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AN INVESTIGATION OF THE ROLE OF METALLOTHIONEINS IN PROTECTION AGAINST THE ACUTE TOXICITY OF THE CADMIUM ION

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Abstract—Pretreatment of female rats with a low dose of Cd^{2+} protects them against a subsequent, normally lethal dose of the same cation, and also induces the synthesis of hepatic cadmium-thionein. This protection, however, is maximal 1–3 days after pretreatment and then decreases, whereas both the increased content, and capacity for the synthesis of the metallothionein are maintained. Because of this increased capacity for cadmium–thionein synthesis a greater percentage of a subsequent dose of Cd^{2+} is retained in the liver of the pretreated animal, than in the non-pretreated control. Uptake of the cation into other organs (i.e. heart, kidney, pancreas and spleen), however, is unaffected by the pretreatment.

Although Cd^{2+} is bound more strongly by the apoprotein, thionein, than is Zn^{2+}, pre-induction of hepatic zinc–thionein by restriction of food intake, does not lead to increased resistance to the toxic cation.

These observations suggest that pre-induced metallothioneins do not have a significant role in the protection against the acute toxicity of Cd^{2+}.

Pretreatment of experimental animals with a low dose of Cd^{2+} protects them against a subsequent, normally lethal dose of the cation [1, 2], and also induces the synthesis of cadmium–thionein (metallothionein*) in the livers and kidneys [2]. Similarly, protection against the Cd^{2+}-induced injury to the testis [18, 19] is achieved by pretreatment with small amounts of Cd^{2+} or larger amounts of Zn^{2+} [19, 20]. Since excess Zn^{2+} also stimulates the production of zinc–thionein in the liver [12], the induced synthesis of a metallothionein seems to be common to all of these instances. The possibility that the induction of cadmium–thionein synthesis may provide a protective mechanism against the toxic cation was suggested by Piscator [7] (see also Friberg *et al.* [2]). This concept has been developed by Nordberg [21] in an explanation of the protection by a preliminary low dose of Cd^{2+} against the necrotizing effect of Cd^{2+} on the mouse testis.

If, as present evidence suggests, thionein synthesis occurs only in response to the inducing cation, and the metal binding sites are fully-saturated at all times [22, 23], the possibility considered by Nordberg [21] that the content of partially saturated cadmium–thionein is increased in the testes of the Cd^{2+}-pretreated mice, seems unlikely. The same argument applied to the suggestion [2] that the preliminary low-level exposure induces the synthesis of cadmium–thionein, which is then available to bind a larger dose. An alternative possibility is that the protective effect of pretreatment is due to 'priming' of the synthetic mechanism, such that formation of cadmium–thionein occurs without lag on subsequent exposure to Cd^{2+}, and thus results in the accumulation of more of the cation in the liver, but less in other organs. These considerations led to the present investigation of the relationship between Cd^{2+}-pre-treatment, cadmium thionein synthesis and Cd^{2+}-toxicity.

* The name 'metallothionein' was given by Vallee and co-workers to a low mol. wt. metalloprotein that contained Cd^{2+} and Zn^{2+} as the principal bound cations, and which was isolated initially from horse kidney [3, 4, 5], and later from human kidney [6]. With the isolation of similar proteins from the livers and kidneys of experimental animals after exposure to Cd^{2+} [7–13], Hg^{2+} (Jakubowski *et al* [14] and Zn^{2+} [12, 15] and the realization of their possible physiological function in the control of the metabolism of certain essential trace metals [16, 17], there is now considerable confusion in terminology. In an attempt to overcome this confusion, in the present paper the term 'thionein' is used (as defined by Vallee and co-workers) for the apoprotein moiety. The prefix 'metallo' is employed in the same sense as in 'metalloprotein' and, when the principal bound cation is known, the metallothionein is defined specifically as a complex of the particular metal with the apoprotein; e.g. zinc–thionein; cadmium–thionein.

MATERIALS AND METHODS

Chemicals. Polyethylene glycol (mol. wt 6000) was obtained from Koch–Light Laboratories, Colnbrook, Bucks, Tris ('Trizma' base) from Sigma Chemical Co., London and Sephadex G75 from Pharmacia (Great Britain) Ltd., London. Radioactive $^{109}CdCl_2$ was obtained from the Radiochemical Centre, Amersham, Bucks, as a solution in 0·1 N HCl. For use in the animal experiments, this was supplemented with carrier Cd^{2+} in 0·15 M acetate buffer to give solutions of pH 5·4, and specific activities of 21·4, 42·75 and 85·5 μCi/mg Cd^{2+}. All other chemicals were either Aristar reagents or of analytical grade.

Animals. Female rats (170–190 g body wt) of the Porton strain were maintained on M.R.C. 41B diet and, for the collection of urine and faeces were housed in metabolism cages. Toxicity of Cd^{2+} was determined by the method of Weil [24]. Pretreatment with Cd^{2+} was done by i.v. injection of either a single

(1·0 mg Cd^{2+}/kg) or increasing doses (1·0, 2·5 and 3·98 mg Cd^{2+}/kg) at 48 hr intervals of solutions of $CdCl_2$, made isotonic with NaCl. Cadmium chloride labelled with $^{109}Cd^{2+}$ was administered as a single intravenous injection to both pretreated and control rats, the animals being killed at intervals thereafter. In some experiments, blood was removed from the heart into a heparinized syringe after the thorax had been opened under ether. The animals were then killed with excess ether and the appropriate organs removed for analysis. When blood was not collected, the rats were killed by decapitation.

