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BINDING OF CADMIUM IONS BY RAT LIVER AND KIDNEY

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Abstract—Most of the bound Cd^{2+}, which accumulates in the livers and kidneys of adult rats after the subcutaneous injection of $CdCl_2$ (2·2 μmoles/100 g body wt) is recovered as a single, heat-stable fraction from the soluble components of these tissues. Although this fraction in either organ also binds Zn^{2+}, Cd^{2+} does not displace Zn^{2+} from any of the normal, soluble Zn^{2+}-metalloproteins.

The proteins of liver and kidney that accumulate Cd^{2+} do not appear to be identical, but have properties in common and after, but not before removal of the bound cations are rich in —SH groups. At least in the male rat, these Cd^{2+}-binding proteins are not normal components of these tissues but are synthesized in response to the uptake of the foreign cation. In the liver, this synthesis seems to be controlled at the translational level, since it is inhibited by cycloheximide, but not by actinomycin D. Also in the liver, the synthesis of the same protein is induced by excess Zn^{2+}, but not by Co^{2+}, Ni^{2+} and Pb^{2+} and it is possible that the "binding proteins" normally function in the control of Zn^{2+}-metabolism. Their induction by Cd^{2+} and also by Hg^{2+}, thus may be a consequence of the similarities in the chemical properties of these cations and of Zn^{2+}.

CADMIUM, when administered either subcutaneously or orally, is known to accumulate in the livers and kidneys of experimental animals.[1–5] In a previous study of the biochemical effects of Cd^{2+} in the rat and the mouse[5] it was shown, in agreement with the earlier work of Piscator[4] with the rabbit, that most of the Cd^{2+} that was accumulated and retained in each of these organs was bound to a single, soluble cytoplasmic protein fraction, provisionally identified as a metallothionein. At least in the liver, it appeared that the synthesis of this protein was either induced, or stimulated greatly in response to the uptake of the cation. Evidence in support of the former of these alternatives is presented in this paper.

MATERIALS AND METHODS

Animals. Male and female rats of the laboratory "hooded" strain were maintained as described previously.[5] Rhabdomyosarcomas were induced in female rats by implantation of finely powdered metallic cadmium into the thigh muscle.[6]

Chemicals. Sephadex G 75 and G 200 were obtained from Pharmacia (Great Britain) Ltd., London and DE 52 cellulose from W & R Balston (Modified Celluloses) Ltd., Maidstone, Kent. Actinomycin D and cycloheximide were purchased from Sigma Chemical Co., Ltd., London and *p*-chloromercuribenzoate from Koch–Light Laboratories Ltd., Colnbrook, Bucks. All other chemicals were of Analar grade. Solutions for injection were made isotonic with NaCl and sterilized by filtration through Swinnex filters (Millipore (U.K.) Ltd, Wembley, Middlesex). Stock solutions of cycloheximide and actinomycin D were made in 1,2-dihydroxypropane or ethanol, and stored in the dark at 4°. Fresh dilutions (1:100 or 1:200) of these solutions were prepared in 154 mM NaCl for each series of injections.

Radioactive chemicals. [1-^{14}C]-L-Leucine (62·5 mc/mmol) and $^{63}NiCl_2$ were obtained from the Radiochemical Centre, Amersham, Bucks. The latter was supplemented with carrier $NiCl_2$ to give a 0·1 mM solution that contained a 0·145 $\mu c/\mu$ atom Ni.

Amino acid incorporation into liver tissue in vitro. This was done as described previously.[5] The incubation medium contained 0·25 μc[1-^{14}C-]L-leucine/ml.

Determination of radioactivity. Samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer. Protein solutions that contained $^{63}Ni^{2+}$ were digested with HNO_3, the digests being made up to volume (1 ml) with water and portions (0·2 ml) transferred to counting vials. These solutions were evaporated to dryness *in vacuo* over KOH and P_2O_5, the residues being dissolved in formic acid (0·5 ml) at 60–70° to decompose any NO_3^- anions before the addition of the scintillant.

Enzyme assays. Neutral proteinase and cathepsin activities were measured with casein and haemoglobin, respectively as substrates by the methods of Lundquist[7] and Barrett.[8] The liver tissue was homogenized in 3 vol. 0·9% (w/v) NaCl solution, the homogenate was then alternately frozen in liquid N_2 and thawed in a water bath at 25°, the cycle being repeated four times. After this treatment the suspension was centrifuged, first at 10,000 *g* for 10 min and then at 90,000 *g* for 60 min, the supernatant fraction being assayed for enzyme activities.

Preparation of cell sap fractions of liver and kidney. The animals (200–250 g body wt) were injected subcutaneously with Cd^{2+} (2·2 μmoles/100 g body wt) and killed by cervical dislocation at least 24 hr later. In the initial experiments the excised liver or kidney tissue was homogenized in 3–5 vol. medium A (250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris–HCl buffer, pH 7·4). In later work, this medium was replaced by phosphate-buffered saline (PBS) (125 mM NaCl and 20 mM phosphate buffer, pH 7·2). The homogenates were centrifuged at 10,000 *g* for 10 min and then at 105,000 *g* for 90 min. The final supernatant solutions usually were fractionated immediately, but could be stored frozen at −20° without alteration in the distribution of Cd^{2+}.

Gel filtration and column chromatography. Gel filtration was done with either NaCl (154 or 170 mM) or 50 mM phosphate buffer, pH 7·2 as eluant at 4° on Sephadex G 75 or G 200 columns, the dimensions of which are given in the text. The initial samples, and pooled eluates were concentrated to the appropriate volume either by dialysis against solid sucrose, or in a Diaflo cell (Amicon Ltd., High Wycombe, Bucks.). DE-52 Cellulose was used in 10 × 1 cm columns and equilibrated with either 20 mM phosphate buffer, pH 7·2 or 1 mM Tris–HCl buffer, pH 8·4. Before chromatography the sample was dialysed for 30 hr against three changes, each of 100 vol. of the appropriate buffer.

Dialysis. Removal of Cd^{2+} by dialysis against 50 mM glycine–HCl buffer, pH 2·2, and uptake of cations by equilibrium dialysis of the Cd^{2+}-free apoprotein (see Results section) were done in closed containers under N_2, all solutions being flushed with the gas before use. In the latter experiments, unbound cations were removed by further dialysis against 50 mM acetate buffer, pH 6·0. The Visking dialysis tubing was kept for 20 min in boiling water, and then washed thoroughly with glass-distilled water before use.

