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LONG-TERM TURNOVER OF CADMIUM METALLOTHIONEIN IN LIVER AND KIDNEY FOLLOWING A SINGLE LOW DOSE OF CADMIUM IN RATS

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Summary

Rats were injected subcutaneously on two consecutive days with $CdCl_2$, and sampled animals, killed at monthly intervals from 1 to 6 months thereafter, exhibited the presence of Cd,Zn-thionein in both the liver and kidney. At 6 months, hepatic thionein was present as the two major polymorphic forms previously demonstrated in short term Cd-injection studies. [^{35}S]cysteine incorporation studies showed that both polymorphic forms of thionein underwent continual turnover at similar rates throughout the study. The slow hepatic and renal turnover of Cd, therefore, was not due to a highly stable form of Cd-thionein, but apparently due to an inefficient mechanism for excretion of Cd from these tissues. The Cd/Zn ratio of hepatic thionein remained relatively constant, suggesting that continual thionein induction results in a long-term hepatic trapping of Zn by thionein, but the ratio for renal thionein showed a marked increase during the course of the study.

Introduction

It is well known that animals administered cadmium salts retain the metal ion for long periods of time in various tissues [1]. The ions are complexed to

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metallothionein, a low molecular weight cytoplasmic protein with an unusually high cysteine content [2—5]. In short term studies, the half-life of hepatic thionein induced by Cd in rats has been reported to be 3.5 [6] or 4.2 days [7], whereas Cd^{2+} itself shows a negligible hepatic turnover. Renal Cd-thionein has been reported to have a turnover half-life of 3.7 days [6]. Chen et al. [7] have shown that Zn^{2+}, which invariably is complexed to Cd-induced thionein, exhibits a turnover of about 4 days which is much shorter than that observed for Cd but longer than when zinc alone is used to induce thionein.

At present, results of these short term studies have been simply extrapolated to longer periods of time. It has been suggested but not proven that the negligible turnover of hepatic Cd with time is not due to a highly stable Cd-thionein complex, but is the result of a rebinding of Cd to thionein chains as the protein molecules are turned over [6]. The present study was initiated to measure Cd-thionein turnover over an extended period of time to determine if the complex does indeed continue to turnover in both liver and kidney long after a single exposure to Cd. A second objective involved assessment of changes in the Zn content of the protein with time as a means of evaluating long-term effects of elevated tissue levels of Cd on Zn metabolism. Such information is essential to obtaining a complete understanding of Cd metabolism and the toxic effects of Cd in vivo after extended periods of time.

Materials and Methods

Chemicals. [^{35}S]cysteine (specific activity, 13 Ci/mmol) was purchased from New England Nuclear. Sephadex G-75 was obtained from Pharmacia Fine Chemicals, and DEAE-cellulose (DE-52) was from Whatman. All other chemicals employed were analytical reagent grade.

Analytical. Concentrations of zinc and cadmium were determined using a Jarrell-Ash Model 850 atomic absorption spectrophotometer. Absorbance at 250 and 280 nm was measured using a Gilford model 250 spectrophotometer. [^{35}S]cysteine incorporation was measured by counting 1-ml aliquots dissolved in 10 ml Aquasol LSC cocktail in a Packard Tricarb liquid scintillation spectrometer.

Induction and isolation of metallothionein. 30 Charles River rats (weighing 150—200 g) were injected subcutaneously on two consecutive days with either physiological saline or 2 mg Cd/kg body weight as $CdCl_2$. The rats were fed commercial rat chow ad libitum. At the end of months 1, 2, 3, 4 and 6 after injection, three control and three Cd-treated animals were pulse-labeled with [^{35}S]cysteine (20 μCi/animal) 2 h prior to being killed by decapitation. The livers and kidneys were immediately removed and placed into physiological saline before weighing. The tissues were minced in 4 vol. 10 mM potassium phosphate (pH 7.8) at 4°C and homogenized in a glass-Teflon Potter-Elvehjem homogenizer. The resulting homogenates from each group were pooled and centrifuged at 4°C for 15 min at 27 000 $\times g$ in a Sorvall RC-5 centrifuge. The supernatant material was decanted and heat-treated in a shaker water-bath at 60°C for 10 min, and cooled in ice for 1 h. This material was centrifuged at 27 000 $\times g$ for 15 min prior to being lyophilized.

Freeze-dried material was dissolved in 10—15 ml 10 mM phosphate buffer

(pH 7.8) and chromatographed on a 5 × 80 cm G-75 Sephadex (fine) column equilibrated and eluted with 10 mM phosphate buffer (pH 7.8). In some cases, the metallothionein fractions were further purified by DEAE-cellulose column chromatography following concentration by lyophilization and dialysis in a Bio-Rad hollow fiber 50 ml beaker. Protein fractions were eluted from the DEAE-cellulose column using a linear gradient of 0—0.050 M NaCl in 0.01 M Tris-HCl (pH 8.6). Pooled protein fractions corresponding to forms I and II were further characterized by gel electrophoresis using 7.5% polyacrylamide gels [8].

