Separation of Two Forms of Rabbit Metallothionein by Isoelectric Focusing

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Rabbits were given repeated injections of cadmium chloride. Cadmium- and zinc-containing protein fractions were obtained from the livers of these animals by precipitation procedures and Sephadex G-75 chromatography. The protein thus obtained showed several characteristics similar to those of the earlier described protein metallothionein. Further separation by isoelectric focusing showed two main protein peaks with isoelectric points at 3.9 and 4.5 respectively. Amino acid analysis of these two forms showed similar content of most amino acids [residues per cent.: cysteine (28%), aspartate (8%), threonine (5–6%), serine (12%), glycine (7%), alanine (13%), methionine (2%), isoleucine (2%)] but with a small difference in content of lysine (12 and 13% respectively), proline (9 and 5% respectively) and glutamate (2 and 4% respectively). The two forms of the protein both contained cadmium, but only the one with *pI* 4.5 contained also significant amounts of zinc.

A protein with a high content of metals such as cadmium, zinc and sometimes mercury has been characterized by Kägi & Vallee (1960, 1961) and Pulido *et al.* (1966). They named this protein metallothionein, and the metal-free form thionein. These investigators isolated the protein from normal kidneys of horses and human beings. A protein with similar properties has also been isolated by Piscator (1964) from the livers of rabbits that had been exposed to cadmium by repeated injections. Both the protein from the rabbit liver (Piscator, 1964) and the one from human and horse kidney (Pulido *et al.*, 1966) showed at least two forms by chromatographic and electrophoretic examination. A more detailed characterization of these two forms, however, has not yet been reported. To study the rabbit protein further, the method of isoelectric focusing (Vesterberg & Svensson, 1966) was used.

Materials and Methods

Materials

Animals. Fourteen rabbits of both sexes and with a body weight range of 2.6–3.9 kg were used.

Treatment of animals. Rabbits were kept in separate cages and supplied with pellets, hay, carrots and tap water. The Cd-exposed group comprised 10 rabbits. They were given sterile iso-osmotic, pyrogen-free solutions of CdCl₂ (0.82 mg of CdCl₂/ml and 4.1 mg of CdCl₂/ml) as subcutaneous injections with intervals of 2–3 days between injections. A total

amount of 10 mg of Cd/kg body wt. was given during the 6 weeks of exposure (six doses each of 0.5 mg of Cd/kg followed by seven doses each of 1 mg of Cd/kg). The animals were then left untreated for 2 weeks before being killed. They were killed by exsanguination with heart puncture under pentobarbital anaesthesia. The livers were removed immediately and the weights recorded. A sample of fresh tissue was taken for metal analysis and the rest of the organ was frozen at a temperature of –70°C.

Four rabbits constituted the control group. They received repeated subcutaneous injections of iso-osmotic saline (1 ml) and were killed and dissected just as the Cd-treated group.

Chemicals. All chemicals were of high analytical grade. Cadmium chloride was from Mallinckrodt's Chemical Works, U.S.A.; tris was from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.; NaCl, HCl, KBr, NH₃ and NaN₃ were from E. Merck A.G., Darmstadt, West Germany; Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was from Farbwerke Hoechst A.G., Frankfurt (M), West Germany. A Protein Calibration Kit with proteins of specified molecular weights (cytochrome *c*, mol.wt. 12500; chymotrypsinogen, mol.wt. 25000; ovalbumin, mol.wt. 45000; albumin, mol.wt. 67000) was purchased from Boehringer und Soehne G.m.b.H., Mannheim, West Germany. Carrier ampholytes for isoelectric focusing (Ampholine, pH3–5 and pH6–8) were from LKB-Produkter, 161 25 Bromma, Sweden.

To prevent metal contamination, all buffers, dilutions etc. were prepared with deionized water, if not otherwise stated.

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Equipment. Jacketed columns for Sephadex chromatography and Sephadex G-75 (fine grade) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Columns for isoelectric focusing as well as a fraction collector (Ultron-Rac LKB 7000) and Uvicord (LKB 4701 A) absorptiometer (254 nm) with recorder (LKB 6520 A) were from LKB-Produkter, 161 25 Bromma, Sweden. For ultrafiltration the Diaflo 402 cell and UM-2 filter were obtained from Amicon, N.V., The Hague, The Netherlands. For centrifugation a Wifug Clinic was used. Perkin-Elmer atomic-absorption spectrophotometers (models 303 and 403) were used for metal analysis. For dialysis Visking Nojax tubes (23/32; 24/32) from Union Carbide, Chicago, Ill., U.S.A. were used.

