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STUDIES ON THE TOXICITY AND METABOLISM OF CADMIUM-THIONEIN

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Abstract—Cadmium, when bound to thionein from either rat or rabbit liver, was 7 to 8 times more toxic for the rat than was ionic Cd^{2+}. Zinc-thionein was not only non-toxic at a dose level of 2.4 mg protein-bound-Zn^{2+}/kg, but also protected the animals against a subsequent, normally lethal dose of cadmium-thionein. In contrast with the free cation, Cd^{2+} administered intravenously as the metallothionein accumulated to its highest concentration in the kidney and, at a lethal dose, caused severe tubular damage. After administration of non-lethal doses of $^{109}Cd^{2+}$-labelled cadmium-thionein to rats thionein-bound-$^{109}Cd^{2+}$ accumulated in the kidneys, but in animals that were dosed with either the ^{35}S- or ^{3}H-labelled metalloprotein, little or none of the radioactive isotope was recovered in the renal metallothionein at 48 hr. The ^{35}S and ^{3}H isotopes, however, were incorporated into high molecular weight proteins of the kidney soluble fraction and also were excreted in the urine, both as the metallothionein and as smaller, diffusible molecules. *In vitro*, ^{3}H-labelled cadmium-thionein was degraded to acid-soluble products by homogenates of rabbit kidney cortex. It is suggested that parenterally administered cadmium-thionein is taken up by the renal tubules and catabolized, probably by the lysosomes of the tubular cells, with the liberation of Cd^{2+} ions. These cations, if present in sufficiently high concentration, cause acute renal damage. Exposure of rats, with high hepatic concentrations of cadmium-thionein, to the hepatotoxins, carbon tetrachloride and retrorsine, did not cause the transfer of Cd^{2+} from the liver to the kidney.

Since appreciable amounts of Cd^{2+} can be stored in the mammalian liver and kidney as cadmium-thionein without toxic manifestations [1], concentrations of the inducible metalloprotein below certain limits usually are regarded as biologically inert. Recent reports, however, have indicated that Th-Cd^{2+}*, when administered to the rat [2] and mouse [3], is 5–7 times more toxic than the "free" cation. Also when given in this protein-bound-form, Cd^{2+} accumulates preferentially in the kidney, where it produces severe tubular damage. Nordberg *et al.* [3] conclude that this renal damage is the main cause of death, and regard the accumulation of Cd^{2+} in the kidney to be in agreement with an earlier hypothesis of Piscator [4], that cadmium-thionein is synthesized in the liver, transported in the blood and accumulated by glomerular filtration followed by tubular reabsorption in the kidney. In apparent agreement with this hypothesis Tanaka *et al.* [5] observed rapid accumulation of thionein-bound $^{109}Cd^{2+}$ in the kidneys of rats after injection of $^{109}Cd^{2+}$-labelled cadmium-thionein. In short-term studies, with appropriately labelled preparations of rat liver cadmium-thionein, Cherian and Shaikh [6], however, observed some catabolism of the protein within 3 hr. As a result of this degradation there was unequal incorporation of the Cd^{2+} and protein moieties of the administered hepatic cadmium-thionein into the renal metalloprotein.

The results of the present work confirm the high toxicity of the metalloprotein, but suggest that Cd^{2+}, liberated from the latter, is the renotoxic agent. The catabolism of an intravenously administered, non-lethal dose of cadmium-thionein, as implied by the short-term experiments of Cherian and Shaikh [6], appears to be complete within 48 hr, and only the Cd^{2+} of this metalloprotein is retained in the metallothionein of the kidney.

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* Abbreviation: Th-Cd^{2+} = thionein-bound-Cd^{2+}.

MATERIALS AND METHODS

Chemicals. Radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks, Sephadex G75 and DEAE Sephadex A-25 from Pharmacia (Great Britain) Ltd., 75 Uxbridge Road, London W5 5SS, BioGel P10 from Bio-Rad Laboratories, Valley Road, St. Albans, Herts, and polyethyleneglycol (mol. wt 6000) from Koch–Light Laboratories Ltd., Colnbrook, Bucks, SL3 OBZ. Retrorsine was provided by Dr. A. R. Mattocks of this Unit.