Results

Rats injected subcutaneously with either $CdCl_2$ or saline were killed in groups of three after 1, 2, 3, 4 and 6 month intervals. The cadmium contents of the liver and kidney pooled supernatants of these animals are shown in Table I. There was no measurable change in the hepatic Cd concentration during the 6 month period after injection of Cd, whereas the renal Cd level increased 2-fold between the first and sixth month.

Soluble protein fractions prepared from the crude homogenates of the ten groups were separately chromatographed on Sephadex G-75 columns to determine the amount of [^{35}S]metallothionein present in the samples and the elution patterns of the Cd^{2+}. The elution profile for the liver supernatants from the 3-month group is given in Fig. 1. In this figure, the profile of the Cd-injected groups is shown together with that of a comparable saline-injected group. Similar profiles were generated for the other time points and even after 6 months (Table II) the hepatic Cd is still complexed to the low molecular weight metallothionein. In order to establish that the major Cd-binding component was metallothionein, low molecular weight Cd elution fractions from the 6-month group were pooled and chromatographed on a DEAE-cellulose column (Fig. 2). The Cd-binding component eluted as two major fractions with peak tube conductances of 0.5 and 0.8 mmho. Both fractions contained bound Cd and Zn, with the metal ion peaks being coincident with the 250 nm protein peaks. The two forms of the metalloprotein were dialyzed against 5 mM potas-

TABLE I

CADMIUM CONTENT OF LIVER KIDNEY IN CONTROL AND Cd-INJECTED RATS

Values represent averages of Cd content per animal of pooled supernatant material of three animals per group. No comparable determinations for Zn were performed.

	Total supernatant hepatic Cd (μgatoms)		Total supernatant renal Cd (μgatoms)	
Month	Injected	Control	Injected	Control
1	1.36	0.0009	0.178	0.0030
2	1.41	0.0005	0.284	0.0030
3	1.33	0.0003	0.311	0.0003
4	1.42	0.0011	0.418	0.0017
6	1.33	0.0115	0.427	0.0035

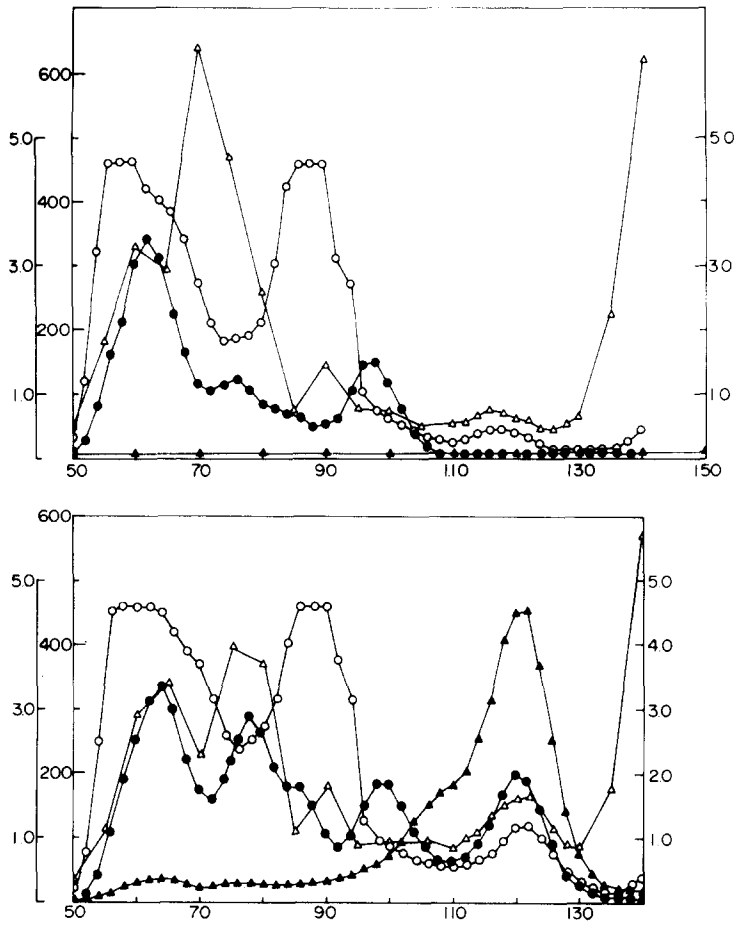


Fig. 1. Elution profile from a Sephadex G-75 column for liver supernatant fractions from control (a) and cadmium (b) injected rats at 3 months showing the low molecular weight Cd-Zn peaks and ^{35}S label. (○) represents A_{250}, (△) represents ^{35}S cpm/ml, (●) ppm Zn, (▲) ppm Cd.

sium phosphate, (pH 7.8), concentrated by lyophilization, and subjected to electrophoresis in 7.5% polyacrylamide gels (Fig. 3). The form I fraction gave a single Coomassie-blue-stained protein band with an R_f of 0.4, whereas the form II fraction showed a major band with an R_f of 0.6. Thus, both the ion-exchange elution and electrophoresis behavior strongly indicate that the 6-month hepatic Cd component is Cd,Zn-thionein, as indicated by others [2].