Preparation of metallothionein

Homogenization. Homogenization was performed with an Ultra-Turrax (JKAA-Werk, Staufen, Germany) at +4°C. For each preparation two livers were thawed and homogenized in a prechilled medium consisting of 0.01 M-tris buffer, pH 8.0, in 0.05 M-NaCl and 100 mg of NaN_3/l .

Precipitation procedures. (a) Rivanol precipitation. A 5% solution of Rivanol in water was added to the homogenate (pH 7.0–7.5) with an equal volume of each solution (Piscator, 1964). After 1 h at +4°C, centrifugation was performed at 5000g for 10 min. (b) Precipitation with KBr. A small volume of a saturated solution of KBr in water was added to the supernatant (pH ~7) after Rivanol precipitation (Matthaeus & Matheka, 1963). Potassium bromide solution was added until no further precipitation took place, at which time a further drop of the KBr solution was added. The mixture thus obtained was left for 1 h at +4°C and then was centrifuged for 10 min at 5000g. (c) Ethanol precipitation. This was performed at +4°C with prechilled (–20°C) 95% (v/v) ethanol. After ethanol had been added with constant stirring, prechilled (–20°C) chloroform was added (Kägi & Vallee, 1960), the mixture was left overnight at +4°C and then centrifuged for 20 min at 5000g. The proportions of homogenate, ethanol and chloroform were 1:1.05:0.08 (by vol.).

Dialysis. Deionized water with pH adjusted to 7 with NH_3 was used for dialysis at a temperature of +4°C. The water was changed four times during the 12 h dialysis.

Ultrafiltration. Concentration was performed in an atmosphere of N_2 at a flow rate of 130 ml/h with the application of an appropriate pressure [0.3–0.4 MN/m² (40–60 lb/in²)] to obtain this flow rate. The ultrafiltration was performed at about +14°C.

Sephadex G-75 chromatography. Samples of the non-diffusible material and of the non-ultrafiltrable material were applied to a column with flow adaptors at both ends. The column (859 mm × 50 mm) was

packed with Sephadex G-75 (fine grade), equilibrated with 0.01 M-tris buffer, pH 8.0, in 0.05 M-NaCl, and fractions of 20 ml volume were collected at a flow rate of 92 ml/h (Fig. 1). For removal of ampholytes, pooled fractions from isoelectric focusing were applied to a column (364 mm × 25 mm) packed with Sephadex G-75 (fine grade) equilibrated with 0.01 M-tris buffer, pH 8.0, in 0.05 M-NaCl. Samples were run from the bottom to the top of the column at a flow rate of 14 ml/h. The fraction volume was 5 ml.

During periods of frequent separations the columns were kept at continuous flow to prevent bacterial growth. When the columns were stored a buffer containing 0.01% (w/v) NaN_3 was used to prevent bacterial growth. The chromatographic separations were performed at about +13°C.

Isoelectric focusing. This was carried out in principle as described by Vesterberg & Svensson (1966) in a 110 ml column equipped with a double cooling jacket (LKB-Produkter), by using a sucrose density gradient (0–50%, w/v). The following carrier ampholytes (Ampholine, 40%, w/v) were used: in the dense solution, 2 ml of pH 3–5; in the less-dense solution, 0.2 ml of pH 3–5 and 0.2 ml of pH 6–8. On the bottom and in the central tube (anode) 20 ml of 50% (w/v) sucrose containing 0.7 ml of 1 M- H_2SO_4 was used. At the cathode 8 ml of water containing 0.05 ml of ethylenediamine was added. A potential of 500 V was used first for 24 h to establish the pH gradient. After this time the sample (1.3 ml) was injected into the column at a place where the pH was estimated to be about 6. The experiment was then continued for 24 h with a potential of 600–700 V (2–3 mA).

The contents of the column were collected in 2 ml fractions. The pH determination (precision ± 0.01 pH unit) was made at +4°C. A sample (0.4 ml) from each fraction was taken for protein determination, spectrophotometry and metal analysis. The remaining 1.6 ml portions from each fraction were pooled (forms I and II) as indicated in Fig. 2. The ampholytes were removed by chromatography on Sephadex G-75.