Analytical methods. Protein was estimated by a Biuret method [7] with bovine serum albumin as standard. Thiol groups were detected with Ellman's reagent [8]. The Cd^{2+} and Zn^{2+} cations were determined by atomic absorption, tissue samples being digested by the method of Thompson and Blanchflower [9]. Urine was analysed for Cd^{2+} by the method of additions [10]. Radioactive isotopes in solution were measured in a Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 3324), the samples being incorporated into either Instagel (Packard Instrument Co., Caversham, Bucks), or a toluene-2-ethoxyethanol solution of chemical scintillators [11]. Corrections for colour-quenching of ^{35}S and ^{3}H activities in urine samples were determined by the channels-ratio method. Contents of $^{115m}Cd^{2+}$ in whole organs were determined in a J & P Modular

Counter (J & P Engineering (Reading) Ltd., Cardiff Road, Reading, Berks).

Induction and isolation of metallothioneins. Cadmium-thionein was induced in the livers of female Wistar rats (200–250 g body wt) and rabbits (New Zealand White × Sandy Half Lop, first generation progeny; 2.5–3.5 kg body wt) by repeated subcutaneous (s.c.) injection every 48 or 72 hr of increasing doses (0.5–4.0 mg/kg body wt) of Cd^{2+} as a solution of $CdCl_2$ made isotonic with NaCl over periods of 3–6 weeks. The animals were killed by either cervical dislocation (rabbits) or decapitation (rats), usually 48 hr after the last injection of the cation, their liver (200–300 μg Cd^{2+}/g wet wt) being homogenized, in 100–150 g portions, with 2 vol of 20 mM Tris–HCl buffer, pH 8.0 in a Waring blender at 4° for about 2 min. Each homogenate was centrifuged at 10,000 *g* for 10 min, the supernatant solution being treated with ethanol and chloroform [12] to remove haemoglobin and other proteins of higher mol. wt. The ethanol concentration then was increased to 75% (v/v) to precipitate the metallothionein fraction. This was dissolved in 10 mM Tris–HCl buffer, pH 8.0 (10 ml), and dialysed for 20 hr at 4° against two changes of the same buffer (2 l) to remove ethanol, then against polyethyleneglycol at 4° to reduce the volume to about 5 ml. Cadmium-thionein was recovered from this solution by gel filtration on Sephadex G75 (see Fig. 1) and was purified by further gel filtration on Bio-Gel P10 (Fig. 2; see Bremner and Davies [12]). The product (e.g. peak c in Fig. 2) was concentrated, de-salted by dialysis against distilled water and frozen-dried.

Preparations of rabbit liver zinc-thionein (5.5% Zn^{2+}) and of cadmium-thionein, in which the apoprotein was labelled with either ^3H or ^{35}S, were isolated by the same method after the administration of either Zn^{2+} (4 × 4.0 mg Zn^{2+}/kg, as $(CH_3COO)_2Zn$ at 24-hr intervals), or two s.c. doses of Cd^{2+} (1.5 mg/kg) followed intravenously (i.v.), 3 hr after the second dose of Cd^{2+}, by (a) L-[^{35}S]cysteine hydrochloride (150 μCi/μmole; 250 μCi/ml; 1.0 ml), (b) L-[3,3′-^{3}H]-cystine hydrochloride (630 μCi/μmole; 1 mCi/ml; 0.25 ml) or (c) L-[U-^{3}H]proline (500 μCi/μmole; 600 μCi/ml; 1.0 ml). These animals were killed at 20 hr (group a), 5 hr (group b) and 17 hr (group c) after the administration of the labelled amino acids. Yields and specific activities were as follows:

Preparation	Labelled precursor amino acid	Yield: Protein (mg)	Yield: Thionein-bound Cd^{2+} (μg)	Sp. act. (cpm/μg Cd^{2+})
a	L-[^{35}S]cystine	5.35	450	2050
b	L-[3,3′-^{3}H]cystine	9.70	800	1230
c	L-[U-^{3}H]proline	8.49	680	300

For use in some of the following experiments, these preparations were supplemented with unlabelled cadmium-thionein.