Analytical methods. Iron, Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} were measured by atomic absorption, quantitative analyses being done on wet-ashed samples.[9] The

distribution of cations in fractions from separations by gel filtration and column chromatography was followed qualitatively, the results being expressed in the figures in arbitrary units (per cent absorption). Protein was determined by the method of Lowry *et al.*[10] and —SH groups with Ellman's[11] reagent. Disk electrophoresis was done as described by Davis.[12]

Isolation of the Cd^{2+}-binding proteins of liver and kidney. On gel filtration on a Sephadex G 75 column (70 × 4 cm) of an approximately 4-fold concentrated sample (8–10 ml) of liver cell sap, protein-bound Cd^{2+} was eluted immediately after the

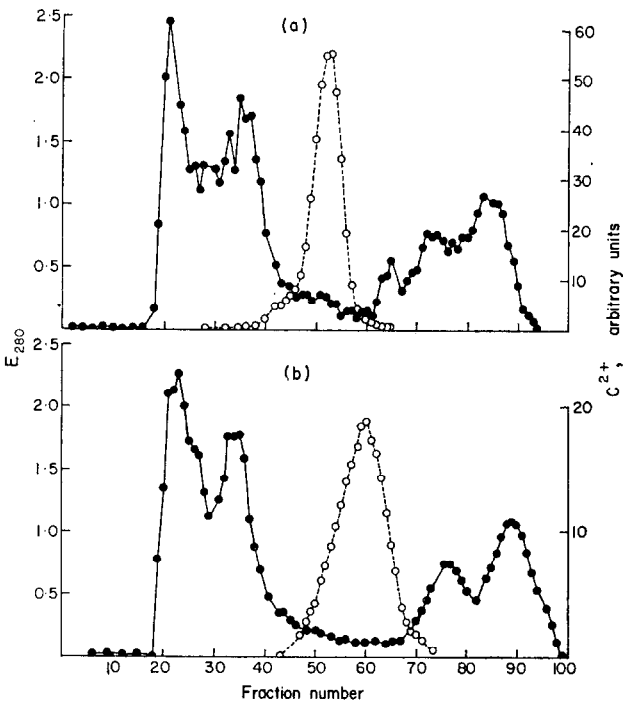

FIG. 1. Separation of the Cd^{2+}-binding protein from the cell sap of (a) liver and (b) kidney of the male rat, 72 hr after the subcutaneous injection of $CdCl_2$ (2·2 μmoles/100 g body wt). The cell sap fractions (15 ml) were concentrated approximately 4-fold, and applied to columns (70 × 4 cm) of Sephadex G 75. The columns were eluted with 154 mM NaCl at 4°, at a flow rate of 17·5 ml/hr. Fractions (5 ml) were collected and analysed for Cd^{2+} (○– – –○) and protein (E_{280}; ●——●).

oxyhaemoglobin (λmax 540–542 and 576–578 nm) as a single sharp peak, which coincided with a minimum in the E_{280} elution profile (Fig. 1a). After concentration of the combined Cd^{2+}-containing fractions, further purification was achieved by chromatography on two columns of DE 52 cellulose, the first being eluted with 20 mM phosphate buffer, pH 7·2, and the second with 1 mM, 250 mM and 1·0 M Tris–HCl buffers, pH 8·4, as described by Kägi and Vallee.[13,14] Although the Cd^{2+}-binding protein was eluted from the first column with the void volume (Fig. 2), contaminating proteins were retained and could be eluted subsequently with an increasing salt (NaCl) gradient in the phosphate buffer. On the second column, the binding protein behaved similarly to horse kidney metallothionein,[14] and was eluted as a sharp peak with the change from 1 to 250 mM Tris buffer.

In some earlier preparations in which larger volumes of liver cell sap were used, high molecular weight proteins were removed with little or no loss of Cd^{2+}, by a preliminary dialysis for 18 hr at 4° against an equal volume of a saturated solution of $[NH_4]_2SO_4$ adjusted to pH 7·2 with NH_4OH. After centrifugation, the supernatant solution was dialysed for 24 hr against three changes of 20 mM phosphate buffer, pH 7·4 (10 vol), concentrated about 7 times and fractionated as described above. Also effective was the fractionation method used by Kägi and Vallee[13] for the concentration of the Cd^{2+}-protein, metallothionein, from horse kidney. Later, it was found that the Cd^{2+}-binding protein of rat liver was heat-stable, and thus considerable purification was possible by the introduction of a heat-denaturation step (10 min at

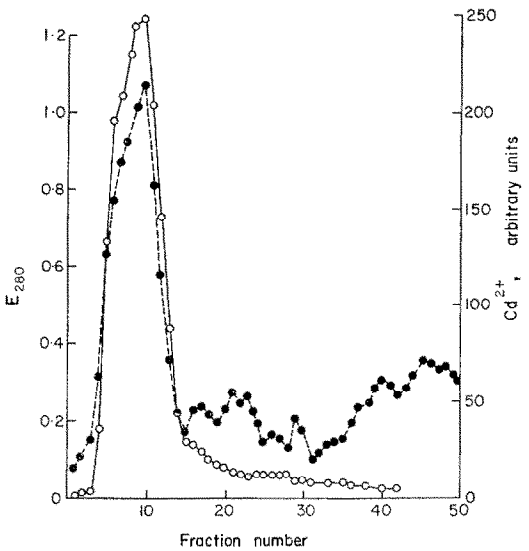

FIG. 2. Chromatography of a crude preparation of the Cd^{2+}-binding protein from rat liver on DE 52 cellulose. The column (10 × 1 cm) was eluted with 20 mM phosphate buffer, pH 7·2, at a flow rate of 12·5 ml/hr. Fractions (2 ml) were collected and analysed for Cd^{2+} (○——○) and protein (E_{280}; ●– – –●).

85°), which removed about 90 per cent of the total protein of the cell sap. During gel filtration of the 10–15 times concentrated supernatant fraction, light brown, rose-pink and yellow bands were resolved in this order on the column, the Cd^{2+}-binding protein being eluted immediately before the third coloured component.