The samples were not stored during the first step of the preparation. After thawing of the livers, homogenization, precipitation procedures and dialysis were performed immediately after one another. Thereafter samples were frozen rapidly by letting the liquid sample fall dropwise into liquid N_2 , whereby small pieces of ice formed immediately. The frozen material was then stored at –70°C for various times between a few days and several weeks.

Analytical methods

Estimation of molecular weight. Several reference proteins of known molecular weight were fractionated on a column (364 mm × 25 mm) packed with Sephadex

G-75 (fine grade). To obtain a standard curve relative elution volumes (V/V_0) were plotted versus the logarithm of the known molecular weights (Fig. 3) as described by Andrews (1965).

Protein determination and spectrophotometry. Protein content of the fractions from isoelectric focusing was determined by a modification of the biuret method described by Piscator (1962). Diluted samples (200 μ l of sample + 2 ml of 0.9% NaCl) were used for the biuret analysis and also for measurements of extinction at 250 and 280 nm. A Beckman DB spectrophotometer was used for spectrophotometric determinations. For registration of absorption spectra this instrument was connected to a Perkin-Elmer recorder model 165.

Metal analysis. Samples of the fractions from isoelectric focusing were diluted (200 μ l of sample + 5 ml of water) for cadmium and zinc analysis. All metal analyses (whole tissue, pellets, precipitates, supernatants and fractions from chromatography and isoelectric focusing) were performed by atomic-absorption spectrophotometry as described by Nordberg *et al.* (1971a).

Amino acid analysis. After removal of ampholytes

samples of 0.5 mg of protein were taken from forms I and II for amino acid analysis. After hydrolysis for 24 or 72 h quantitative amino acid analysis was performed by ion-exchange chromatography. Samples were treated by oxidation for determination of cysteine. The mentioned methods have been described in detail by Eaker (1970) and Moore (1963). The analyses were performed at the laboratory for amino acid analysis, Department of Biochemistry, Uppsala University, Uppsala, Sweden.

Results

Piscator (1964) showed that a protein very similar to metallothionein could be isolated from the livers of rabbits that had received repeated injections of cadmium chloride. It has been shown that exposure to cadmium facilitates the isolation of low-molecular-weight metal-containing proteins (Shaikh & Lucis, 1970; Nordberg *et al.*, 1971a). Therefore to get appropriate amounts of metallothionein from the livers, the rabbits were intensively exposed to cadmium. An interval of 2 weeks between last exposure and death was chosen to give the cadmium

Scheme 1. Purification of metallothionein

time to be incorporated into the low-molecular-weight proteins of the liver, presupposing mechanisms for 'incorporation of cadmium' in the rabbit similar to those found in mouse liver (Nordberg *et al.*, 1971a).

The mean cadmium concentration in the livers of the cadmium-exposed group was $176 \mu\text{g/g}$ wet wt. (range $144\text{--}197 \mu\text{g}$) and the mean zinc concentration was $119 \mu\text{g/g}$ wet wt. (range $93\text{--}224 \mu\text{g}$). In the control group the corresponding values were $0.2 \mu\text{g}$ of Cd/g wet wt. (range $0.1\text{--}0.4 \mu\text{g}$) and $46 \mu\text{g}$ of Zn/g wet wt. (range $42\text{--}50 \mu\text{g}$). Concentrations of cadmium and zinc in the livers of the cadmium-exposed rabbits thus were similar to those used by Piscator (1964).

Different variations of the methods described by Piscator (1964) and Pulido *et al.* (1966) for preparation of metallothionein were tried on the homogenates from the livers. Since no important differences were found in these studies, they will not be reported in detail. Direct treatment of homogenates with Rivanol followed by precipitation with potassium bromide and separation on Sephadex G-75 gave approximately the same results as primary centrifugation, precipitation with ethanol-chloroform, dialysis and separation on Sephadex G-75. For details of the procedures used, see the Materials and Methods section.