$^{115m}Cd^{2+}$- and $^{109}Cd^{2+}$-labelled cadmium-thionein (950 and 5000 cpm/μg Cd^{2+}) were prepared from the livers of female rats (250 g body wt) that were dosed s.c. with Cd^{2+} (1.0 mg/kg body wt), followed after 4 days by the i.v. injection of $^{115m}Cd^{2+}$ (20 μCi and 250 μg Cd^{2+}/animal) or $^{109}Cd^{2+}$ (25 μCi and 250 μg Cd^{2+}/animal), the animals being killed after a further 24 hr.

Metabolism of cadmium-thionein in vivo. The labelled preparations of cadmium-thionein were injected i.v. as solutions in 0.15 M NaCl into either rats or rabbits at the dose levels given in the "Results" section. The rats were kept in metabolism cages, for the collection of two 24-hr samples of urine, and then were killed. Rabbits were killed after 48 hr. Soluble fractions were prepared from homogenates of the kidneys (or of the kidney cortex) in 3 vol, 20 mM Tris–HCl buffer, pH 8.0, and were fractionated by gel filtration on columns (85 × 2.3 cm) of Sephadex G75 at a flow rate of 17 ml/hr. In one experiment (see "Results" section) the concentrated non-diffusible components of the urine also were fractionated by gel filtration on a column (85 × 1.5 cm) of Sephadex G75 at a flow-rate of 12 ml/hr.

Metabolism of rabbit liver cadmium-thionein by rabbit kidney cortex in vitro. Cortical tissue from the kidneys of an adult female rabbit was homogenised at 0° in 8 vol of Krebs–Ringer-phosphate (pH 6.0) in a motor-driven homogenizer [14]. Portions of the homogenate (4.5 ml), with (experimental) and without (control) the addition of a solution of [3,3′-^{3}H]cysteine-labelled cadmium-thionein (984 cpm/μg Cd^{2+}; 80 μg Cd^{2+}/ml; 0.5 ml), were shaken at 37° with free access of air, the pH of each suspension being maintained between 5.8 and 6.4 by the addition of 0.1 N NaOH. At intervals, experimental and control suspensions were transferred to ice and the metallothionein solution (0.5 ml) added to the latter. The suspensions were centrifuged and portions (2.1 ml) of the supernatant solutions were treated with a 20% (w/v) aqueous solution of trichloroacetic acid (0.7 ml) and centrifuged after 15 min at 0°. Samples of the supernatant solutions (1.5 ml) were treated with concentrated HCl (0.05 ml) and extracted three times with water saturated ether (4.0 ml) to remove trichloroacetic acid. After the removal of residual ether at 40°, measured volumes (1.0 ml) of the aqueous solutions were incorporated into Instagel (10.0 ml) and assayed for ^3H activity.

Similar experiments were done in Krebs–Ringer phosphate (pH 7.4) with slices (0.3 × 0.3 × 4.0 mm; 200 mg wet wt tissue) of kidney-cortex tissue, that were prepared in a McIlwain and Buddle [15] tissue-chopper. A soluble fraction was prepared from residual tissue fragments after incubation for 4 hr with the metallothionein, and fractionated by gel filtration on a column (85 × 1.5 cm) of Sephadex G75. Portions of each fraction (2.0 ml in vol) were analysed for Cd^{2+} and ^3H.

Toxicity measurements and histology. LD_{50}-values were determined by the method of Weil [16] in young (100 g body wt) male rats of the Porton strain. For i.v. injections, the metallothioneins were dissolved in 0.154 M NaCl. Solutions of Cd^{2+} were prepared by dilution of 0.103 M $CdCl_2$ with 0.154 M NaCl. Mixtures of bovine serum albumin and Cd^{2+} (60:1, by wt) were prepared from solutions of the protein and of Cd^{2+}, both in 0.154 M sodium acetate buffer, pH 5.8, and were adjusted to pH 7.0–7.2 with 0.1 N NaOH. "Cadmium-serum" (92 μg Cd^{2+}/ml) was obtained as described by Weinzierl and Webb [17]. For histology, organs (kidney, liver, heart, lung and

brain) were removed from the animals immediately after death, and fixed in formol–alcohol. Preparations, mounted in paraffin wax, were sectioned at 5 μm and stained with haematoxylin-eosin.