Analytical methods. Tissues were weighed and homogenized in 5 or 10 vols. deionized water in an Ultra-Turrax homogenizer (Janke & Kunkel, IKA Products, Belmont, Surrey). Duplicate samples (1·0 or 2·0 ml) of the homogenates and of urine were digested with a mixture of Aristar $HClO_4$ (s.g. 1·70) and Aristar HNO_3 (1 : 4, v/v) by the method of Thompson and Blanchflower [25]. Blood and plasma were weighed into the digestion vials. Faeces were homogenized in water (50 or 100 ml) in an M.S.E. homogenizer (MSE Scientific Instruments, Manor Royal, Crawley, Sussex), operated at full speed for at least 3 min.; portions of the homogenates (2–5 ml) were then digested as above. After digestion, the dry inorganic residues were dissolved in 5% (v/v) HCl (5·0 ml) for analysis and measurement of radioactivity.

Metal analyses were made by atomic absorption with a Perkin–Elmer Model 306 spectrophotometer. Radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 3324), the samples (1·0 ml) being incorporated into Instagel (10·0 ml; Packard Instruments Ltd., Caversham, Bucks). The counting efficiency for $^{109}Cd^{2+}$ was 65%.

Metallothioneins and other soluble proteins were separated from the tissue homogenates by gel filtration on columns (85 × 2·5 cm or 83·5 × 1·5 cm) of Sephadex G75 at 4°. Portions of the homogenates, either in water or in Tris buffered (0·01 M, pH 8·0) saline (0·1 M), were centrifuged first for 10 min at 10,000 *g* and then for 1 hr at 105,000 *g*. The supernatant fractions were concentrated by dialysis against polyethylene glycol, clarified by centrifugation (10 min 5000 *g*) and volumes, equivalent to 1·0–2·0 g initial wet wt tissue, applied to the columns. These were eluted with a solution of 0·1 M NaCl in 0·01 M Tris–HCl buffer, pH 8·0, at flow rates of either 17 ml/hr (for the larger column) or 7·0 ml/hr. The absorbance at 254 nm of the eluates was monitored with an LKB Uvicord Recording Spectrophotometer (LKB Instruments Ltd., Addington Road, South Croydon, Surrey) and 5 ml or 2·5 ml fractions were collected. These were analysed for $^{109}Cd^{2+}$, Cd^{2+} and, in some experiments, Zn^{2+}, without digestion. Urinary protein was determined by the method of Piscator [26].

RESULTS

Effect of Cd^{2+}-preatment on the toxicity of Cd^{2+}. Rats, pretreated with Cd^{2+} (1·0 mg/kg) were resistant to a subsequent, normally lethal dose of the same cation (4·5 mg/kg). As in mice[27], protection induced by the pretreatment was maintained for at least 3 days, and then decreased with time (Table 1).

Table 1. Effect of Cd^{2+}-pretreatment on the toxicity of Cd^{2+}

Days after pretreatment with 1 mg Cd^{2+}/kg	Mortality after i.v. administration of 4·5 mg Cd^{2+}/kg	Content of Cd^{2+} in cadmium thionein of liver (μg Cd^{2+}/g wet wt)
1	2/8	11·3
3	2/8	9·7
7	4/8	10·6
10	5/8	11·6
Controls (no pretreatment)	8/8	0·0

At intervals of 1, 3, 7 and 10 days after pretreatment with Cd^{2+} (1·0 mg/kg; see 'Materials and Methods') groups of 8 female rats (170–190 g body wt) were dosed intravenously with 4·5 mg Cd^{2+}/kg. Mortality during the following 36 hr was recorded. Cadmium–thionein was separated at the same intervals from the cytosol of the pooled livers from two additional pretreated animals by gel-filtration on Sephadex G75.

During the 14-day period of the experiment, the liver spleen, pancreas, adrenals and kidneys of the pretreated animal remained histologically normal, and proteinuria was not increased relative to the untreated control.

Cadmium-thionein was not detectable in the liver of the non-pretreated control rat, but was induced by pretreatment with Cd^{2+}. The content of this protein (≡ 10–11 μg Cd^{2+}/g wet wt liver) in the pretreated animals showed some variation, attributed to differences between individual animals but, in contrast to the resistance against toxicity, did not decrease with time (Table 1). Also, 9 days after pretreatment, incorporation of a subsequent dose of $^{109}Cd^{2+}$ into cadmium–thionein of the liver was much greater in the pretreated rat than in the untreated control (Fig. 1). Since incorporation of $^{109}Cd^{2+}$ into the metallothionein under these conditions is a measure of further synthesis of the protein, and not of cation exchange [28], it follows that the loss of protection within 10 days of pretreatment (Table 1) was not correlated with either decreased levels of cadmium–thionein (Table 1), or the loss of the increased capacity for the synthesis of the metalloprotein.