The following results are from a preparation carried out as described in Scheme 1. Half of the homogenate from two livers was used for the preparation of the material described below, the other half being used for the above-mentioned comparative studies. After homogenization the main part of the liver proteins and cellular elements were precipitated by Rivanol and the excess of this substance was removed in the precipitation with potassium bromide. Haemoglobin was subsequently removed by precipitation with ethanol-chloroform. The Rivanol precipitate contained 11%, the potassium bromide precipitate

3% and the precipitate after ethanol-chloroform 3% of the cadmium originally present in the homogenate. For zinc the corresponding values were 25, 3 and 16%. At dialysis chloroform droplets precipitated in the dialysis tubes. To avoid contamination with this chloroform, they were discarded together with the surrounding water phase. This procedure gave rise to some loss of material (about 10%). However, 71% of the total amount of cadmium and 40% of zinc present in the crude homogenate remained before concentration in the ultrafiltration-cell, during which step no losses occurred.

When material was separated on Sephadex G-75, the total recovery was 90–100% of the input. Fractions indicated in Fig. 1 contained 60% of the cadmium and the same percentage of the zinc calculated on the amount applied to the column. (Thus 43% of the cadmium in the crude homogenate was recovered in these low-molecular-weight protein fractions. The corresponding value for zinc was 24%.) These fractions were taken for further treatment by ultrafiltration (see the Materials and Methods section), and the buffer was exchanged for 0.9% sodium chloride with pH adjusted to 7–8 with tris. After this step the protein concentration was 15.8 mg/ml , the E_{250}/E_{280} ratio was 18, the cadmium concentration $583 \mu\text{g/ml}$ and zinc concentration $218 \mu\text{g/ml}$. The molecular weight of this metallothionein was estimated as 9000–10000 on a calibrated Sephadex G-75 column (Fig. 3).

Preparation by the schedule described above, but from the liver of a control rabbit not exposed to cadmium, yielded a much smaller amount of protein/g of liver than from the cadmium-treated animals. The yield of zinc was poor and the cadmium content was so low that it was difficult to analyse appropriately without losing too much material.

One part of the material obtained from the cad-

Fig. 1. Sephadex G-75 separation of proteins of non-diffusible material and non-ultrafiltrable material

Column dimensions were $859 \text{ mm} \times 50 \text{ mm}$. Elution was carried out with 0.01 M -tris buffer– 0.05 M -NaCl, pH 8.0. Fractions (20 ml) were collected. E_{250} was recorded in a Uvicord with 3 mm light-path and is plotted versus elution volume. Fractions 58–70, indicated by the bold line, were taken for further study.

mium-treated rabbits was used for toxicological studies and another part was washed with deionized water in an ultrafiltration cell and used for further characterization by isoelectric focusing. Preliminary experiments in which the protein was added at the start of the isoelectric focusing procedure proved unsatisfactory. The reason for this is not quite clear, but it is probable that a part of the metal-containing proteins became exposed to an acidic pH in the lower end of the column before being transported to their *pI*. Too low a pH dissociates the metals, which may then be redistributed in a complex manner. Therefore we introduced the sample at a neutral pH in a pre-established gradient. By this method distinct peaks were obtained.

Two major fraction groups containing metals and protein were obtained by the isoelectric focusing procedure. The isoelectric point was 3.9 for form I and 4.5 for form II (Fig. 2). The recovery of cadmium was 81% and of zinc 97% of the metal put into the column. Fig. 2 shows that both forms I and II contain cadmium, but only form II contains significant amounts of zinc.

With the conventional biuret procedure the Ampholine gives a low background, but with the modified method a somewhat higher background was obtained because the Ampholine was partly precipitated together with the protein and poly-

peptides. Even if an estimated background corresponding to a concentration of 0.5 mg of protein/ml was subtracted from the values presented in Fig. 2, the absolute values could be subject to some error. It is evident, however, that the protein curve seen in Fig. 2 is divided in the same two main fraction groups as the metal content and the E_{280} readings.

The method for separation of proteins from Ampholine after isoelectric focusing has been described recently (Vesterberg, 1969). In accord with recommendations included there a procedure with chromatography on Sephadex G-75 was used in this study, and permitted a simultaneous estimation of the molecular weight, which was found to be 9000–10000 for both forms (Fig. 3).

Metal analysis of forms I and II showed that the total molar amount of metal was about the same in both forms. Form I contained cadmium and zinc in a molar ratio of 50:1 and form II in a ratio of 1:1.2. Form I contained 11% (w/w) cadmium and 0.1% of zinc, and form II 5% cadmium and 4% zinc, when the protein content was calculated from the amino acid analysis.