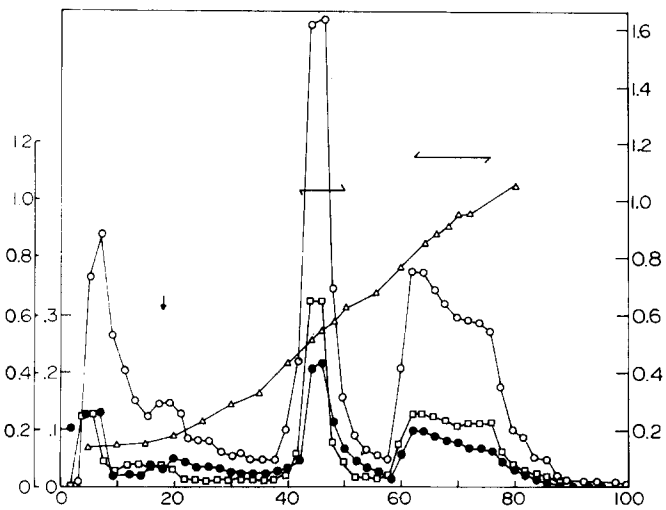
As can be seen in Table II at all time points after Cd injection, there was still an enhanced incorporation on [^{35}S]cysteine into thionein in the experimental groups as measured by a 2-h pulse of [^{35}S]cysteine prior to killing. [^{35}S]cysteine/Cd ratios were calculated by dividing the difference in peak tube radioactivities (cpm) of the Cd and saline groups by the peak tube Cd concentration. The ratios in the liver varied between 30 and 70 for the five different time groups. A similar situation existed in kidneys, and renal metallothionein ^{35}S/Cd ratios were calculated to vary between 160 and 350 for the five time points. The

TABLE II

HEPATIC AND RENAL THIONEIN Cd AND Zn CONCENTRATIONS AND Cd/Zn RATIOS

Results were obtained for liver and kidney thionein over a 6 month period. Values for Cd and Zn represent metal concentration (μg/ml) obtained in the peak tube of the G-75 Sephadex thionein fraction elution volume (see Fig. 1). Calculation for ^{35}S/Cd were made after taking ^{35}S decay into account and subtracting cpm values in control G-75 thionein elution volume peak from the cpm values in the Cd-injected G-75 thionein volume peak. This cpm value was divided by the G-75 thionein elution volume peak Cd concentration (μg/ml).

Month	Liver				Kidney			
	Cd	Zn	Cd/Zn (mol/mol)	^{35}S/Cd	Cd	Zn	Cd/Zn (mol/mol)	^{35}S/Cd
1	42	2.2	1.10		—	—	—	—
2	4.0	2.0	1.16	63	0.60	0.20	1.74	330
3	4.5	2.0	1.31	32	1.50	0.18	4.83	185
4	4.0	2.5	0.93	71	1.00	0.04	14.5	160
6	4.0	2.0	1.16	25	0.36	0.02	10.4	350



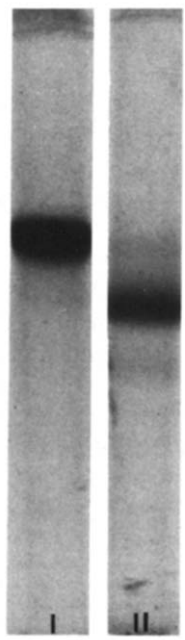


Fig. 2. DEAE-cellulose column chromatography profile for low molecular weight Cd peak fraction from the Sephadex G-75 column profile at 6 months showing two distinct peaks (I) and (II) coincident with the 250 nm protein peaks. ○ represents ppm Cd, □ represents ppm Cd, ● represents A_{250}, △ represents conductivity. Arrow (↓) indicates gradient initiation and (⟵⟶) indicates peak fractions pooled for electrophoresis.