Effect of Cd^{2+}-pretreatment on the incorporation of $^{109}Cd^{2+}$ by different tissues of the rat. To induce higher levels of hepatic cadmium–thionein, rats for these experiments were pretreated at intervals of 48 hr with 1·0, 2·5 and 3·98 mg Cd^{2+}/kg (see 'Materials and Methods'). The effects of this pretreatment on Cd^{2+}-toxicity were similar to those of the single, low-level dose (1·0 mg/kg; Table 1). Thus, at 48 hr after the final dose of the pretreatment schedule (day zero of the experiment), the animals were resistant to a subsequent dose of 5·0 mg Cd^{2+}/kg (the highest tested). This protection also was not maintained and, in rats that were kept for 9 days after pretreatment, a dose of 4·0 mg Cd^{2+}/kg killed 4 out of 5 animals.

To investigate whether the presence of pre-synthesized cadmium–thionein in the liver affected the distribution of a subsequent dose of Cd^{2+}, control and pretreated rats were given either 0·5, 1·0 and 2·0 mg $^{109}Cd^{2+}$/kg (Table 2) or 1·0 mg $^{109}Cd^{2+}$/kg (Table 3) by intravenous injection and killed at suitable intervals thereafter. Accumulation of $^{109}Cd^{2+}$ in the livers, but not in other tissues, of these pretreated animals was greater than in the controls. As shown

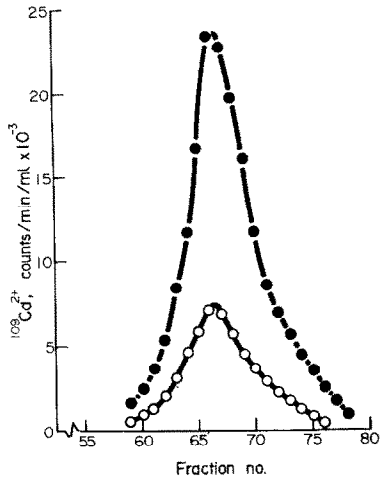

Fig. 1. Incorporation of $^{109}Cd^{2+}$ into the metallothionein fraction of the liver of the normal rat and of the Cd^{2+}-pretreated rat. Three female rats were pretreated with Cd^{2+} (1·0 mg/kg). Nine days after pretreatment these animals, together with 3 controls were given $^{109}Cd^{2+}$ (1·0 mg Cd^{2+} and 42·75 μCi $^{109}Cd^{2+}$/kg.) i.v. and killed after a further 6 hr. Cytosols were prepared from the pooled livers of the pretreated and control groups and portions of these (≡ 2·0 g wet wt tissue) were fractionated by fel filtration on a column 85 × 2·5 cm) of Sephadex G75 (see 'Materials and Methods'). The graphs show the distribution of $^{109}Cd^{2+}$ in the cadmium–thionein region of the elution profile of the cytosol from the normal rat (——○——) and the Cd^{2+}-pretreated rat (——●——). Apart from the probable replacement of Zn^{2+} in zinc thionein, which is present in low concentration in the liver of the normal female rat (see text and Fig. 4), incorporation of $^{109}Cd^{2+}$ into the metallothionein under these conditions is a measure of new protein synthesis, not of cation exchange.

by the results of Table 2 (dose level, 1·0 mg ^{109}Cd/kg) and Table 3, this increased hepatic uptake was observed at either 48 hr or 9 days after pretreatment, i.e. when the rats were fully resistant and only partially resistant, respectively, to Cd^{2+}.

Intravenously administered Cd^{2+} is known to be cleared rapidly from blood [29, 30] and, in the present experiments, only low levels of $^{109}Cd^{2+}$ remained in the blood of both control and pretreated rats at 6 hr (Table 2). Even at this time most of the $^{109}Cd^{2+}$ in the blood was associated with the cells although, in the normal animal, greater amounts were bound in the plasma as the dose increased (Table 2). In control (non-pretreated) animals the contents of $^{109}Cd^{2+}$ in whole blood were slightly greater at 48 hr than at 6 hr, whereas, in pretreated rats, there was little difference with time. (Table 2). In both groups, plasma levels of $^{109}Cd^{2+}$ usually were very low at this time (48 hr).

Incorporation of $^{109}Cd^{2+}$ into heart (Table 2), spleen and pancreas (Table 3) at 6 hr was unaffected by pretreatment of the rats with Cd^{2+}. Incorporation by the kidneys at the lower dose levels (0·5 and 1·0 mg Cd^{2+}/kg; Table 2), however, was somewhat less in the pretreated than in the non-treated animals. Apart from blood plasma and the liver of the control rat, incorporation of $^{109}Cd^{2+}$ into the different tissues at this time (6 hr) was proportional to the dose (Table 2). After 48 hr, the tissue levels were still dose-dependent, but proportionality was not maintained (Table 2). Incorporation of $^{109}Cd^{2+}$ into the brain was extremely low; differences in the levels of the isotope in this tissue of the control and Cd^{2+}-pretreated animals at 6 hr and 48 hr (Table 2, c.f. Table 3) are likely to be within the range of experimental error.

