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THE TIME-COURSE OF CADMIUM-THIONEIN SYNTHESIS IN THE RAT

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Abstract—After the intravenous injection of Cd^{2+} (1.6 mg/kg body wt) the cation accumulates to slightly greater concentrations in the liver and kidneys of the 10-week-old female Wistar rat, than in these organs of the 10-week-old male. Ionic Cd^{2+} is more toxic for the female than for the male, the LD_{50} values being 1.4 (1.2–1.7) and 2.2 (1.9–2.6) mg Cd^{2+}/kg body wt, respectively. This difference in toxicity is not correlated with differences in rates of hepatic cadmium-thionein synthesis. In both sexes, uptake of Cd^{2+} by the liver is complete within 1 hr. In the male rat there is a lag phase of 3–4 hr between the administration of Cd^{2+} and the onset of the induced synthesis of thionein in the liver. Once this synthesis occurs, the content of the metallothionein increases rapidly, Cd^{2+} being transferred to the apoprotein from proteins of high molecular weight that provide the initial binding sites for the cation in the soluble fraction of the liver. At a dose level of 1.6 mg Cd^{2+}/kg, synthesis of the metallothionein is mainly complete within 8 hr and appears to be unaffected by age; both the length of the lag phase and subsequent rate of formation of the hepatic metalloprotein in the 80-week-old male rat being the same as those in the 10-week-old animal. In contrast with the male, approximately 5 per cent of the total Cd^{2+} in the soluble fraction of the liver of the female rat is bound as the metallothionein within 1 hr after the administration of the cation. This incorporation of Cd^{2+} into the metalloprotein is attributed to replacement by Cd^{2+} of Zn^{2+} in zinc-thionein, which is present in low concentration in the liver of the normal female rat. This initial replacement is followed by a slow increase in the content of thionein-bound Cd^{2+} during the following 2–3 hr. Thereafter, presumably due to stimulation by the Cd^{2+}-induced messenger, the rate of synthesis increases rapidly to a maximum, which is at least equal to, and occurs at the same time, as that in the male. At 24 hr after the administration of Cd^{2+} to the male rat, the content of Zn^{2+} in the hepatic metallothionein is similar to that of Cd^{2+}. Replacement of this Zn^{2+} by Cd^{2+} may account for the immediate incorporation of the latter cation into the hepatic metallothionein that occurs when the animals are given a second dose of Cd^{2+}. After this initial replacement the synthesis of thionein, which has been primed by the first dose of Cd^{2+}, occurs without lag on exposure to the second, and both Zn^{2+} and Cd^{2+} are incorporated into the metallothionein. Intravenous injection of 1.6 mg Cd^{2+}/kg body wt also leads to a rapid accumulation of the cation in the kidney, but does not induce the synthesis of the metallothionein in this organ of either the male or female rat during the following 48 hr.

The synthesis of the metallothionein, cadmium-thionein, which occurs in the livers and kidneys of experimental animals in response to the administration of Cd^{2+}, is known to be inducible [1, 2, 3], and to require the induction of m-RNA [4, 5, 6]. There is, however, little or no precise information about the time-course of this synthesis. Nordberg *et al.* [2], for example, from measurements of the concentration of thionein-bound Cd^{2+} in the livers of mice at 20 min, 4 hr and 1, 6 and 18 days after the subcutaneous injection of Cd^{2+} (3.0 mg/kg body wt), concluded that there was a lag of at least 24 hr between the administration of the cation and the onset of synthesis of the metalloprotein. It seems possible, however, that this long lag phase may have been due to the use of a high dose of Cd^{2+} as, at least in the rat, the injection of 3.0 mg Cd^{2+}/kg can be inhibitory to hepatic protein synthesis [7].