RESULTS

Preparation and properties of the metallothioneins. Cadmium-thionein preparations that were isolated by gel filtration on Sephadex G75, after preliminary fractionation of the soluble components of the livers of either Cd^{2+}-exposed rabbits of rats with ethanol, were free from contaminating polysaccharide (c.f. [18]) and contained 7.0–7.6% Cd^{2+}. Further purification by gel filtration on Bio-Gel P10 increased the Cd^{2+} content to 8.0–8.4%. In both gel-filtration steps some Cd^{2+} was associated with minor protein components of higher mol. wt (Figs. 1 and 2). Although these were not investigated in detail, all contained –SH groups that were detectable after replacement of Cd^{2+} by H^+, and probably were polymers of the low mol. wt metallothionein. In addition to Cd^{2+}, cadmium-thionein usually, if not invariably, also contains Zn^{2+} (e.g. [2]) which is bound less firmly by the protein moiety [12]. That polymerization occurs through partial loss of Zn^{2+}, followed by intermolecular disulphide formation from the liberated –SH groups is suggested by the decrease in Cd^{2+}/Zn^{2+} ratio with molecular size of the three metalloprotein fractions (a, b and c) from the Bio-Gel P10 column (Fig. 2).

Although the preparations of cadmium- and zinc-thioneins, that were used in the toxicity and metabolic studies, appeared homogeneous by gel filtration (e.g. Fig. 2) they could be resolved into the "a" and "b"

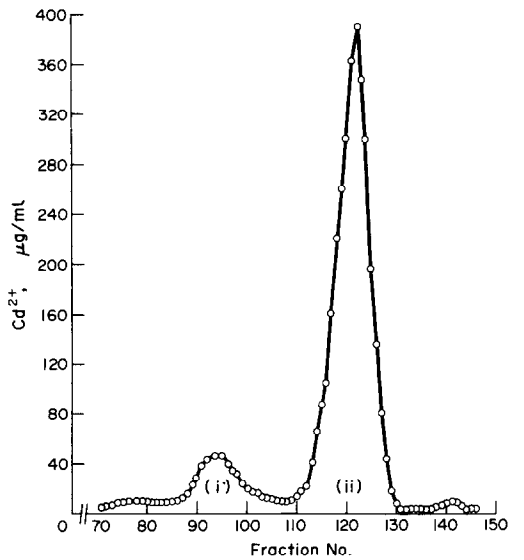

Fig. 1. Isolation of rabbit liver cadmium-thionein by gel filtration on Sephadex G75. The metallothionein-rich fraction that was obtained by precipitation with ethanol (see "Materials and Methods") was applied to a column (85 × 5 cm) of Sephadex G75, equilibrated with 20 mM Tris–HCl buffer, pH 8.0. The column was eluted with the same buffer at a flow-rate of 23 ml/hr and fractions of 7.5 ml in volume were collected.

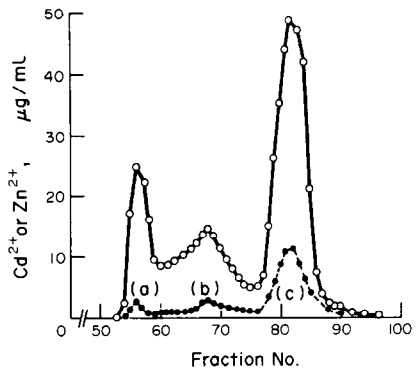

Fig. 2. Purification of cadmium-thionein from rabbit liver by gel filtration on Bio Gel P10. The product (peak "b") from the Sephadex G75 column (see Fig. 1) was concentrated and applied to a column (113 × 4.5 cm) of Bio Gel P10, equilibrated with 20 mM Tris–HCl buffer pH 8.0. The column was eluted with the same buffer at a flow-rate of 25 ml/hr. The eluate fractions (7.5 ml in volume) were analysed by atomic absorption for Cd^{2+} (—○—) and Zn^{2+} (—●—). The molar ratios of Cd^{2+}:Zn^{2+} in the resolved fractions were (a) 4.8:1, (b) 3.5:1 and (c) 2.4:1.

components by chromatography on DEAE-Sephadex A25 [19].