Since this work was completed Shaikh and Lucis[15] have described the isolation of two Cd^{2+}-binding proteins from the soluble fraction of rat liver by gel filtration on Sephadex G 75 followed by chromatography on DEAE Sephadex with a tris buffer gradient (1–200 mM) as eluant. Each of these proteins appeared homogeneous on disk electrophoresis.

None of the procedures that were used in the present work, however, which were applicable also to the isolation of the binding protein of rat kidney, yielded preparations that were homogeneous. On disk electrophoresis of 100–200 μg samples of the proteins, for example, usually three major and three minor bands were resolved.

Effects of actinomycin D and cycloheximide on the synthesis of the binding protein

Actinomycin D. Male rats (250 g body wt) were injected i.p. every 24 hr for 5 days with actinomycin D (10 μg/100 g body wt), Cd^{2+} (2·2 μmoles/100 g body wt) being injected 2 hr before the last dose of the antibiotic and the animals killed 24 hr later.

Cycloheximide. Injections of the antibiotic (100 μg/100 g body wt) were made at 2 hr intervals over a period of 6 or 8 hr, Cd^{2+} (2·2 μmoles/100 g body wt) being injected at 0·5 hr and the animals killed at 8·0 or 8·5 hr.

RESULTS

Properties of the binding proteins from rat liver and kidney

Although the Cd^{2+}-binding proteins of liver and kidney were located similarly in the elution profiles that were obtained on gel filtration of cell sap preparations on either Sephadex G 200 or G 75 (e.g. Figs. 1a and 1b), no common protein component was observed on disk electrophoresis of the recovered fractions. Nevertheless, the partially purified preparations from both sources had properties in common with one another and also with metallothionein, the Cd^{2+}-containing protein of horse renal cortex, previously studied by Kägi and Vallee.[13,14] Both the liver and kidney proteins contained Zn^{2+} in addition to Cd^{2+}, together with small amounts of Cu^{2+}, but no detectable content of iron. The Cd^{2+} cation was more firmly bound than Zn^{2+} and was retained completely on dialysis of the preparations against 20 mM acetate buffer, pH 5·0, but was removed quantitatively by dialysis against either 0·3 mM *p*-chloromercuribenzoate in 20 mM sodium phosphate buffer, pH 7·4, or 50 mM glycine–HCl buffer, pH 2·2. After, but not before dialysis under the latter conditions, the protein from both tissues contained free —SH groups; 2·1 moles —SH/g atom bound cation being liberated for example, from a preparation (LS III) from male rat liver (14 weeks after the injection of Cd^{2+}) and which contained approximately equimolar concentrations of Zn^{2+} (2·8 per cent) and Cd^{2+} (5·1 per cent). On further dialysis of this preparation for 24 hr against acetate buffer, pH 6·7, the free —SH groups were oxidized, the capacity for Cd^{2+} re-binding being reduced in proportion to the loss of —SH. On a molar basis, binding of $^{63}Ni^{2+}$ by these "apoprotein" preparations, that contained free —SH groups, was twice as great as that of Cd^{2+}.

Under similar conditions of dialysis at pH 2, Kägi and Vallee[13] observed the liberation of three —SH groups/g atom $\Sigma Cd^{2+} + Zn^{2+}$ from metallothionein of horse kidney. These authors also record that approximately one in every three or four amino acid residues of this protein, which lacked tyrosine and tryptophane, was cysteine, whilst proline, serine and lysine were next on order of abundance.

Thus far the binding proteins from either rat liver or kidney have not been obtained sufficiently pure to warrant detailed amino acid analysis. All partially purified preparations that were examined, however, had a (bound) SH content equivalent to 7·5–10% cysteine, whilst a deficiency of aromatic amino acids was inferred from the absorption spectra, which fell continuously from 220–300 nm; with only a slight shoulder at 282·5 nm.

Cd^{2+}-binding proteins of the cell sap of normal male rat liver and kidney

Although Cd^{2+} (6·45 μg/ml) was accumulated when a cell sap preparation of normal male rat liver was dialysed to equilibrium against 50 μM Cd^{2+} in 0·2 M

acetate buffer, pH 6·0, the cation was not firmly bound to protein, and the elution profiles that were obtained by gel filtration of the sample on either Sephadex G 200 or G 75 were devoid of peaks corresponding to the Cd^{2+}-binding protein in the liver cell sap of Cd^{2+}-injected animals. The same results were obtained with protein fractions that were isolated from the cell sap of normal liver and kidney by precipitation with $[NH_4]_2SO_4$ as described by Kägi and Vallee[13] for the isolation of metallothionein. Both of these preparations bound Cd^{2+} when dialysed against an acetate-buffered (pH 6·0) solution of $CdCl_2$ (50 μM), but the cations were eliminated when the proteins were fractionated on Sephadex G 75.

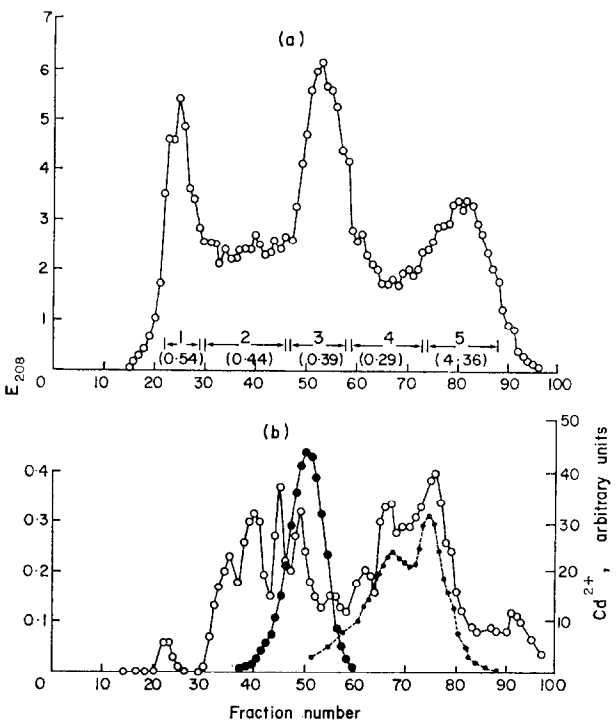

FIG. 3. Cadmium binding by the proteins of the cell sap of normal male rat liver. A concentrated preparation (13 ml) of the cell sap was subjected to gel filtration on a column (75 × 4 cm) of Sephadex G 200 with 154 mM NaCl as eluant at a flow rate of 16 ml/hr. Fractions (5 ml) were collected and, after being analysed for protein (E_{280} values; ○——○), combined to give the 5 samples as indicated in Fig. 3a. These were concentrated by dialysis against solid sucrose and portions of each then dialysed first against 0·1 M sodium acetate, adjusted to pH 6·5, until free from sucrose, then against equal volumes of 50 μM $CdCl_2$ in the same acetate solution for 48 hr, and finally against 3 changes, each of 2 l. of 0·02 M acetate, pH 6·5. The residual solutions were analysed quantitatively for protein and Cd^{2+}, the results (in parentheses) being expressed in μg Cd^{2+}/mg protein.