After intraperitoneal administration of Cd^{2+} (1.0 mg/kg) to the rat, Sabbioni and Marafante [8] found the accumulation of the hepatic metalloprotein to have begun, but to be incomplete at 6 hr, the earliest time at which analyses were made. Previously Shaikh and Lucis [9] had observed synthesis of the apoprotein, as measured by the incorporation of ^{14}C-labelled cysteine, to occur between 5 and 24 hr after the injection of Cd^{2+}, whilst Squibb and Cousins [10] had concluded that, as actinomycin D did not inhibit the formation of the metallothionein when administered 3 hr after the cation, the pre-induction of the necessary m-RNAs was complete within this time.

The work summarized in this paper was done to examine in more detail the time course of cadmium-thionein synthesis in the liver of the rat and to determine whether this synthesis (a) occurred at the same time in the kidney and (b) was influenced by either sex or age.

MATERIALS AND METHODS

Male (10-week-, and 80-week-old) and female (10-week-old) rats, of the Wistar strain, were maintained on a standard laboratory diet (41B). The animals were given 1.6 mg Cd^{2+}/kg by i.v. injection (tail vein) of a sterile solution of $CdCl_2$ (3.2 mg Cd^{2+}/ml),

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made isotonic with NaCl, and were killed in groups of 2 or 4 at intervals during the following 48 hr. The liver and kidneys were removed from each rat immediately after death and, after being weighed, were frozen in liquid N_2 and stored at −20° until processed.

For the determination of thionein-bound Cd^{2+}, either the whole kidneys, or equal weights of tissue from the livers of each group of rats, were combined and homogenized in 4 vol of a solution of 135 mM NaCl in 20 mM Tris HCl buffer, pH 8.0. The homogenate was centrifuged in a refrigerated (+4°) centrifuge for 10 min at 10,000 *g*, the supernatant fraction being re-centrifuged for 1 hr at 105,000 *g*. The soluble fraction was removed, concentrated about 5-fold by dialysis against solid polyethylene glycol (mol. wt 6000; Koch–Light Laboratories Ltd., Colnbrook, Bucks) at 4°, and diluted with the homogenization medium to contain the equivalent of 1 g original wet wt tissue/ml, a correction being applied for losses during centrifugation (i.e. in the unwashed sediments) which, from preliminary experiments, were estimated to be about 6 per cent. A portion of this final solution (0.8–1.2 ml, usually 1.0 ml) was fractionated by gel filtration at room temperature (20–25°) on a column (85 × 1.5-cm) of Sephadex G75 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) with a solution of 50 mM NaCl in 10 mM Tris–HCl buffer, pH 8.0, that contained 0.01% (w/v) NaN_3, as eluant. The column was operated at a flow-rate of 8 ml/hr and fractions of 2.3 ml volume were collected. Under these conditions, high mol. wt proteins were eluted at the void volume (v_0 = 56 ml) of the column and the metallothionein at a V_e/V_0 ratio of 2.0 (see Fig. 1).

Concentrations of Cd^{2+} were determined in the liver and kidneys of each animal by atomic absorption with a Perkin–Elmer Model 306 Spectrophotometer, the tissue samples (0.3–0.4 g wet wt) being digested and prepared for analysis by the method of Thompson and Blanchflower [11]. The Cd^{2+} and Zn^{2+} cations were determined in the eluates from the Sephadex G75 column by direct analysis, i.e. without digestion. Recovery of either cation in the eluate was always greater than 95 per cent of amount applied to the column, and usually was quantitative.

Toxicity (LD_{50}) of Cd^{2+} was determined by the method of Weil [12].

RESULTS

Hepatic and renal uptake of Cd^{2+}. As shown in Table 1, there was no difference in the liver weight, expressed as a percentage of the body weight, of male and female rats at 10 weeks of age, but the kidneys of the female were relatively larger than those of the male. In males, aged 80 weeks, the weights of both the liver and kidneys, relative to the body weight, were significantly less than at 10 weeks of age. These organ:body weight ratios were unaffected by the administration of Cd^{2+} during an experimental period of up to 48 hr.