In the liver of the control rat at 6 hr a greater percentage of $^{109}Cd^{2+}$ was bound as the dose was decreased from 2·0 to 0·5 mg/kg (Table 2). This suggests the saturation of some component with high affinity for Cd^{2+} and present at a concentration sufficient to bind about 1·5 μg Cd^{2+}/g wet wt tissue. Such high affinity binding might occur by displacement of Zn^{2+} from zinc–thionein. This was present in the liver of the control female rat at a concentration equivalent to about 1·0 μg Zn^{2+}/g wet wt tissue (Fig. 4) and thus, by cation replacement, could accumulate 1·7 μg Cd^{2+}/g wet wt tissue. In the liver of the Cd^{2+}-pretreated rat, such displacement would have occurred during pretreatment and, as observed experimentally (Table 2), further uptake (of $^{109}Cd^{2+}$) would be proportional to the dose.

In the liver of the normal rat, cadmium–thionein synthesis occurred rapidly after administration of Cd^{2+} by the i.v. route; appreciable amounts of the metallothionein were present at 6 hr and, as calculated from the results of Fig. 2, there was little further increase in content (i.e. 18%) during the following 12 hr. Nevertheless, the greater accumulation of $^{109}Cd^{2+}$ in the liver of Cd^{2+}-pretreated rat (Tables 2 and 3) was correlated with the presence of pre-synthesized cadmium–thionein and, at least at 18 hr, binding by the high mol. wt protein fraction of the cytosol was reduced (Fig. 2a, 2b). Increased binding of $^{109}Cd^{2+}$ occurred also in other components of the cytosol which, on gel filtration, were eluted before the cadmium–thionein (Figs. 2a, 2b). Since uptake of $^{109}Cd^{2+}$ into cadmium–thionein under the conditions of these experiments, occurs not by displacement of Cd^{2+} that is already present, but by further synthesis of the metallo-protein [28], it follows that the greater incorporation of $^{109}Cd^{2+}$ into the liver of the Cd^{2+}-pretreated rat was due to more rapid synthesis of the metal-binding protein. This, however, did not cause Cd^{2+} to be diverted from other tissues to the liver (Tables 2 and 3), but resulted in the retention in the latter organ of a greater percentage of the dose. Thus the faecal excretion of $^{109}Cd^{2+}$ was decreased in the Cd^{2+}-pretreated rat (Table 4).

Synthesis of cadmium–thionein in the control kidney *in vivo*, in contrast with isolated cells of kidney cortex *in vitro* [31], seemed to be preceded by a long lag phase. At 18 hr after the administration of 1·0 mg $^{109}Cd^{2+}$/kg, for example, the metallothionein was not present in the kidney cytosol. At this time, as at 6 hr, essentially all of the $^{109}Cd^{2+}$ in the soluble fraction of the control kidney was bound by the high molecular weight proteins (Fig. 2c). Cadmium–thionein, however, was present in the kidneys of the Cd^{2+}-pretreated rat and, on subsequent dosing with $^{109}Cd^{2+}$, the isotope was incorporated into the metallothionein within 6 hr (Fig. 2c, 2d). Although the presence of cadmium–thionein in the kidney did not increase the

Table 2. Uptake of $^{109}Cd^{2+}$ by different tissues of the Cd^{2+} pretreated female rat, 48 hr after pretreatment and of the non-pretreated control

Tissue	Contents of $^{109}Cd^{2+}$ (ng/g wet wt) Dose level of $^{109}Cd^{2+}$ (mg/kg) 0·5				1·0				2·0			
	6 hr		48 hr		6 hr		48 hr		6 hr		48 hr	
	Control	Pretreated	Control	Pretreated	Control	Pretreated	Control	Pretreated	Control	Pretreated	Control	Pretreated
Blood	11·4 ± 0·4	11·3 ± 0·2	15·2 ± 2·8	11·6 ± 0·8	19·2 ± 0·3	22·0 ± 2·2	28·9 ± 3·2	19·8 ± 1·1	44·5 ± 2·0	50·3 ± 6·0	58·1 ± 1·6	48·8 ± 0·4
Plasma	1·4 ± 0·4	3·6 ± 0·5	2·9 ± 0·4	0·27 ± 0·06	4·6 ± 0·3	6·0 ± 0·2	0·39 ± 0·01	1·00 ± 0·14	19·4 ± 3·5	16·3 ± 0·8	1·21 ± 0·27	1·80 ± 0·05
Brain	6·0 ± 0·6	6·5 ± 0·7	8·1 ± 0·7	5·9 ± 0·4	12·1 ± 0·7	11·1 ± 2·1	15·4 ± 0·5	9·7 ± 0·1	24·7 ± 1·7	23·8 ± 2·1	28·1 ± 2·8	23·1 ± 0·5
Liver	3079 ± 179	3124 ± 20	2394 ± 143	3995 ± 431	4648 ± 0	6243 ± 257	4024 ± 67	5620 ± 129.	6035 ± 165	12300 ± 300	6530 ± 145	11064 ± 20
Kidney	536 ± 47	430 ± 1	506 ± 13	427 ± 7	1076 ± 68	894 ± 36	1164 ± 94	911 ± 33	2087 ± 103	2102 ± 137	2619 ± 128	2388 ± 97
Heart	147 ± 3	145 ± 6	128 ± 4	125 ± 7	272 ± 4	237 ± 9	239 ± 10	209 ± 22	500 ± 5	537 ± 22	536 ± 17	597 ± 18