The u.v.-absorption spectra for the two forms are seen from Fig. 4.

Table I shows the results of the amino acid analysis. The molecular weight of the metal-free protein (thionein) was calculated from this analysis to be

Fig. 2. Results of isoelectric focusing of metallothionein prepared as described in Scheme 1

Fractions (2ml) were collected and the measurements indicated were performed on each fraction. (a) Concentration of protein. (b) $\square$ — $\square$, Concentration of cadmium; $\blacksquare$ — $\blacksquare$, concentration of zinc; $\bullet$ — $\bullet$, E_{280} ; $\blacktriangle$ — $\blacktriangle$, E_{250} ; —, pH at +4°C.

Fig. 3. Estimation of molecular weight of metallothionein

Line obtained by plot of relative elution volume (V/V_0) versus $\log(\text{mol.wt.})$ of proteins with known molecular weight (see the text for details). Separation was carried out on a column (364 mm $\times$ 25 mm) of Sephadex G-75. Elution was with 0.01 M-tris buffer in 0.05 M-NaCl, pH 8.0.

about 6000 in the present study, under the assumption that only one residue of the amino acids with the lowest values was present. The calculation has been done from the amino acid alanine, which gives accurate values both at 24h and 72h hydrolysis, assuming 8 residues of alanine.

Discussion

Earlier experiments indicated that cadmium is largely bound to haemoglobin in the blood of cadmium-exposed rabbits (Carlson & Friberg, 1957). It was therefore logical to try to improve the purification used earlier on rabbit liver (Piscator, 1964) by a haemoglobin-precipitation step. Only a small amount of cadmium occurred in the haemoglobin precipitate. This finding is in accord with the recent observation that, in blood in animals treated for a long time with cadmium (Nordberg *et al.*, 1971b), little cadmium is found in fractions corresponding to haemoglobin. In the study on haemolysate by Carlson & Friberg (1957) about 40% of the cadmium was diffusible and this part, which could have been bound to metallothionein, was not further studied. In the liver large amounts of metallothionein are present and this protein has a much greater affinity for cadmium than does haemoglobin. This may be the explanation for the small amounts of cadmium precipitated together with haemoglobin in the present study. The methods used for the first steps of purification are similar to

Fig. 4. U.v.-absorption spectra after removal of ampholytes from forms I and II of metallothionein prepared as described in Fig. 2

A Beckman DB spectrophotometer with a Perkin-Elmer no. 165 recorder was used.

the one earlier described (Piscator, 1964) and avoid expensive equipment such as ultracentrifuges or other large-volume, high-speed centrifuges.

The yield of cadmium in different steps of the preparation was good in comparison with earlier reports on metallothionein preparation from kidney cortex. After dialysis 71% of the original cadmium was recovered, compared with 44% (horse kidney: Kägi & Vallee, 1960) or 25% (human kidney: Pulido *et al.*, 1966), and after chromatography on Sephadex G-75 43% was recovered compared with 19 and 11% respectively.

Owing to the high resolving power of the isoelectric focusing method it was possible to obtain a nearly complete separation of two forms of metallothionein in the present investigation. Metallothionein from human kidney, which also showed a single peak on gel filtration, has been separated incompletely into three components by preparative electrophoresis by Pulido *et al.* (1966). Fig. 2 shows that in the present study one could interpret the zinc and protein peak at 92 ml as a third form, but, because this peak contained only a minor part of the total protein, it was not further studied.

The u.v.-absorption spectra of the two forms (Fig. 4) show a maximal difference from each other at about 250 nm. The most likely explanation for this difference is the different cadmium content in the two forms, as it has been shown by Kägi & Vallee (1961) that the cadmium-mercaptide bond is re-

Table 1. *Amino acid composition of rabbit hepatic metallothionein forms I and II and of horse hepatic metallothionein*

Except where noted the values are averages from 24h and 72h hydrolysis.