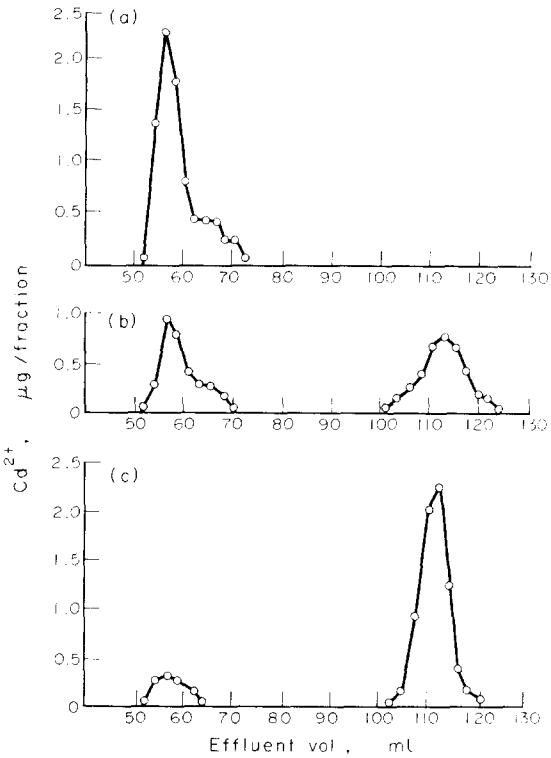

Fig. 1. Time course of cadmium-thionein synthesis in the liver of the 10-week-old male rat. The graphs show the distribution of Cd^{2+} in the eluate fractions (2.3 ml vol) obtained by gel filtration of the liver cytosol (≈ 0.8–1.0 g wet wt tissue) at (a) 2, (b) 6 and (c) 24 hr after the i.v. administration of Cd^{2+} (1.6 mg/kg) to the male rat. For experimental details see Materials and Methods. The total amounts of Cd^{2+} that were applied to the Sephadex G75 columns were (a) 8.0, (b) 6.9 and (c) 8.0 μg, and the recoveries were 97.5, 95.6 and 100%, respectively.

Uptake of Cd^{2+} into the liver of the 10-week-old male rat was complete within 1 hr after the i.v. injection of 1.6 mg Cd^{2+}/kg; thereafter there were no differences, other than those attributed to variations between animals, in either content or concentration of Cd^{2+} during the following 48 hr (Table 2). In females of the same age the pattern was similar, although the concentration, but not the content, of Cd^{2+} in the liver was greater than in the males (Table 2). Also in females, in contrast with the males, this

Table 1. Liver and kidney weights (per cent of body weight) of male and female rats at 10 weeks, and of male rats at 80 weeks of age

Sex	Age (weeks)	Body wt (g)	Liver (Per cent of body wt)	Kidney (Per cent of body wt)
Male	10	310 ± 33 (30)*	3.47 ± 0.06 (30)*	0.57 ± 0.01 (30)*
Female	10	210 ± 20 (28)	3.52 ± 0.07 (28)	0.66 ± 0.01 (28)
Male	80	595 ± 46 (8)	2.82 ± 0.09 (7)	0.43 ± 0.02 (7)

* Values in parenthesis are the numbers of animals.

Table 2. Contents of Cd^{2+} in the livers and kidneys of male and female rats at different times after the administration of 1.6 mg Cd^{2+}/kg body wt

Sex	Age (weeks)	Time after adminis-tratn. of Cd^{2+} (hr)	Cd^{2+} content in whole organ (μg) Liver	Kidney	Cd^{2+} concentration (μg/g wet wt) Liver	Kidney
Male	10	1	171.5 ± 17.0 (4)*	6.4 ± 0.9 (4)*	16.4 ± 0.4 (4)*	4.1 ± 0.6 (4)*
	10	2–8	171.9 ± 11.8 (14)	8.9 ± 1.2 (12)	15.7 ± 1.0 (14)	4.9 ± 0.6 (12)
	10	16–24	178.0 ± 8.3 (8)	9.9 ± 1.0 (6)	15.7 ± 2.1 (8)	5.5 ± 0.6 (6)
	80	1–24	244.6 ± 9.7 (10)	–	14.5 ± 1.1 (10)	—
Female	10	1	146.8 ± 9.5 (4)	7.9 ± 1.5 (4)	22.5 ± 1.1 (4)	5.9 ± 1.4 (4)
	10	2–8	148.6 ± 11.2 (12)	8.3 ± 0.7 (12)	21.2 ± 1.3 (12)	6.0 ± 0.6 (12)
	10	16–24	144.4 ± 6.0 (6)	12.4 ± 1.1 (6)	18.9 ± 0.5 (6)	9.5 ± 1.0 (6)