Female rats (170–190 g body wt) were made resistant to Cd^{2+} by i.v. injection at 3 day intervals of increasing doses of Cd^{2+} (1·0, 2·5 and 3·98 mg/kg). After a further 48 hr these animals, in groups of 4, together with the same number of untreated controls were given (i.v.) mixtures of $^{109}Cd^{2+}$ and Cd^{2+} such that the animals in the 3 groups of each series received the same dose of $^{109}Cd^{2+}$ (42·75 μCi/kg) and 0·5, 1·0 and 2·0 mg total Cd^{2+}/kg. At 6 and 48 hr, two rats from each group were killed and tissues removed for analysis. The results are mean values of analyses, each in duplicate, on tissues from two animals.

Table 3. Uptake of $^{109}Cd^{2+}$ by different tissues of the Cd^{2+}-pretreated female rat, 9 days after pretreatment, and of the non-pretreated control

Tissue	$^{109}Cd^{2+}$ content (ng/g wet wt tissue) 6 hr Control	6 hr Pretreated	18 hr Control	18 hr Pretreated
Liver	4105 ± 5	5668 ± 32	4048 ± 52	5560 ± 20
Spleen	403 ± 10	388 ± 10	379 ± 17	407 ± 12
Kidney	1123 ± 44	1032 ± 70	1132 ± 32	1051 ± 33
Pancreas	1024 ± 26	1022 ± 16	953 ± 13	955 ± 13
Brain	34 ± 2	30 ± 1	31 ± 0	33 ± 3
Heart	191 ± 1	234 ± 6	205 ± 8	205 ± 8

Female rats (170–190 g body wt) were pretreated by i.v. injection at 3 day intervals of increasing doses of Cd^{2+} (1·0, 2·5 and 3·98 mg/kg). Nine days after the last pretreatment dose the animals, together with the untreated controls, were given $^{109}Cd^{2+}$ (1·0 mg/kg) by i.v. injection. Two animals from each series were killed at 6 hr and 18 hr and the tissues listed below were removed for analysis. Results are mean values of analyses, each in duplicate, on tissues from two rats.

renal uptake of $^{109}Cd^{2+}$ (Tables 2 and 3), it affected the distribution of the cation. Thus relative to the normal kidney, binding of $^{109}Cd^{2+}$ by the soluble, high mol. wt protein fraction was decreased (Figs. 2c, 2d), whilst recovery of $^{109}Cd^{2+}$ in the cytosol was increased from 51% (of the total renal $^{109}Cd^{2+}$) in the control to 79% in the pretreated animal.

These results suggested that, although uptake of $^{109}Cd^{2+}$ by other tissues (e.g. heart, spleen and pancreas) was unaffected by Cd^{2+}-pretreatment, the presence of pre-synthesized cadmium–thionein might lead, nevertheless, to an altered distribution of the cation. As shown by the absence of the characteristic cadmium–thionein peak from the elution profiles of the soluble fractions (Fig. 3; c.f. Fig. 2), however, no evidence was obtained for the presence of significant concentrations of the metalloprotein in these organs, even from the pretreated animal. The results of Fig. 3a and Fig. 3c, in particular, indicate that the spleen and pancreas, which also accumulate Cd^{2+} (Table 3; [29, 30]) do not have the capacity of either the liver or the kidney for the synthesis of the cadmium–binding protein. The possibility that limited synthesis of this protein occurs in these organs, however, is not excluded. According to Kägi (quoted by Kägi *et al.* [32]), for example, a metallothionein (presumably zinc–thionein) is present in nearly every tissue of the body, whilst Davies and Bremner [33] have observed the *de novo* formation in isolated tissue from the rat

Table 4. Excretion of $^{109}Cd^{2+}$ in urine and faeces and of urinary protein after administration of $^{109}CdCl_2$ to normal and Cd^{2+}-pretreated female rats.

Dose of $^{109}Cd^{2+}$ (mg/kg)	$^{109}Cd^{2+}$ excretion (ng/animal) Faeces Pretreated 24 hr	Faeces Pretreated 48 hr	Faeces Controls 24 hr	Faeces Controls 48 hr	Urine Pretreated 24 hr	Urine Pretreated 48 hr	Urine Controls 24 hr	Urine Controls 48 hr
0·5	1360	5490	4970	13330	2·56	4·60	1·10	1·80
1·0	3590	15730	21560	36510	3·07	6·01	3·28	5·44

The methods of pretreatment and of $^{109}Cd^{2+}$ administration were as described in the legend to Table 2. The $^{109}Cd^{2+}$ was given 48 hr after the termination of pretreatment. Results are mean values for 2 animals.