Toxicity of the metallothioneins. In the rat, the LD_{50} values for the rat and rabbit hepatic cadmium-thioneins were 0.28 (0.22–0.35) mg. Th-Cd^{2+}/kg, and 0.32 (0.25–0.37) mg Th–Cd^{2+}/kg, respectively, whereas that of ionic Cd^{2+} was 2.24 (1.78–2.82) mg Cd^{2+}/kg. The difference in toxicities of the rat and rabbit metallothioneins may have been due to differences in either purity or the relative proportions of Cd^{2+} and Zn^{2+}, and probably is not significant. Zinc-thionein was not only non-toxic at 2.4 mg protein bound Zn^{2+}/kg but also protected the animals against a subsequent, normally lethal dose (0.4 mg Th–Cd^{2+}/kg) of cadmium-thionein. Protection against 1.0 mg Th–Cd^{2+}/kg also occurred when the animals were pretreated with either ionic Cd^{2+} (2.0 mg/kg) or cadmium-thionein (0.16 mg Th–Cd^{2+}/kg).

Administration of bovine serum albumin with Cd^{2+} had no effect on the toxicity of the cation. In such mixtures however, the cation was not firmly bound by the protein since, on dialysis against 0.154 M NaCl, more than 95 per cent of the former was diffusible. "Cadmium-serum", in which the cation is complexed more firmly, but with both proteins and diffusible molecules [16] was not toxic at doses of less than 1.5 mg (bound) Cd^{2+}/kg.

Rats that were given a lethal dose of cadmium-thionein did not display the ataxia of gait and weakness of the hind limbs that were early responses to the administration of a toxic dose of Cd^{2+}. Initially, no toxic manifestations were observed but, after 12 hr, the animals became increasingly lethargic and died, usually between 24 hr and 30 hr. In animals that were examined immediately after death from a lethal dose of Th–Cd^{2+}, Cd^{2+} was present in all tissues that were analysed (Table 1), a low but reasonably constant concentration being found in the brain. No abnormalities were detected, however, in the brains, as well as the lungs, livers and hearts of these animals on histological examination. In the kidneys the concentration of Cd^{2+} was much higher than in any other

Table 1. Distribution of Cd^{2+} in tissues of the male rat at death after the administration of lethal doses of cadmium-thionein

Sources of cadmium-thionein	Dose (mg thionein-bound Cd^{2+}/kg)	Time of death (hr after administration)	Cd^{2+} concn (μg/g wet wt tissue) Brain	Heart	Liver	Kidney	Lung
Rabbit liver	2.06	26			4.82	11.50	
	2.06	30			3.50	9.90	
	1.58	24	0.14	0.36	2.94	15.80	0.53
	1.00	26	0.14		2.10	11.20	0.42
	1.00	29	0.13	0.32	2.64	10.24	
	0.63	22	0.13	0.36	1.95	10.52	0.29
Rat liver	0.40	27	0.10	0.38	2.04	11.90	
	0.40	25	0.17		3.84	10.86	
	0.40	29	0.13	0.32	3.14	10.74	

Cadmium-thionein was isolated from the livers of Cd^{2+}-treated female rabbits and rats, the metalloprotein from the latter source being labelled with $^{115m}Cd^{2+}$ (see "Materials and Methods"). The preparations were dissolved in isotonic NaCl solution and administered to young (100 g body wt) male rats by i.v. injection. The results recorded above were obtained on tissues that were removed from animals immediately after death. Animals in which death occurred, but was not observed, were discarded. Cadmium contents of the weighed whole organs from rats that were dosed with the [$^{115m}Cd^{2+}$]-cadmium-thionein were determined from measurements of the radioactivity in a well-shaped NaI scintillation counter. Concentrations of Cd^{2+} in the organs from animals that received rabbit liver cadmium-thionein were determined by atomic absorption after acid-digestion of the weighed tissue-samples.

organ, and appeared to be more dependent on survival time, than on dose (Table 1). The lining epithelial cells of the proximal convoluted tubules were necrotic and, in many regions, had disintegrated to leave a bare basement membrane. Most of the tubules contained abundant basophilic material but, as observed previously in mice [3], there was little or no local inflammatory response and no spread of basophilic material outside the tubules. Other parts of the nephrons (distal tubules and glomeruli), as well as interstitial connective tissue and blood vessels, appeared normal. Such tubular damage was not observed in the kidneys of rats that either were given a lethal dose of the cation alone, or were killed 72 hr after the injection of "cadmium-serum" (1.0 mg Cd^{2+}/kg body wt). Also in the latter animals the cation accumulated to a higher concentration in the liver (13.1 ± 0.2 μg/g wet wt) than in the kidney (3.6 ± 0.4 μg/g wet wt).