	Rabbit metallothionein				To nearest integer		Horse metallo-thionein (from Kägi, 1970) (residues %)
	Form I		Form II		Form I	Form II	
	Residues/ mol.§	Residues %	Residues/ mol.§	Residues %			
Lysine	6.7	11.8	8.7	13.2	7	9	10.4
Cysteic acid*	15.1	26.6	19.0	28.9	15	19	33.2
Aspartic acid	4.8	8.4	5.2	8.0	5	5	5.0
Threonine†	3.0	5.2	3.9	5.8	3	4	4.1
Serine†	6.7	11.8	8.5	12.9	7	9	11.5
Glutamic acid	1.3	2.4	3.0	4.5	1	3	4.0
Proline	5.2	9.1	3.3	5.0	5	3	5.4
Glycine	4.1	7.2	4.3	6.5	4	4	9.6
Alanine	8.0	14.1	8.0	12.1	8	8	8.9
Methionine	1.0	1.8	1.0	1.6	1	1	1.6
Isoleucine‡	1.0	1.7	0.9	1.4	1	1	0.9
Arginine	0.0	0.0	0.0	0.0	—	—	2.8
Valine	0.0	0.0	0.0	0.0	—	—	2.4
Leucine	0.0	0.0	0.0	0.0	—	—	0.4
Calculated mol.wt. of thionein					5608	6616	6600

* Oxidation. † Extrapolated to zero hours hydrolysis. ‡ 72h hydrolysis only.

‡ Calculated on the basis of 8 residues of alanine/mol. || % of total number of residues in molecule.

sponsible for the specific absorption of metallothionein at 250nm. Actually the absorption spectrum for form I, which contains almost exclusively cadmium, is very similar to the spectrum obtained by Kägi & Vallee (1961) for cadmium-thionein.

The reason for the difference in isoelectric point between the two forms is not quite clear at the present. The possibility that differences in isoelectric point between different forms of metallothionein could be due to differences in metal content was discussed by Pulido *et al.* (1966). They found in their preparation from human kidney that the metal content was smaller in one of their metallothionein components and stated that this fact could explain the difference in electrophoretic mobility between the different forms. In the present study the total molar metal content was nearly the same in both forms, but form I contained predominantly cadmium and form II about equal molar amounts of cadmium and zinc, thus suggesting that different metals influence the *pI* differently. Another explanation for the small difference in *pI* would be a small difference in amino acid composition. There seems to be a difference between the two forms especially with respect to

lysine, proline and glutamic acid contents (Table 1). Lysine and glutamic acid, which each carry a group with a free charge, must be particularly suspected. Lysine, which has a fully ionized amino group, can explain the higher isoelectric point for form II. The free carboxyl groups of glutamic acid are only partly ionized at this pH and have thus probably less influence on *pI*. The fact that form II contains much more zinc than form I may be explained also by the differences in amino acid composition. A comparison of the amino acid composition of this rabbit metallothionein with the one reported by Kägi (1970) for horse metallothionein (Table 1) shows an absence of arginine, valine and leucine in the rabbit protein and indicates a species difference. The molecular weight of rabbit thionein was calculated to be about 6000 from the amino acid analysis. Of course any multiple of 6000 would also be a possible molecular weight of the protein, but the estimation of the molecular size on the Sephadex G-75 column points to a molecular weight of 9000–10000 for metallothionein, which makes it most probable that 6000 is the right one for thionein. The estimation of molecular weight on Sephadex G-75 is unsatisfactory at these

low molecular weights, partly because of the unavailability of marker proteins with lower molecular weight than the tested protein. The estimation in the present study could be used only as an approximate upper limit. This estimation has been included to demonstrate the similarity between the rabbit protein and metallothionein from other species, when subjected to estimations of molecular weight on Sephadex. Kägi & Vallee (1961) found the molecular weight to be 10000 for horse metallothionein, Pulido *et al.* (1966) found 10500 for human metallothionein and Wisniewska *et al.* (1970) found 11200 for metallothionein from rat kidney by the same method. The molecular weight obtained by amino acid analysis in the present study of rabbit metallothionein is similar to the one reported for horse hepatic metallothionein by Kägi (1970) (see Table 1). It thus seems that the molecular weights of metallothionein from different species were similarly low. The implication is that this protein could probably be filtered through the glomeruli and, if present in blood, act as a transport protein for cadmium and possibly other metals in the body. A cadmium-binding protein with a small molecular size, possibly identical with metallothionein, has recently been demonstrated in the blood of mice exposed repeatedly to cadmium (Nordberg *et al.*, 1971b). An account of possible mechanisms for cadmium accumulation in humans and other mammals, in which the subject of metallothionein as a transport protein is given much attention, is that by Friberg *et al.* (1971).

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