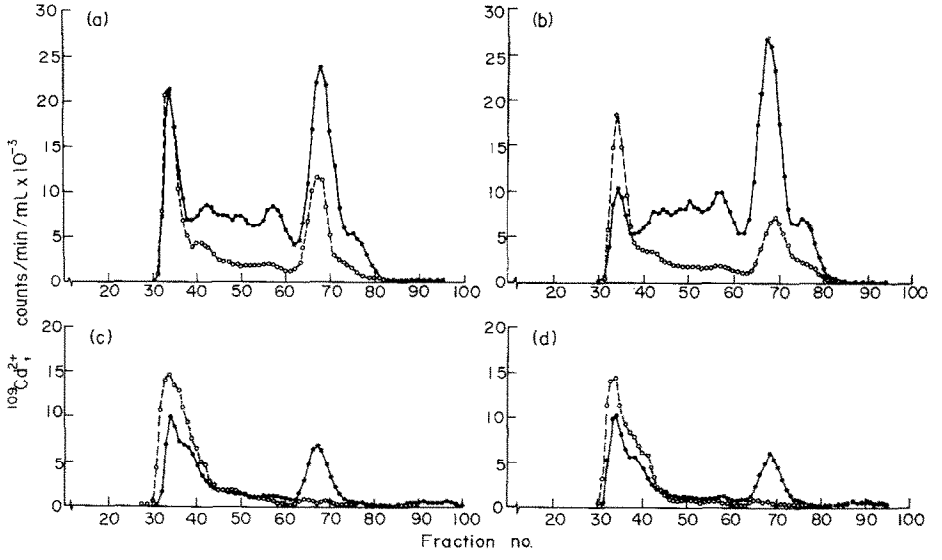

Fig. 2. Distribution of $^{109}Cd^{2+}$ in the liver and kidney cytosols from control and Cd^{2+}-pretreated rats. The concentrated cytosols (≡2·0 g wet wt tissue*, see 'Materials and Methods') were prepared from liver (Fig. 2a, 2b) and kidney (Fig. 2c, 2d) of control (—○—) and Cd^{2+}-pretreated (—●—) female rats at 6 hr (Fig. 2a, 2c) and 18 hr (Fig. 2b, 2d) after the i.v. administration of $^{109}Cd^{2+}$ (47·5 μCi and 1·0 mg Cd^{2+}/kg), and were fractionated by gel filtration on a column (85 × 2·5 cm) of Sephadex G75 at a flow rate of 17 ml/hr. Fractions (5 ml) were collected and analysed for $^{109}Cd^{2+}$. The pretreated animals were given 1·0, 2·5 and 3·98 mg Cd^{2+}/kg by i.v. injection at 3 day intervals, the $^{109}Cd^{2+}$ being administered 48 hr after the last pretreatment dose.

* The equivalent of 1·1 g wet wt tissue of the control liver was used for the fractionation in Fig. 2b.

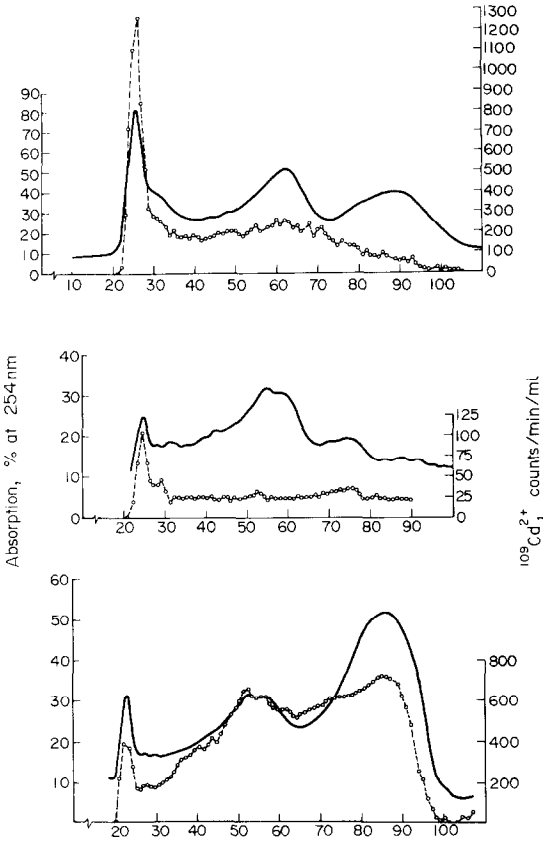

Fig. 3. Distribution of $^{109}Cd^{2+}$ in the cytosols of (a) spleen (b) heart and (c) pancreas after administration of $^{109}Cd^{2+}$ to Cd^{2+}-pretreated female rats. The animals were pretreated with Cd^{2+} and dosed with $^{109}Cd^{2+}$ as described in the legend to Fig. 2. They were killed after 18 hr, the tissues being removed, frozen in liquid N_2 and stored at −20° until processed. Cytosols were prepared as described under 'Materials and Methods' and were fractionated by gel filtration on a column (85 × 2·5 cm) of Sephadex G75 (see legend to Fig. 2) Absorbance at 254 nm, ——; $^{109}Cd^{2+}$, ---O---.

pancreas of a protein, similar to zinc–thionein in its behaviour on gel filtration, in response to Zn^{2+}-accumulation *in vitro*.

