493–496, University Park Press, Baltimore

Bremner, I. & Marshall, R. B. (1974) *Br. J. Nutr.* **32**, 293–300

Bremner, I. & Young, B. W. (1976) *Biochem J.* **155**, 631–635

Bremner, I. & Young, B. W. (1977) *Chem.–Biol. Interact.* **19**, 13–23

Davis, B. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427

El-Gazaar, R., Boyle, J., Cornicelli, J., & Petering, H. G. (1978) *Biochem. Pharmacol.* **27**, 2001–2008

Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77

Fridovich, I. (1974) *Adv. Enzymol.* **41**, 35–97
Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147–159
Hartmann, H.-J. & Weser, U. (1977) *Biochem. Biophys. Acta* **491**, 211–222
Irons, R. D. & Smith, J. C. (1977) *Arch. Environ. Contam. Toxicol.* **6**, 193–202
Jocelyn, P. C. (1972) *The Biochemistry of the SH Group*, p. 261, Academic Press, New York
Kägi, J. H. R. & Vallee, B. L. (1960) *J. Biol. Chem.* **235**, 3460–3465
Kägi, J. H. R. & Vallee, B. L. (1961) *J. Biol. Chem.* **236**, 2435–2442
Kägi, J. H. R., Himmelhoch, S. R., Whanger, P. D., Bethune, J. L., & Vallee, B. L. (1974) *J. Biol. Chem.* **249**, 3537–3542
Kimura, M., Otaki, N., Yoshika, S., Suzuki, M., Horinchi, N. & Suda, T. (1974) *Arch. Biochem. Biophys.* **165**, 340–348
Koch, J., Wielgus, S., Shankara, B., Shaw, III, C. F., & Petering, D. H. (1980) *Biochem. J.* **189**, 95–104
Layne, E. (1957) *Methods Enzymol.* **3**, 447–454
Margoshes, M. & Vallee, B. L. (1957) *J. Am. Chem. Soc.* **79**, 4813–4814
Miller, M. L., Murthy, L., Basam, C. R. & Petering, H. G. (1974) *Am. J. Anat.* **141**, 23–32
Nordberg, G. F., Nordberg,, M., Piscator, M. & Vesterberg, O. (1972) *Biochem. J.* **126**, 491–498
Oh, S. H., Deagen, J. J., Whanger, P. D. & Weswig, P. H. (1978*a*) *Bioinorg. Chem.* **8**, 245–254
Oh, S. H., Deagen, J. J., Whanger, P. D. & Weswig, P. H. (1978*b*) *Am. J. Physiol.* **234**, E282–E285
Perry, Jr., H. M. (1976) in *Trace Elements in Human Health and Disease*, vol. 2 (Prasad, A. S., ed.), chapter 43, Academic Press, New York
Petering, H. G. (1978) *Environ. Health Perspect.* **25**, 141–145
Petering, D. H., Koch, J., Wielgus, S. & Shaw, C. F., III (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1349
Probst, G. S., Bousquet, W. F. & Miya, T. S. (1977) *Toxicol. Appl. Pharmacol.* **39**, 51–60
Richards, M. P. & Cousins, R. J. (1976) *J. Nutr.* **106**, 1591–1599
Rupp, H. & Weser, U. (1974) *FEBS Lett.* **44**, 293–297
Rydén, L. & Deutsch, H. F. (1978) *J. Biol. Chem.* **255**, 519–524
Shaikh, Z. A. & Smith, J. C. (1977) *Chem.-Biol. Interact.* **19**, 161–171
Sobocinski, P. Z., Canterbury, W. J., Mapes, C. A. & Dinterman, R. E. (1978) *Am. J. Physiol.* **234**, E399–E406
Tsunoo, H., Kino, K., Nakajima, H., Hata, A., Huang, I.-Y. & Yoshida, A. (1978) *J. Biol. Chem.* **253**, 4172–4174
Webb, M. (1972) *Biochem. Pharmacol.* **21**, 2751–2765
Weser, U., Rupp, H., Donay, F., Linnemann, F., Voelter, W., Voetsch, W. & Jung, G. (1973) *Biochem. J.* **139**, 127–140
Winge, D. R. & Rajagopalan, K. V. (1972) *Arch. Biochem. Biophys.* **153**, 755–762
Winge, D. R., Premakumar, R. & Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys.* **170**, 242–252
Winge, D. R., Premakumar, R. & Rajagopalan, K. V. (1978) *Arch. Biochem. Biophys.* **188**, 466–475