The remainder of fraction 4 (Fig. 3a) was treated with a solution of dithiothreitol (21 mg) in 60 mM Tris–HCl buffer, pH 7·8 (5 ml). After 30 min at room temperature, the solution was dialysed anaerobically against distilled water (2 × 2 l.) for 4 hr, then against 0·1 mM $(CH_3COO)_2Cd$ in 0·1 M acetate, pH 6·5 for 16 hr and finally against 3 changes, each of 1 l. of 0·1 M acetate pH 6·5 for 24 hr. The final solution (24·5 ml) was concentrated against sucrose and fractionated (Fig. 3b) by gel filtration on a 22×2 cm column of Sephadex G 75 with 154 mM NaCl as eluant. The eluted fractions (2 ml vol) were analysed for protein (E_{280}; ○——○) and Cd^{2+} (●– – –●). The figure also shows the elution profile of a sample of a preparation of the binding protein from the Cd^{2+}-injected rat, when run on the same column and analysed for Cd^{2+} only (●——●).

In a further experiment, a preparation of the cell sap from normal male rat liver was separated by gel filtration on Sephadex G 200 into five fractions (Fig. 3a). Of these, the fifth bound the highest concentration of Cd^{2+} (4·36 μg Cd^{2+}/mg protein), whereas fraction four which, in its position in the elution profile, corresponded to the Cd^{2+}-binding protein in the cell sap of the liver of the Cd^{2+} injected animal (cf. e.g. Fig. 1a), bound the least (0·29 μg Cd^{2+}/mg protein). As —SH groups are involved in Cd^{2+}-binding, the remainder of fraction four was reduced with dithiothreitol before addition of Cd^{2+}. After this treatment two Cd^{2+}-containing components were separated by gel filtration of the product on Sephadex G 75 (Fig. 3b). Neither of these, however, was identical with the authentic Cd^{2+}-binding protein (Fig. 3b). These observations indicated that the Cd^{2+}-binding protein, at least in rat liver, was not a normal tissue component, but was synthesized (induced) in response to the uptake of the toxic cation. Evidence in support of this conclusion was obtained by the results summarized in the following sections.

Cd^{2+}-binding in relation to the distribution of Zn^{2+} in the soluble fractions of rat liver and kidney

As mentioned above, the Cd^{2+}-binding proteins of liver and kidney also contained considerable amounts of Zn^{2+}. In untreated, control animals a sex-linked difference was observed in the distribution of Zn^{2+} in the liver cell sap. Gel filtration on Sephadex G 75 of the soluble liver proteins of the normal male rat, for example, gave three major Zn^{2+} containing fractions (I, II and III, Fig. 4a). These were present also in the cell sap from the liver of the female rat, together with an additional minor component (Zn^{2+}-protein IV, Fig. 4b), the location of which in the elution profile was coincident with that of the Cd^{2+}-binding component of the liver cell sap from both the Cd^{2+} injected male (Fig. 1a) and female (Fig. 5).

As shown in Fig. 5, accumulation of Cd^{2+} in the liver of the female rat in response to the subcutaneous injection of $CdCl_2$, also led to an increase in Zn^{2+}-content of the Zn^{2+}-protein IV fraction. Thus 24 hr after the injection of Cd^{2+} the Zn^{2+} content (at the maximum of the Zn^{2+}-protein IV peak in the elution profile) was increased from 0·58 to 1·90 μg Zn^{2+}/mg protein, whilst the ratio (by weight) of Cd^{2+}:Zn^{2+} was approximately 2:1. After 48 hr (not shown in the figure) the Zn^{2+} content was increased relative to that of Cd^{2+} (Cd^{2+}:Zn^{2+} = 1·2:1) and after 23 days the contents of Zn^{2+} (3·65 μg Zn^{2+}/mg protein) and of Cd^{2+} (3·78 μg Cd^{2+}/mg protein) were almost equal. Although in these experiments the protein contents of the extracts applied to the Sephadex columns were only approximately the same, the results of Fig. 5 show that with the uptake of Zn^{2+} by the Cd^{2+}-binding component, the Zn^{2+}-contents of the other Zn^{2+} containing proteins (e.g. II and III) of the cell sap decreased. A very pronounced fall in the Zn^{2+} contents of these proteins was observed also in the liver cell sap of female rats bearing primary rhabdomyosarcomata, that had been induced by intramuscular implants of finely powdered metallic cadmium. The accumulation of excessively high concentrations of Cd^{2+} in the livers and kidneys (e.g. 400 and 180 μg Cd^{2+}/g wet wt tissue, respectively) of these animals, in which there is a continual slow feed of Cd^{2+} into the body fluids as the metallic implants dissolve, has been described by Heath and Webb.[9] Further examination of the cell sap from the livers of these animals showed that Cd^{2+} was bound by the same protein fraction (i.e. Zn^{2+}-protein IV) as in the livers of rats injected subcutaneously with