Toxicity of Cd^{2+} for the starved rat. Although the preceding results had shown no relationship between resistance to Cd^{2+} and cadmium–thionein levels, it was possible that induction of thionein synthesis by a cation of lower binding affinity (e.g. Zn^{2+}, [4, 5]) might have a protective function. To investigate this, and, at the same time, to avoid competitive interactions between cations, which are possible in animals pretreated with excess Zn^{2+}, hepatic zinc–thionein was induced by restriction of food intake [15]. In agreement with the observations of Bremner *et al.* [15], starvation of female rats for 24 hr resulted in a 3·4-fold increase in the zinc–thionein content of the liver cytosol (Fig. 4). This increased concentration of zinc-thionein (≅ 3·8 μg Zn^{2+}/g wet wt tissue), however, did not protect against Cd^{2+}-toxicity, the LD_{50} values for the cation in these animals being identical with that in normally-fed controls.

DISCUSSION

The above results lead to the following conclusions; (1) The tolerance produced in rats by a low dose of Cd^{2+} is maximal 1–3 days after pretreatment and then decreases with time (Table 1), whereas both the increased content (Table 1) and induced capacity for the synthesis of hepatic cadmium–thionein (Fig. 1) are maintained. (2) Because of this increased capacity for cadmium–thionein synthesis, accumulation of Cd^{2+} in the liver is greater in the pretreated rat than in the untreated control (Tables 2 and 3). This is due to the retention of a greater percentage of the dose (Table 4), and uptake of the cation by other tissues is unaltered (Tables 2 and 3). (3) Although Cd^{2+} is bound more strongly by the apoprotein, thionein, than is Zn^{2+}, rats in which the levels of hepatic zinc-thionein have been increased by starvation (Fig. 4) show the same susceptibility to Cd^{2+} as controls on normal diet. Thus, although synthesis of cadmium-thionein probably explains why experimental animals tolerate a larger amount of Cd^{2+} given as multiple doses at frequent intervals, than when administered as a single injection [2], it seems that neither pre-induction of this protein (which also 'primes' the synthetic mechanism such that further synthesis occurs rapidly in response to a subsequent dose), nor of zinc–thionein, has a protective function against the immediate toxicity of the Cd^{2+} cation. This conclusion is supported by the observation [27] that pretreatment with other metals (e.g. In^{3+}, Mn^{2+}), which do not

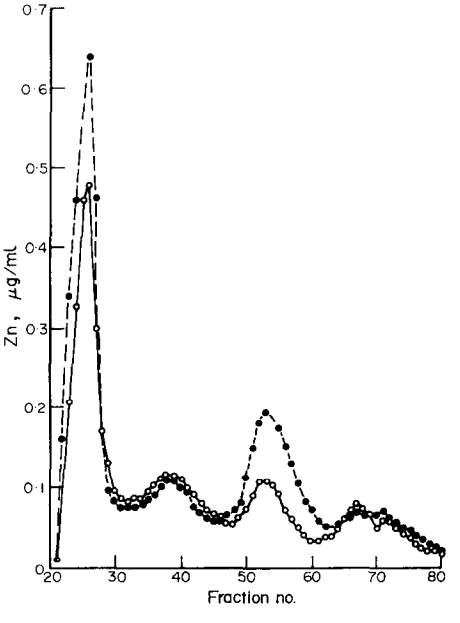

Fig. 4. Effect of starvation on the content of zinc-thionein in rat liver. Concentrated cytosols were prepared (see 'Materials and Methods') from the livers of control and starved female rats and portions of these (≡0·5 g wet wt tissue) were fractionated by gel filtration on a column (83·5 × 1·5 cm) of Sephadex G75 at a flow rate of 7 ml/hr. Fractions (2·5 ml) were collected and analysed for Zn^{2+}. Control liver, ——O——; liver from the starved rat, ——●——. The metallothionein (zinc–thionein) was eluted from this small column between fractions 51 and 57 (c.f. Fig. 2).

induce the synthesis of a metallothionein, also protects against Cd^{2+}.

Suzuki and Yoshikawa [34] have observed that the Zn^{2+} cation (3·3 μg/g wet wt tissue), which also occurs in the hepatic metallothionein of the Cd^{2+}-pretreated (0·3 mg Cd^{2+}/kg) rat, is displaced by Cd^{2+} within 2 hr after subsequent dosing with a larger amount of Cd^{2+} (3·0 mg/kg). From this the authors conclude that replacement of Zn^{2+} by Cd^{2+} in the pre-synthesized metalloprotein provides a mechanism whereby Cd^{2+} accumulates, and is immobilized, more rapidly in the liver of the pre-treated, than in the non-pretreated animal. The results of the present work, however, show that an increase in the (Cd^{2+}-free) zinc-thionein content of the liver of the normal female rat to a concentration equivalent to at least 4 μg Zn^{2+}/g wet wt tissue does not protect against the toxicity of Cd^{2+}.