* Values in parenthesis are the numbers of animals.

dose of Cd^{2+} (1.6 mg/kg) killed a number of the animals, usually between 8 and 24 hr after injection. Determination of the toxicity of Cd^{2+} in these 10-week-old animals confirmed that the females were more susceptible to the cation than were the males, the 24 hr LD_{50}-values being 1.4 (1.2–1.7) mg Cd^{2+}/kg and 2.2 (1.9–2.6) mg Cd^{2+}/kg, respectively.

The concentration of Cd^{2+} in the liver of the male rats at 80 weeks of age was similar to that at 10 weeks although, as the organ (mean wt = 16.8 g) was about 1.6 times larger in the older animals, the total content of the cation was greater (Table 2). Nevertheless, a smaller proportion of the dose was retained in the livers of these rats, since their liver weights, relative to body weights, were lower than in the younger animals (Table 1). No difference was observed however in the toxicity of Cd^{2+} for males of these two age groups.

As in the liver, Cd^{2+} was taken up rapidly by the kidneys of both sexes. In the 10-week-old animals the renal concentration of Cd^{2+} remained reasonably constant between 1 hr and 8 hr after treatment, but then increased, the increase being greater in the females than in the males (Table 2).

In male rats that had been dosed once with Cd^{2+} (1.6 mg/kg), administration of a second dose of the cation after 24 hr was followed by the rapid uptake of further amounts of Cd^{2+} into the livers and kidneys. In both organs, however, uptake from the second dose, which also was complete within 1 hr, was almost double that from the first. Thus the content of Cd^{2+} in the liver was increased from 178.0 ± 8.3 μg [8] to 474.6 ± 27.3 μg [8] and in the kidney from 9.9 ± 1.0 μg [6] to 28.6 ± 2.3 μg [7].

Time course of cadmium-thionein synthesis. Cadmium-thionein was not present in the liver of the young male rat during the first 3 hr after the injection of a single dose of Cd^{2+} (1.6 mg/kg), and all of the Cd^{2+} in the soluble fraction was bound by the high mol. wt proteins that were eluted at or near the void volume of the Sephadex column (Fig. 1). After 6 hr approximately half, and after 24 hr almost all, of the Cd^{2+} in the soluble fraction was bound in the metallothionein (Fig. 1). More complete data on the time-course of the synthesis of the metalloprotein are given in Table 3. In this table the Cd^{2+} contents of the high mol. wt protein fraction and of the metallothionein have been expressed as percentages of the total

Table 3. Time course of cadmium-thionein synthesis in the liver of the male and female rat after the intravenous injection of Cd^{2+} (1.6 mg/kg)

Time after injection of Cd^{2+} (hr)	Male Per cent of total Cd^{2+} of soluble fraction in High mol. wt protein fraction	Metallothionein	Female High mol. wt protein fraction	Metallothionein
1	100 (100)*	0 (0)	95.4	4.6
2	100	0	92.7	7.3
3	100	0	89.1	10.9
4	73.8 (63.6)	26.2 (36.4)	87.5	12.5
5	67.9	32.1	39.5	60.5
6	45.6	54.4	45.9	54.1
8	10.3 (8.9)	89.7 (91.1)	16.0	84.0
16	8.6	91.4	13.3	86.7
24	10.6 (11.8)	89.6 (88.2)	13.7	86.3
48	2.9	97.1	4.5	95.5

* The values in brackets are for 80-week-old male rats.

The livers were removed from the animals at the times shown in the table and were fractionated as described in Materials and Methods. The results, other than those given in brackets, are for 10-week-old rats.