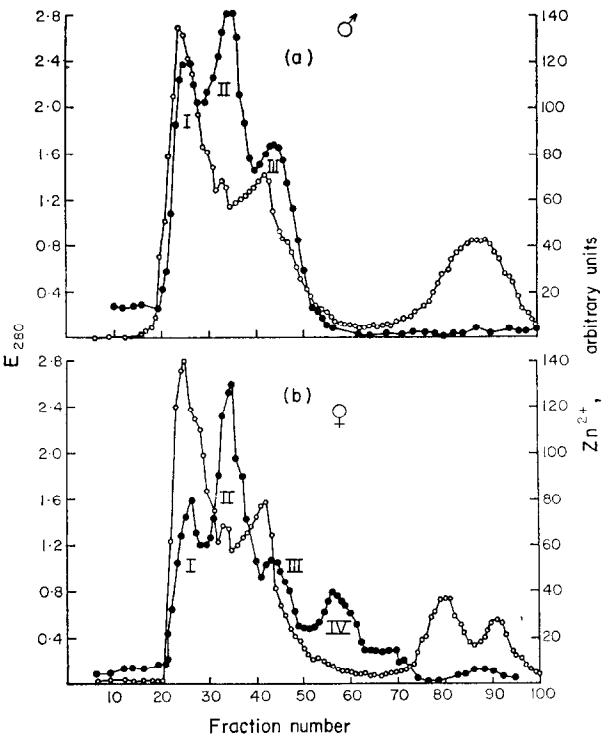

FIG. 4. Distribution of Zn^{2+} in the cell sap fractions of (a) male and (b) female rat liver. The tissues were homogenized in 3 vol. medium A and the concentrated cell sap fractions (10 ml; see Materials and Methods section) applied to 75 × 4 cm columns of Sephadex G 75. The columns were eluted with 154 mM NaCl, the eluted fractions (5 ml) being analysed for Zn^{2+} (●——●) and protein (E_{280} ○——○).

ionic Cd^{2+}. Accumulation of Cd^{2+} (8·7 μg/mg protein) was accompanied by a large increase in the Zn^{2+}-content of this fraction (e.g. 11·9 μg Zn^{2+}/mg protein) and the binding of Cu^{2+} (0·7 μg/mg protein) but not of iron. In contrast to the liver, neither the Zn^{2+}-protein IV, nor an analogous Cd^{2+}-binding protein was present in the soluble fraction of the metal-induced tumours.

The Zn^{2+}-protein IV, although absent from the cell sap of the normal male rat, was present in the livers of animals after injection of additional Zn^{2+} (Fig. 6). A preparation of this component was isolated, 72 hr after injection of Zn^{2+}, by fractionation of the liver cell sap first on Sephadex G 75 and then on DE 52 cellulose. At the same time the Cd^{2+}-binding protein, which also contained Zn^{2+}, was separated by the same methods from the liver cell sap of rats injected 72 hr previously with Cd^{2+}. On gel filtration of a mixture of the two on Sephadex G 75, a single peak of Zn^{2+} was obtained (Fig. 7). Furthermore, disk electrophoresis showed that both fractions had the same protein composition. A difference was observed, however, in the apparent stability of the binding protein when induced by the two cations. Thus, in male rats that had been injected 14 months previously with Zn^{2+}, Zn^{2+}-protein IV was absent from the liver cell sap, whereas it was still present, and contained both bound Cd^{2+} and Zn^{2+}, in animals that had been injected at the same time with Cd^{2+}.

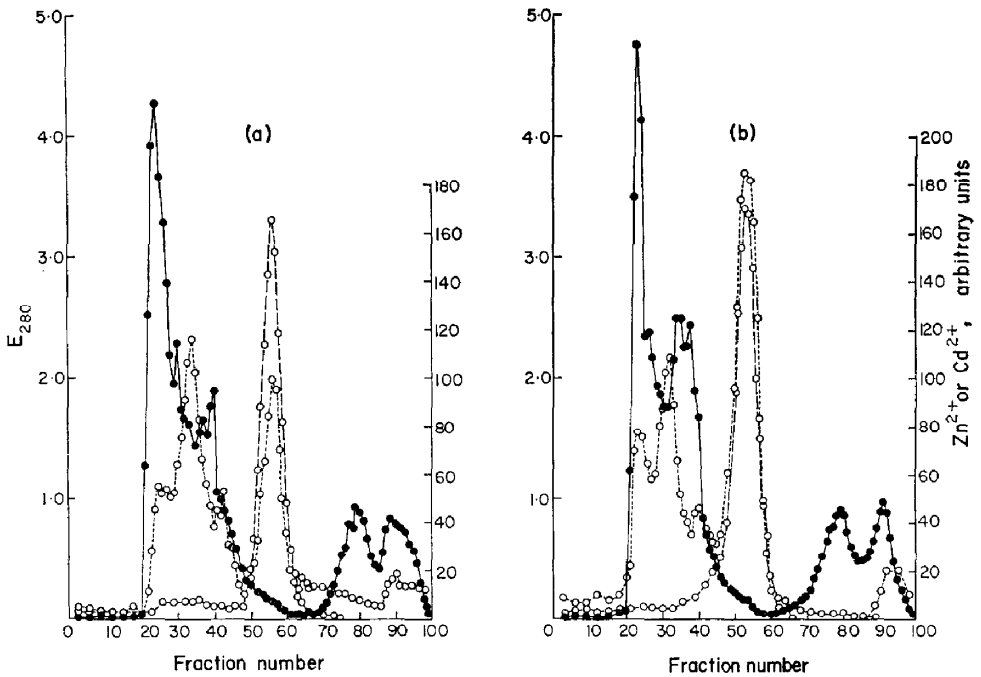

FIG. 5. Distribution of Zn^{2+} and Cd^{2+} in the cell sap fractions from the livers of female rats (a) 24 hr and (b) 23 days after the subcutaneous injection of $CdCl_2$ (2·2 μmoles/100 g body wt). The tissue extracts were prepared and fractionated on columns of Sephadex G 75 as described in the legend of Fig. 4. The eluted fractions were analysed for protein (E_{280} ●——●), Zn^{2+} (○.....○) and Cd^{2+} (○– – –○). The results of the metal analyses are given in arbitrary units (per cent absorption), the absorption of a standard solution of Zn^{2+} (2·5 μg/ml) being 1·25 times greater than that of a standard solution of Cd^{2+} of the same concentration.