Metabolism of cadmium-thionein in vivo. In agreement with the work of Cherian and Shaikh [6] and of Tanaka *et al.* [5], injection of $^{109}Cd^{2+}$-labelled cadmium-thionein (0.25 mg Th–Cd^{2+}/kg) into the rat led to the accumulation of $^{109}Cd^{2+}$ in the kidney as a metallothionein. If, however, rats were dosed with the cadmium-thionein preparations, the protein moieties of which were labelled with ^{35}S or ^{3}H, very little radioactivity was recovered in the renal metallothionein (Table 2, Fig. 3). The metalloprotein that was isolated from the kidneys 48 hr after the i.v. injection of [^{35}S]cysteine labelled cadmium-thionein (2050 cpm/μg Cd^{2+}) for example, had only 55 cpm/μg Cd^{2+}. In this, as in the experiment with L-[U-^{3}H]proline-labelled cadmium-thionein (Fig. 3), radioactivity was distributed throughout the elution profile, the greatest amount being present in the high mol. wt proteins, a pattern of distribution that suggests metabolism of the thionein moiety and partial re-utilization of the component amino acids.

A similar pattern of ^{3}H-distribution was observed in the soluble fraction of the kidney after the administration of rabbit liver cadmium-thionein, labelled with L-[3,3′-^{3}H]-cysteine (100 μg Cd^{2+}; 1230 cpm/μg Cd^{2+}) to the rabbit, with the exception that, in addition to ^{3}H, some Cd^{2+} also was located in the proteins of higher mol. wt. This fraction, however, had only 144 cpm/μg Cd^{2+}, and thus did not contain a polymer of the administered metallothionein.

After injection of rats with the labelled cadmium-thioneins, a variable percentage of the protein label, together with some Cd^{2+}, was excreted in the urine

Table 2. Renal incorporation and urinary excretion of Cd^{2+} and "protein" in rats after the administration of rabbit liver cadmium-thionein

Administered cadmium-thionein: Radioactive precursor amino acid	Sp. act. (cpm/μg Cd^{2+})	Total cpm	Total Cd^{2+} (μg)	Urinary excretion: Total cpm	Total Cd^{2+} (μg)	Renal cadmium-thionein: Total cpm	Cd^{2+} (μg)	Specific activity (cpm/μg Cd^{2+})
L-[^{35}S]cysteine	2050	442800	216	88032 (19.9)*	32.4 (15.0)†	1795 (0.41)*	32.9 (15.2)†	55
L-[^{35}S]cysteine	2050	369000	180	42470 (11.5)*	6.7 (3.7)†	—	—	—
L-[U-^{3}H]proline	303	57270	189	44645 (77.9)*	18.8 (10.0)†	610 (1.1)*	21.1 (11.2)†	29

* Per cent of total counts administered to three rats.

† Per cent of total Cd^{2+} administered to three rats.

The labelled preparations of cadmium-thionein, in doses of 240–290 μg thionein-bound Cd^{2+}/kg body weight were administered i.v. to groups of three female rats (230–250 g body wt) which were then housed in metabolism cages for the collection of urine. The animals were killed after 48 hr and the cadmium-thionein was isolated from the pooled kidneys of each group (see Figs. 3 and 4). Measurements of radioactivity and of Cd^{2+}-concentration in the urine and column fractions were made as described in "Materials and Methods".