Table 4. Time-course of synthesis of, and contents of Cd^{2+} and Zn^{2+} in, the metallothionein in the liver of the 10-week-old male rat after the second of two doses of Cd^{2+}

Time after administration of the second dose of Cd^{2+} (hr)	High mol. wt protein fraction Cd^{2+}	Metallothionein Cd^{2+}	Metallothionein Zn^{2+}
	(μg/g wet wt liver)		
0*	0.9 (10.8)†	6.9 (89.2)†	3.4
1	6.7 (29.4)	16.1 (70.6)	1.0
3	2.7 (11.3)	21.3 (88.7)	1.4
4	1.4 (6.0)	22.0 (94.0)	2.0
5	1.4 (5.7)	23.7 (94.3)	3.3

* These animals were given a single dose of Cd^{2+} and killed after 24 hr.

† Per cent of total Cd^{2+} recovered from the soluble fraction.

Male rats were given 1.6 mg Cd^{2+}/kg by i.v. injection. After 24 hr, certain of these animals were given a second dose of Cd^{2+} (1.6 mg/kg) by the same route and were killed in groups of three at intervals, thereafter the livers being removed, pooled and fractionated as described under Materials and Methods.

recovered Cd^{2+} to allow for variations, such as those in Fig. 1, in the amounts of the cation in the different preparations that were applied to the column. The results show that with the onset of cadmium-thionein synthesis, which occurred between 3 and 4 hr (Table 3), the content of the metalloprotein increased rapidly whilst binding of the cation by the large proteins of the cytosol was decreased proportionately (see also Fig. 1). Synthesis appeared to be mainly complete within 8 hr, and there was only a very slight increase in cadmium-thionein content during the next 40 hr (Table 3). During this time, however, the capacity for synthesis of the metalloprotein was maintained. Thus, when male rats were given a second dose of Cd^{2+}, 24 hr after the first, cadmium-thionein synthesis occurred without a lag phase (Table 4). The metallothionein that was induced in the liver by the first dose of Cd^{2+} also accumulated Zn^{2+} and, at 24 hr, the content of the latter cation (3.4 μg or 51.9 ng ions) was similar to that of the former (6.9 μg or 62.3 ng ions; Table 4). Uptake of Cd^{2+} from the second dose into the hepatic metallothionein of the once-pretreated animal caused an initial displacement of part of this Zn^{2+} (Table 4). With the further synthesis of thionein, however, both the Cd^{2+} and Zn^{2+} contents of the metalloprotein fraction increased (Table 4).

In 80-week-old male rats the time-course of the synthesis of the hepatic protein was similar to that in the younger animals (Table 3). In the liver of the female, however, about 5 per cent of the Cd^{2+} in the soluble fraction of the liver was present as the metallothionein at 1 hr after the administration of the cation (Table 3). During the next 2–3 hr, which corresponded to the lag phase in the liver of the male rat, the content of thionein-bound Cd^{2+} slowly increased by approximately 2.5 times and then increased rapidly to a maximum between 5 and 6 hr.

Although Cd^{2+} was present in the kidney, shortly after the administration of the cation to the animal, at a concentration only slightly less than that in the liver (Table 2), no synthesis of cadmium-thionein occurred in the organ of the male or female rat within 48 hr.