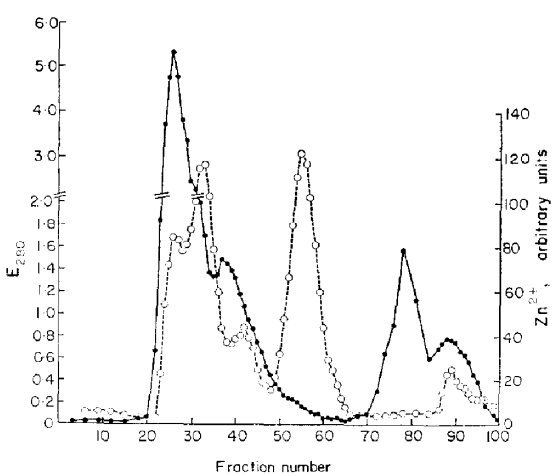

FIG. 6. Distribution of Zn^{2+} in the cell sap fraction of the liver of the male rat 72 hr after the intraperitoneal injection of $(CH_3COO)_2Zn$ (6·4 μmoles/100 g body wt). A 3-fold concentrated sample (10 ml) of the cell sap (see Materials and Methods section) was applied to a 75 × 4 cm column of Sephadex G 75. The column was eluted with 170 mM NaCl, fractions (5 ml) being collected at a flow rate of 18·0 ml/hr and analysed for protein (E_{280} ●——●) and Zn^{2+} (○– – –○).

Effects of actinomycin D and cycloheximide on the accumulation and binding of Cd^{2+} in rat liver

In experiments in which rats were injected daily for 3–5 days with actinomycin D (80 μg/kg body wt) and acetoxycycloheximide (250 μg/kg body wt), 0·5 and 4 hr, respectively, before the injection of Cu^{2+} (1·25–2·5 mg Cu^{2+}/kg body wt/day) Gregoriadis and Sourkes[16] observed that the Cu^{2+} content of the liver was increased relative to the appropriate controls, and concluded that protein synthesis was neces-

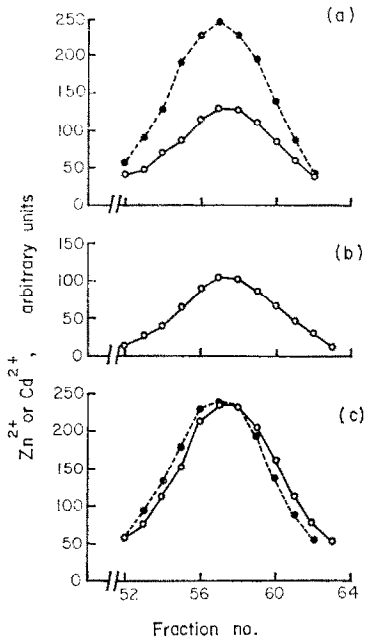

FIG. 7. Identity of Zn^{2+}-protein IV and the Cd^{2+}-binding protein. Preparations of Zn^{2+}-protein IV and the Cd^{2+}-binding protein were isolated from the livers of male rats 72 hr after the subcutaneous injection of $(CH_3COO)_2$ Zn (6·4 μmoles/100 g body wt) and $CdCl_2$ (2·2 μmoles/100 g body wt), respectively, by gel filtration on Sephadex G 75, as shown in Figs. 6 and 1a. Both preparations were purified by chromatography on DE52 cellulose (see Fig. 2) and after concentration, were applied separately (Fig. 7a and 7b) and admixture (Fig. 7c) to a column (22 × 2 cm) of Sephadex G 75. Fractions (2 ml), eluted with 1% (w/v) NaCl, were analysed for Cd^{2+} (●– – –●) and/or Zn^{2+} (○——○). (a) Cd^{2+}-binding protein. (b) Zn^{2+}-protein IV. (c) Mixture of the same volumes of (a) and (b).

sary for the removal of the cation from the tissue. In rat liver, however, although the effects of inhibitory levels of actinomycin D on RNA synthesis persist for at least 24–36 hr,[17] inhibition of amino acid incorporation into protein by acetoxycycloheximide and by cycloheximide are maximal at 30 min to 2 hr after injection and then decrease until, at 12 hr, protein synthesis is normal.[18] It seems, therefore, that the conclusions of Gregoriadis and Sourkes,[16] at least with regard to the effects of acetoxycycloheximide, may be invalid since, in these animals, protein synthesis in the liver probably would have followed a cycle of inhibition and recovery during each 24 hr period. The present experiments, therefore, although modelled on those of Gregoriadis

and Sourkes,[16] were done with the modifications described in the Materials and Methods section. Under these conditions, after treatment with actinomycin D and Cd^{2+}, the main wet wt of the livers was 40 per cent less than that of control animals injected either with saline, or with Cd^{2+} only, whereas the rate of amino acid incorporation into protein of the liver tissue *in vitro* was 30 per cent greater. The concentration of Cd^{2+} in the cell sap fraction of the livers of the actinomycin D-treated animals (0·45 μg Cd^{2+}/mg protein) was also greater than that (0·30 μg Cd^{2+}/mg protein) from the livers of rats injected with Cd^{2+} only. Both preparations, however, gave the same elution profile on gel filtration on Sephadex G 75, the cation being located in a single peak, characteristic of the binding protein.

In contrast, the rate of ^{14}C-L-leucine incorporation into liver protein *in vitro* was reduced by 92 per cent relative to that in control tissue by treatment of the animals with cycloheximide. This essentially complete inhibition of protein synthesis did not prevent the accumulation of Cd^{2+} in the liver. In one experiment, for example, cell sap preparations from 1:3 homogenates of livers of Cd^{2+}-injected control and cycloheximide-treated rats contained 5·4 μg Cd^{2+}/ml (0·24 μg Cd^{2+}/mg protein) and 3·8 μg Cd^{2+}/ml (0·15 μg Cd^{2+}/mg protein), respectively. As shown by these figures, the protein content of the soluble fraction of the livers of the rats injected with Cd^{2+} and cycloheximide was greater than that of animals given Cd^{2+} only. This difference was due, at least in part, to increased amounts of haemoglobin in the former. This was shown by measurements of E_{576}, or of E_{540}, which were taken as a rough estimate of (oxy-)haemoglobin contents. Thus the E_{576} values of liver cell sap preparations from control rats, animals injected with Cd^{2+}, cycloheximide, and Cd^{2+} + cycloheximide, respectively were in the ratio: 1:1·27:1·83:3·30.