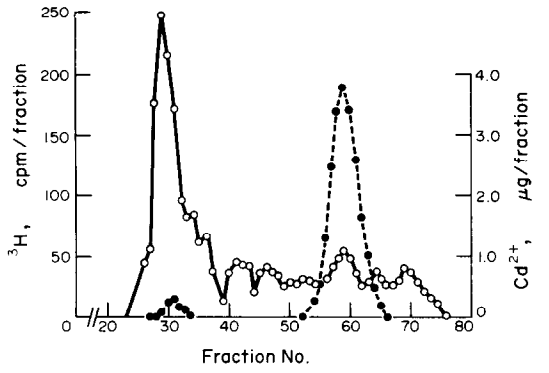

Fig. 3. Distribution of ^{3}H (—○—) and of Cd^{2+} (- -●- -) in the elution profile, obtained on gel filtration of the soluble fraction from the pooled kidneys of 3 rats, 48 hr after the injection of L-[U-^{3}H]-proline-labelled cadmium-thionein (63 μg Cd^{2+}/animal; 303 cpm/μg Cd^{2+}) from rabbit liver.

(Table 2). About 40 per cent of the urinary Cd^{2+} of animals that had been dosed with L-[^{35}S]cysteine-labelled cadmium-thionein (2050 cpm/μg Cd^{2+}) was recovered by gel filtration as the metalloprotein of essentially unchanged sp. act. (1880 cpm/μg Cd^{2+}), but the remainder was present either as the free cation, or as complexes of low mol. wt and, together with 34.5 per cent of the radioactivity, was lost on dialysis against 10 mM Tris HCl buffer, pH 7.8.

Metabolism of cadmium-thionein in vitro. Only limited degradation (<10 per cent) occurred when L-[3,3′-^{3}H]cysteine-labelled cadmium-thionein was incubated for 4.5 hr with slices of kidney cortex. The metallothionein was not incorporated into the tissue fragments, but was recovered, with unchanged sp. act., from the medium at the end of this period. When the labelled metalloprotein was incubated with a kidney homogenate, however, there was a time-dependent hydrolysis to acid-soluble products (Table 3).

Treatment of Cd^{2+}-exposed animals with CCl_4 and retrorsine. Rats that had received total doses of 8 and 32 mg Cd^{2+}/kg body wt by repeated s.c. injection over periods of 5 days and 3 weeks, and which had accumulated high hepatic stores of Cd^{2+} (160–170 and 415–430 μg Cd^{2+}/g wet wt tissue, respectively) were exposed to CCl_4 (32 mg/l air) in inhalation chambers for 4 hr (L. Magos, personal communication) or to retrorsine (32 and 38 mg/kg body wt) by i.p. injection [20], to determine if subsequent liver damage resulted in the liberation of the hepatic metallothionein with the transfer of Cd^{2+} from the liver to the kidney. Neither of these hepatotoxins caused urinary excretion of Cd^{2+} during the following 72 hr and, when the animals were killed after this time, no significant changes were detected in the Cd^{2+}-concentrations of the livers and kidneys. In the experimental groups the histological changes in the livers were similar to those of normal animals that were treated with the hepatotoxins under identical conditions as controls.

Table 3. Degradation of L-[3,3′-^{3}H]cysteine labelled rabbit liver cadmium-thionein in homogenates of rabbit kidney cortex

Period of incubation at 37° (h)	Thionein rendered acid-soluble (cpm/ml)	Hydrolysis (%)
1.0	716	9.6
2.0	1324	17.8
4.5	3074	41.4

L-[3,3′-^{3}H]-cysteine labelled rabbit liver cadmium-thionein was supplemented with a preparation of the unlabelled metalloprotein to a sp. act. of 984 cpm/μg Cd^{2+} and was added to homogenates of rabbit kidney cortex (0.1 g wet wt tissue/ml) to a concentration of 8 μg thionein-bound Cd^{2+}/ml. The suspensions were shaken at 37° for the times shown below and then were processed as described in "Materials and Methods". Hydrolysis of the cadmium-thionein was calculated from measurements of acid-soluble ^{3}H. The results have been corrected for the presence in the substrate of counts (6.6% of the total) that were not precipitated in 5% (w/v) trichloroacetic acid.

DISCUSSION

Low mol. wt proteins are known to pass through the glomerulus of the kidney and to be taken up and catabolized in the convoluted tubular cells. In tubular proteinuria the linked uptake-catabolic function fails, with the result that urinary excretion of these proteins is increased [21].