In agreement with the present results, Yoshikawa [35] has reported that uptake of Cd^{2+} in the livers of male rats after i.p. administration is increased by pretreatment with a small dose of the cation. As, in this work, unlabelled Cd^{2+} was used for both the initial (0·6 mg/kg) and challenging dose (3·0 mg/kg) 24 hr later, it is not possible to distinguish Cd^{2+} accumulated from the second dose, and that taken up from the first. Thus, although the increased uptake of Cd^{2+} by the liver of the pretreated rat is obvious from Yoshikawa's results [34], the evidence for his conclusion that the contents of the cation in other organs (heart, lungs, kidneys, spleen and testes) are decreased, is not clear. The present studies, in which the challenge dose of $^{109}Cd^{2+}$ was administered intravenously, show no significant differences in uptake of the labelled cation by various organs, other than the liver, of pretreated and non-pretreated female rats (Tables 2 and 3). Also in the spleen, pancreas and heart, the intracellular distribution of the cation seems to be unaffected by pretreatment. In the kidney, as in the liver, the distribution of $^{109}Cd^{2+}$ is altered because of the presence of pre-induced cadmium-thionein (Fig. 2c, 2d). Thus, although uptake of $^{109}Cd^{2+}$ by the kidney of the pretreated rat is not increased, less is bound by the particulate cellular components and more by the metallothionein fraction of the cytosol. A similar change in pattern of distribution of $^{109}Cd^{2+}$ has been observed in the testes of mice after pretreatment with repeated small doses of Cd^{2+} [21], and it is possible, therefore, that the testis in common with the liver and kidney, also has the capacity for the inducible synthesis of cadmium-thionein.

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REFERENCES

1. C. J. Terhaar, E. Vis, R. L. Rondabush and D. W. Fassett, *Toxicol. app. pharmac.* **7**, 500 (1965).
2. L. Friberg, M. Piscator and G. F. Nordberg, *Cadmium in the Environment: An Epidemiologic and Toxicologic Appraisal*. Cleveland, Chem. Rubber Co. (1971).
3. Margoshes and B. L. Vallee, *J. Amer. chem. Soc.* **79**, 4813 (1957).
4. J. H. R. Kägi and B. L. Vallee, *J. biol. Chem.* **235**, 3460 (1960).
5. J. H. R. Kägi and B. L. Vallee, *J. biol. Chem.* **236**, 2434 (1961).
6. P. Pulido, J. H. R. Kägi and B. L. Vallee, *Biochemistry* **5**, 1768 (1966).
7. M. Piscator, *Nord. Hyg. Tydskr.* **45**, 76 (1964).
8. Z. A. Shaikh and O. J. Lucis, *Proc. Can. Fed. Biol. Soc.* **12**, 101 (1969).
9. Z. A. Shaikh and O. J. Lucis, *Experientia* **29**, 301 (1970).
10. Z. A. Shaikh and O. J. Lucis, *Archs environ. Health* **214**, 419 (1972).
11. G. F. Nordberg, M. Piscator and B. Lind, *Acta pharmac. tox.* **29**, 456 (1971).
12. M. Webb, *Biochem. Pharmac.* **21**, 2751 (1972).
13. M. Webb, *J. Reproduct. Fert.* **30**, 83 (1972).
14. M. Jakubowski, J. Piotrowski and B. Trojanowska, *Toxicol. app. pharmac.* **16**, 743 (1970).
15. I. Bremner, N. T. Davies and C. F. Mills, *Biochem. Soc. Trans.* **1**, 982 (1973).
16. B. C. Starcher, *J. Nutr.* **97**, 321 (1969).
17. G. W. Evans, P. F. Majors and W. E. Cornatzer, *Biochem. biophys. Res. Commun.* **40**, 1142 (1970).
18. J. Pařízek, *J. Endocrinol.* **15**, 56 (1957).
19. J. Pařízek, *J. Reproduct. Fert.* **1**, 294 (1960).
20. S. A. Gunn, T. C. Gould and W. A. D. Anderson, *Archs Path.* **71**, 274 (1961).
21. G. F. Nordberg, *Environ. Physiol.* **1**, 171 (1971).
22. M. Webb, *Biochem. Pharmac.* **21**, 2767 (1972).
23. I. Bremner and R. B. Marshall, *Brit. J. Nutr.* **32**, 293 (1974).
24. C. S. Weil, *Biometrics* **8**, 249 (1952).
25. R. H. Thompson and W. J. Blanchflower, *Lab. Practice* **20**, 859 (1971).
26. M. Piscator, *Archs environ. Health* **5**, 325 (1962).
27. H. Yoshikawa, *Ind. Health* **8**, 184 (1970).
28. M. Webb, *Biochem. Soc. Trans.* **3**, 632 (1975).
29. M. Berlin and S. Ullberg, *Archs environ. Health* **7**, 686 (1963).
30. G. F. Nordberg and K. Nishiyama, *Archs environ. Health* **24**, 209 (1972).
31. M. Webb and M. Daniel, *Chem-Biol. Interactions* **10**, 269 (1975).
32. J. H. R. Kägi, *Fedn Proc.* **32**, 942 (1973).
33. N. T. Davies and I. Bremner, *Biochem. Soc. Trans.* **2**, 425 (1974).
34. Y. Suzuki and H. Yoshikawa, *Ind. Health* **12**, 141 (1974).
35. H. Yoshikawa, *Ind. Health* **11**, 113 (1973).