Although Cd^{2+} accumulated in the soluble components of the liver of the cycloheximide-treated rat, 75–90 per cent of the bound cation was removed with the coagulated protein on heat-denaturation (85°/15 min). Gel filtration of the original cell sap preparation also showed that, in contrast to the soluble fraction of the livers of animals injected with Cd^{2+} only, the cation was not bound by a single protein of low molecular weight (i.e. the specific binding protein), but was distributed amongst the larger proteins, roughly in parallel to that of Zn^{2+} (Fig. 8, graph 1; cf Fig. 4). It was not possible to determine whether uptake of Cd^{2+} under these conditions, however, caused the displacement of Zn^{2+} from the soluble Zn^{2+}-metalloproteins of the cell sap, since the protein composition of this fraction was very different from that of the liver of the normal animal.

These results, which showed that the production of the specific binding protein in response to the injection of Cd^{2+} did not occur in liver when protein synthesis was blocked by cycloheximide, could be due to the inhibition of the synthesis of either an induced protein, or of enzymes, normally functional in the hydrolysis of Cd^{2+}-carrier proteins. It was possible, for example, that, in the normal animal, Cd^{2+} was transported as a complex with protein of high molecular weight, which was hydrolysed in the liver by lysosomal or other proteinases to yield the heat-stable, binding protein. Inhibition of the activities or synthesis of these enzymes, therefore, might lead to an accumulation of Cd^{2+} in the large protein fraction of the cell sap.

Four 2-hr injections of cycloheximide (1 μg/g body wt) into male rats caused a slight reduction (about 20 per cent) in the neutral proteinase activity, but had no effect on the cathepsin D activity (136 units/g wet wt) of the liver. Although extracts of the

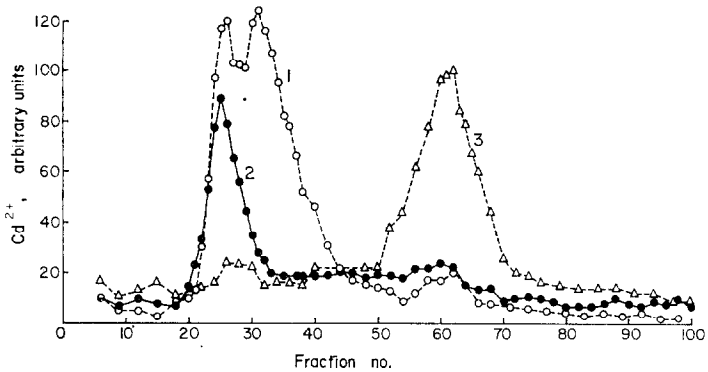

FIG. 8. Inhibition of the synthesis of the Cd^{2+} binding protein by cycloheximide. Four groups of male rats (220–230 g body wt) were injected with (a) cycloheximide (200 μg/ml in 154 mM NaCl; 1 μg/g body wt) at 0, 2, 4 and 6 hr and Cd^{2+} (2·2 μmoles/100 g body wt) at 0·5 hr; (b) cycloheximide at 0, 2, 4 and 6 hr; (c) 154 mM NaCl (0·5 ml/body wt) at the same times, and (d) Cd^{2+} (2·2 μmoles/100 g body wt) at 0·5 hr. All animals were killed at 8 hr. Liver tissue from rats of groups (b) and (c) was homogenized in 3 vol. 154 mM NaCl and the homogenates used for the preparation of proteolytically active extracts (see Materials and Methods section). Cell sap fractions were prepared from homogenates of the livers of animals of groups (a) and (d) in 3 vol. medium A. Portions of these fractions were mixed with equal volumes of extracts (b) and (c) and after the addition of NaN_3 (0·2 mg/ml), incubated for 4 hr at 37°. The pH of each mixture then was adjusted to 5·2 and incubation continued for a further 21 hr. The digests and equivalent volumes of the original extracts (a) and (d) were concentrated by dialysis against solid sucrose and a suitable volume of each ($\equiv$ 10–12 μg Cd^{2+}) applied to a column (22 × 2·4 cm) of Sephadex G 75. The columns were eluted with 154 mM NaCl, fractions of 1·0 ml being collected and analysed for Cd^{2+}.

The graphs show the distribution of Cd^{2+} in the liver cell sap of the cycloheximide-treated rat (1) before (○– – –○) and (2) after (●——●) incubation with the extract of the livers of control (saline-injected) rats (extract c), and (3) in the liver cell sap of the Cd^{2+}-injected rat after incubation under the same conditions (△– – –△). Identical results were obtained when extract (b) was used in place of extract (c).

livers of both these animals and of normal males catalysed the hydrolysis of one of the high molecular weight, Cd^{2+} containing protein fractions of the liver cell sap from the Cd^{2+}—and cycloheximide—treated rats, products similar to the Cd^{2+} binding protein of normal liver were not formed (Fig. 8, graph 2). Instead, all polypeptide-bound Cd^{2+} was diffusible and was eliminated during the concentration of the enzyme-digests. The authentic Cd^{2+}-binding protein, however, was resistant to attack by these enzymes and was recovered, apparently unchanged after incubation with them (Fig. 8, graph 3).

Specificity of the binding protein. The invariable presence of Zn^{2+} in the Cd^{2+}-binding protein, and the identity of the latter with Zn^{2+}-protein IV (Figs. 6 and 7), provided useful markers to determine whether the same protein was synthesized in response to other cations, particularly those such as Ni^{2+}, Co^{2+} and Pb^{2+} for which atomic absorption analysis had lower sensitivity.

The absence of Zn^{2+}-protein IV from the cell sap of the liver of the male rat 24 or 48 hr after the subcutaneous injection of either Ni^{2+} (1·25 mg) or Co^{2+} (2·5 mg) for example, was good evidence that these cations were not accumulated in the liver in the same way as was Cd^{2+}. This was confirmed by concentration and analysis of the combined eluate fractions (e.g. fractions 47–57 in Fig. 1a) from the Sephadex G 75 columns that would be expected to contain the binding protein. Neither cation was

detected, and only the first protein fraction to be eluted from these columns (e.g. fractions 18–24 in Fig. 1a) contained measurable amounts of Co^{2+} (0·033 μg/mg protein) and Ni^{2+} (0·024 μg/mg protein) Also, Zn^{2+}-protein IV was not present in the livers of male rats that either had ingested 1% (w/v) lead acetate in the drinking water for 3 months, or had been injected 48–72 hr previously with $(CH_3COO)_2$ Pb (1 μmole/100 g body wt). In further experiments male rats were injected first with Zn^{2+} or Cd^{2+}, to stimulate the production of the binding protein in the liver and then, after a further 2–4 days, with either Co^{2+} or Ni^{2+}. Even under these conditions only low amounts of the latter cations were found in the Cd^{2+} or Zn^{2+}-labelled binding protein (e.g. 0·06–0·08 μ atom Co or Ni/μ atom Cd or Zn), when these were isolated 24–48 hr later.