The present results, coupled with those of Nordberg *et al.* [3] and of Cherian and Shaikh [6] show that i.v. injected cadmium-thionein also is taken up and catabolized in the kidney; the metallothionein, however, is highly renotoxic, and urinary excretion of the protein occurs even at low dose levels. The renal metabolism of the metalloprotein seems to involve complete degradation of the thionein moiety, followed by at least partial re-utilization of the amino acids. Thus at 3 hr after the administration of ^{35}S-labelled cadmium-thionein to the rat, most of the radioactivity in the soluble fraction of the kidney is present in peptides of lower mol. wt [6] whereas, as shown by the present results, at 48 hr it is associated predominantly with proteins of higher mol. wt. The low levels of radioactivity that are found in the metallothionein of the kidney at this time (e.g. Table 2) probably are due to recycling of the labelled amino acid, rather than to residual precursor protein since, at least in the liver, there is continual turnover of Cd^{2+}-induced thionein [32].

Enzymes which, in kidney cortex homogenates, catalyse the hydrolysis of the protein moiety of cadmium-thionein to acid-soluble products (Table 3), probably originate from lysosomes. *In vivo*, the catabolism of various intravenously administered proteins, that are taken up from the lumen of the proximal renal tubules, appears to be a lysosomal process [23], and a progressive increase in number of these bodies has been observed in the proximal renal tubules of rats exposed to Cd^{2+} in their drinking water [24] or when given a single i.v. injection of cadmium-thionein [25]. As, after gastrointestinal absorption or parenteral administration, Cd^{2+} is transported in blood initially as complexed with plasma proteins [26] particularly serum-albumin (see e.g. [27]), the mechanisms of kidney uptake of "ionic" Cd^{2+} and of thionein-bound-Cd^{2+} may be qualitatively the same.

The observation that, at 3 hr after the administration of the metalloprotein to the rat, only 45 per cent of the Cd^{2+} in the kidney was recovered as cadmium-thionein [6], implies that more than half of the total renal Cd^{2+} at this time is not thionein-bound, and suggests that cation is liberated rapidly from the metallothionein once it reaches the kidney. Although at the low pH of the lysosome, the liberation of Cd^{2+} may precede the catabolism of the protein, it is improbable that the induced synthesis of new thionein would be initiated within 3 hr (see e.g. [28]). Thus the greater incorporation of Cd^{2+} than of thionein into the renal metallothionein, observed by Cherian and Shaikh [6] at this time, may be due to displacement by Cd^{2+} of Zn^{2+} from zinc-thionein, which normally seems to be present at low concentration in the rat kidney (M. Webb, unpublished observations).

Renal damage, that follows the injection of a toxic dose of cadmium-thionein, thus seems to be due to the Cd^{2+} cation and not to the intact metalloprotein. Since, at death from a lethal dose of cadmium-thionein, the renal concentration of Cd^{2+} is only 10–15 μg/g wet wt (Table 1), renal failure is more likely to be explained by the rapid transport and liberation of Cd^{2+} in specific locations in the kidney, than by the total content of the cation in this organ (cf. [26]). The greater toxicity of cadmium-thionein, in comparison with ionic Cd^{2+}, "cadmium-serum" or mixtures of Cd^{2+} and serum albumin, therefore, may be related to preferential absorption, since glomerular filtration favours smaller molecules or to differences in content and strength of binding of the cation in the metalloprotein, which facilitate transport of the cation to the kidney. In this respect thionein may resemble cysteine and penicillamine, both of which increase the kidney uptake and nephrotoxicity of Cd^{2+} [29–31]. The assumption that the liberated Cd^{2+} ion is the toxic species is supported by the observation that pretreatment with ionic Cd^{2+} protects against a normally lethal dose of cadmium-thionein. Such protection, although unexplained, is well known in the toxicology of Cd^{2+} (see e.g. [32]).

The high toxicity of cadmium-thionein suggests that the accumulation of the metalloprotein in the livers of human beings and other mammalian species, environmentally exposed to Cd^{2+} may be potentially dangerous. In man, for example, it is possible that subsequent exposure, either environmental or industrial, to other agents could cause liver damage with the liberation of the metallothionein. Although in Cd^{2+}-exposed rats, that contained high hepatic contents of cadmium-thionein, no evidence was obtained that exposure to the hepatotoxins, carbon tetrachloride and retrorsine, caused the transfer of Cd^{2+} from the liver to the kidney, the rat in this instance may not be an appropriate model for man.

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