DISCUSSION

At dose levels below about 2.5–3.0 μg Cd^{2+}/kg, the incorporation of Cd^{2+} into the metallothionein (cadmium-thionein) of rat liver is known to be correlated with the synthesis of the apoprotein, thionein [3, 8, 9, 10]. The increase in the content of thionein-bound Cd^{2+} that occurs after the administration of the cation to the experimental animal thus provides a convenient method by which the synthesis of the protein can be followed, at least qualitatively. By this and tracer methods it has been shown that production of cadmium-thionein is not immediate, but is preceded by a lag phase, during which the formation of the appropriate mRNAs is assumed to occur [4, 5, 6, 10]. Because of this lag phase which, as shown by the present results (Table 3) lasts for about 3 hr in the liver of the male rat, there is an intracellular redistribution of the cation with time. Thus, after i.v. injection of a single dose of Cd^{2+} (1.6 mg/kg) uptake of the cation by the liver is complete within 1 hr (the earliest time at which measurements were made). During the first 3 hr after injection, however, Cd^{2+} incorporated into the soluble fraction of the tissue is bound by proteins of high mol. wt. which form initial binding sites for the cation. With the onset of thionein synthesis, Cd^{2+} is removed from these proteins and incorporated into the metallothionein the content of which increases rapidly between 4 and 8 hr (Table 3; Fig. 1). Although decreased hepatic protein synthesis, attributed to age-linked alterations in microsomes has been described in the livers of old animals [13], the induction of cadmium-thionein synthesis in the male rat does not seem to be affected by age; both the lengths of the lag phase and subsequent rates of formation of the metalloprotein are the same in 10-week-old and 80-week-old animals (Table 3).

Zinc-thionein, which was not detected in the liver of the male Wistar rat by cation-exchange with Cd^{2+} (Table 3, c.f. Squibb and Cousins [10]), normally occurs in low concentrations in the liver of the female [14]. Replacement of this Zn^{2+} by Cd^{2+} probably explains the incorporation of small amounts of the latter cation into the metallothionein of the liver of the female within 1 hr. Also, during the lag phase that precedes the Cd^{2+}-induced synthesis in the female there is a slow, 2.5-fold increase in the content of the thionein-bound Cd^{2+} (Table 3), possibly controlled by the mRNA's that already exist for the production of the zinc-metalloprotein. Thereafter the rate of synthesis increases rapidly to a maximum, which occurs between 5 and 6 hr after the administration of the cation and is at least equal to that in the male (Table 3). Differences in the rates of hepatic cadmium-thionein formation, therefore, cannot explain the observed difference in the LD_{50} of Cd^{2+} for the male (2.2 mg/kg) and female (1.4 mg/kg) rat.

At 24 hr after the administration of Cd^{2+} to the male rat the induced hepatic metallothionein, which usually also accumulates Zn^{2+} (see e.g. [7]), contained almost equal amounts of these cations (e.g. 52 ng ions Zn^{2+}, 61 ng ions Cd^{2+}; Table 4). As observed by Suzuki and Yoshikawa [15], replacement

of much of this Zn^{2+} seems to occur when the animals are given a second dose of Cd^{2+}, and may contribute to the initial rapid uptake of the latter cation into the hepatic metallothionein that is observed under such conditions (Table 4). Even at 1 hr after administration of the second dose, however, the uptake of Cd^{2+} into the metallothionein (9.2 μg, or 82.1 ng ions/g wet wt liver) is greater than the loss of Zn^{2+} (2.4 μg, or 36.9 ng ions/g wet wt liver) and, during the following 4 hr, the contents of both of these cations in the metalloprotein fraction increase progressively (Table 4). It seems, therefore, that the first dose of Cd^{2+} primes the synthetic mechanism such that further synthesis occurs without lag on exposure to the second. Even in the Cd^{2+}-pretreated animal, however, some of the "new" Cd^{2+} that accumulates in the soluble fraction of the liver after the second dose also is bound initially by the high mol. wt proteins, and is transferred subsequently to the metallothionein (Table 4).

Intravenous injection of a single dose of Cd^{2+} also leads to the rapid incorporation of the cation into the kidney of the rat (Table 2). Under the conditions of the present experiments the renal concentration (and content) of Cd^{2+} was found to increase between 8 and 16 hr, the increase being greater in the female than in the male (Table 2). Throughout the 48-hr period after injection, however, Cd^{2+} that was incorporated into the soluble fraction of the kidney remained bound by the high mol. wt proteins, and there was no synthesis of the metallothionein. As cells of the renal cortex are able to form cadmium-thionein when cultured *in vitro* in the presence of Cd^{2+} [16], it is possible that the threshold concentration of the cation, below which the synthesis of the metalloprotein does not occur *in vivo*, may be higher in the kidney than in the liver.

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