DISCUSSION

After a single subcutaneous injection of Cd^{2+} into the adult rat the cation accumulates, at first rapidly, and then more slowly in the liver and kidney, maximum concentrations being reached after about 13–17 weeks.[5] In both organs most of the bound Cd^{2+} is found in a single fraction of the cell sap (Fig. 1). Although this fraction also binds Zn^{2+}, Cd^{2+} neither displaces this cation, nor is concentrated in any of the normal Zn^{2+} metalloproteins of the cell sap (Fig. 1a, cf. Fig. 4a).

The proteins of liver and kidney that are responsible for the binding of Cd^{2+} and also additional Zn^{2+}, although not identical, have properties in common with one another and with metallothionein, a Cd^{2+}-containing protein from horse kidney.[13,14] In particular, the binding proteins are deficient in aromatic amino acids and after, but not before the removal of the bound cations are rich in —SH groups. Kägi and Vallee[13,14] found that three —SH groups appeared to be involved in the binding of each metal ion by metallothionein, whereas the present results suggest that the cation binding site of the rat liver protein contains only two. The fact that the metal-free apoprotein binds twice as much Ni^{2+} as Cd^{2+} indicates that only one of these groups is necessary for chelation of the former cation, whereas both are required for the specific binding of the latter.

The binding proteins of both rat liver and kidney are heat-stable and can be separated from about 90 per cent of the total protein of the cell sap by an initial thermal denaturation step. In this respect, these proteins resemble ferritin, the iron-storage protein of liver, the isolation of which includes a similar heat treatment.[19] The Cd^{2+} binding protein, however, is separated completely from ferritin by gel-filtration, and even the partially purified preparations from rat liver do not contain iron.

Although the function of metallothionein was not defined by Kägi and Vallee,[13,14] the subsequent isolation of similar proteins from human kidney,[20] from the livers of Cd^{2+}-injected rabbits,[4] and from the livers and kidneys of rats after the intravenous injection of $^{203}HgCl_2$ (Ref. 21) suggests that they can act as defence mechanisms against certain heavy metals. These proteins, however, are not general scavengers of toxic metals, since ions such as Ni^{2+}, Co^{2+} and Pb^{2+} are not accumulated by them.

Recently, Shaikh and Lucis[22] have shown that, within limits, the contents of Cd^{2+}-binding proteins in the livers and kidneys of adult rats increase according to the dose of subcutaneously injected $CdCl_2$. The present results indicate that these binding proteins are not normal components of these tissues, but are synthesized in response to the uptake of the cation. The evidence for this is as follows;

(1) The presence of proteins with high binding affinity for Cd^{2+} has not been demonstrated in cell sap fractions of the normal tissues.

(2) The binding proteins when induced by Cd^{2+}, also bind Zn^{2+} and, on gel filtration of the liver or kidney cell sap, can be detected equally well by the presence of an additional Zn^{2+} peak (e.g. Zn^{2+}-protein IV: Figs. 5 and 6) in the elution profile, as by the presence of Cd^{2+} (Fig. 1a). This Zn^{2+}-protein IV is not present in the cell sap of normal male rat liver (Fig. 4a), although it does occur in low concentration in the liver of the female (Fig. 4b). This apparent sex-linked difference in the distribution of Zn^{2+} in the liver cell sap of the male and female animal may be related to the greater requirements of the former for this cation.[23] Further work is necessary however, to establish this, since the present experiments were done at different times and the dietary intake of Zn^{2+} was not controlled.

(3) In contrast to the results of Shaikh and Lucis,[22] injection of excess Zn^{2+} into male rats causes the appearance of Zn^{2+}-protein IV in the liver (Fig. 6). The subsequent injection of Cd^{2+} into these animals is followed by the accumulation of Cd^{2+} in this protein. Furthermore, animals that contain Zn^{2+}-protein IV in the liver, have increased resistance to the selective toxic effects of the Cd^{2+}-cation.[24]

(4) The production of the Cd^{2+}-binding protein *in vivo*, although unaffected by actinomycin D, is inhibited when protein synthesis is blocked by cycloheximide. Under the latter conditions Cd^{2+} still accumulates in the liver cell sap, but is distributed differently; the cation being bound by proteins of high molecular weight. This distribution is similar to that observed by Nordberg, Piscator and Lind[25] in the liver proteins of mice at short times after the subcutaneous injection of $CdCl_2$ (3 mg Cd^{2+}/kg body wt). One of these larger proteins may be haemoglobin, the content of which is increased in the liver of the cycloheximide-treated, Cd^{2+}-injected rat. In this connection it may be significant that, in the rabbit, Cd^{2+} is transported mainly by the erythrocyte, in which it is bound to haemoglobin.[26] The possibility that the binding protein is a stable Cd^{2+}-containing product of the degradation by lysosomal proteinases of either a carrier, such as haemoglobin, or a primary acceptor protein of high molecular weight, however, seems unlikely, since the activities of these enzymes are unaffected by cycloheximide under the conditions of the present experiments. Thus, although conclusions based on negative results with actinomycin D are open to criticism, it seems from these observations that the Cd^{2+}-binding protein is inducible, and its synthesis is controlled at the translational rather than the transcriptional level. A similar inference has been drawn with regard to ferritin, the synthesis of which is stimulated greatly by high concentrations of serum iron,[27,28] and is inhibited by cycloheximide, but not by actinomycin D.[29] As has been discussed by Miller *et al.*,[29] ferritin acts not only as an iron storage protein, but also as a mechanism for the regulation of iron adsorption and protection against the toxic effects of the "free" cation. It is possible that the Cd^{2+}-binding protein normally functions in a similar way in the control of Zn^{2+} metabolism, and that the induction of this protein by Cd^{2+} and Hg^{2+} (Ref. 21) may be a fortunate consequence of the similarities in chemical properties of these three cations. Persistence of Cd^{2+}, but not of excess Zn^{2+} in the liver, thus could be due to the inhibition by the former cation of the turnover of this protein.

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