Fig. 3. Coomassie-blue-stained polyacrylamide gels of the two Cd-Zn protein peaks shown in Fig. 1. The two bands have R_F values of 0.4 (I) and 0.6 (II), respectively.

enhanced incorporation of [^{35}S]cysteine indicates continued turnover of thionein in both the liver and kidney. Although the ^{35}S/Cd ratios show great variation for the different time points for both organs, the fact that there is enhanced radioactivity in the thionein fraction indicates continued thionein induction. The greater magnitude of the ^{35}S/Cd ratio for the renal thionein fraction compared to the hepatic thionein may reflect differences in tissue uptake of the labeled amino acid, tissue amino acid pool sizes or perhaps varying rates of thionein turnover in the two organs. Oh et al. [9] and Feldman et al. [6] have reported similar turnover rates for liver and kidney Cd-thionein at short-time periods, however, which suggests that the increased renal ^{35}S/Cd ratio is not a reflection of a difference in the turnover rate for the kidney.

Another marked difference between liver and kidney thionein concerned changes in Cd/Zn ratios for these organs (Table II). The hepatic Cd/Zn mol/mol ratio for thionein was found to remain relatively constant at around 1 throughout the study, whereas the Cd/Zn ratio for kidney was found to increase from about 2 to as high as 14.5 over the time points samples. Hepatic Cd,Zn-thionein isolated within 1 week of Cd injection has been previously shown to exhibit a Cd/Zn ratio between 2.0 and 3.0 [10] on a weight basis.

Discussion

Animals injected with $CdCl_2$ accumulate cadmium primarily in the liver and kidney. With time there is a gradual increase in renal Cd, and it has been reported that a corresponding decrease occurs in liver Cd [1,11]. This suggests that a translocation of Cd^{2+} occurs between the liver and kidney. In the present study, it is unclear from which body pool of Cd the kidney derives the metal ions. However, the liver does not appear to be the source since the hepatic Cd concentration remained relatively constant during the 6 month period. On the other hand, slow release of Cd from the injection site to the liver with subsequent release to the kidney following establishment of a stable hepatic Cd pool cannot be excluded. Within each tissue, Cd^{2+} is complexed predominately to thionein as Cd,Zn-thionein. Data presented here indicate that thionein undergoes continued turnover in both tissues. Several months after injection of $CdCl_2$, the metal ions still stimulated the incorporation of [^{35}S]cysteine into hepatic and renal thioneins. It is known that the turnover half-time for liver Cd-thionein is about 4 days [6,7] but Cd^{2+}, unlike Zn^{2+}, exhibits an unmeasurably long half-time [7]. Apparently, the liver is unable to efficiently eliminate Cd^{2+}, thereby leaving the Cd^{2+} to re-induce thionein synthesis. The ability of Cd to induce thionein formation has been demonstrated in several laboratories [1—4,9,12]. Both polymorphic forms of hepatic thionein, I and II, are induced to an equal extent in short-term [2] as well as long-term injection studies (Fig. 3). This is consistent with the similar turnover rate of the two major forms of thionein reported by Feldman et al. [6].

Although the zinc content is somewhat lower in 6-month hepatic thionein than in the metalloprotein isolated after short-term injection studies [10], the presence of Zn in this protein is intriguing. In a study of the zinc content of Cd-induced thionein, Winge et al. [10] found that several factors were involved in Zn binding to Cd-induced thionein. Major factors appeared to involve effects

of Cd^{2+} on intestinal motility and absorption. These effects resulted in an apparent mobilization of Zn^{2+} to the liver where it became complexed to thionein molecules. Another factor was the hepatic Zn trap created by available binding sites on induced thionein. These high-affinity binding sites trap hepatic Zn^{2+}, thereby shifting Zn homeostasis to a point where greater hepatic uptake of Zn occurs. The presence of Zn after several months in hepatic and, to a lesser extent, renal Cd-induced thionein must result from this thionein trapping mechanism.

Despite the turnover of the metallothionein molecule and the apparent shorter half-life for Zn^{2+} compared to Cd^{2+} as reported by Chen et al. [7] in a short-term study, the ratio of Cd to Zn in liver thionein remains constant over the extended periods of time examined in this study. This suggests that zinc continues to be trapped by thionein chains in the liver as they are resynthesized to bind the stable Cd^{2+}.

In the kidney, however, the ratio of Cd to Zn in thionein increases with time. This is due, in part, to the continued influx of Cd^{2+} into the kidney, which may replace Zn^{2+} on metallothionein rather than bind to new sites. Another possibility is that the rebinding of Zn^{2+} to resynthesized chains of thionein could be much lower in the kidney than the liver because of a smaller pool of available Zn^{2+} in the kidney or a more efficient excretion of Zn^{2+} in the urine.

This study has demonstrated that Cd-thionein is, indeed, continually turned over in both hepatic and renal tissue long after an initial single exposure to Cd. The long half-life of Cd within the liver appears to be the result of an inability of hepatocytes to excrete Cd^{2+} prior to its being rebound by thionein chains. In a similar manner, Zn^{2+} are also trapped by hepatic thionein molecules, leasing to a long-term perturbation of hepatic zinc levels in cadmium-exposed animals.

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