Uptake and binding of cadmium and mercury to metallothionein in rat hepatocyte primary cultures

Ronald J. GERSON and Zahir A. SHAIKH*

Division of Toxicology, Department of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

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The administration of inorganic Cd and Hg *in vivo* has been shown to result in markedly different metal concentrations in rat liver. Primary cultures of rat hepatocytes were utilized to gain insight into the dispositional differences between these chemically similar metals. Hepatocyte monolayer cultures were exposed to several concentrations of Cd or Hg (3, 10 and 30 μM) in serum-containing medium for 30 min. The cells were then washed and incubated in fresh medium for the remainder of the experiment. Hepatocytes exposed to Cd accumulated significantly more metal than hepatocytes exposed to equimolar concentrations of Hg. In cells exposed to 3 μM -Cd there was an initial loss of Cd from the hepatocytes when placed in fresh medium, followed by a gradual re-uptake of metal, concomitant with increased binding to metallothionein. In hepatocytes exposed to 3 and 10 μM -Cd, 87 and 77% of the intracellular Cd was bound to metallothionein within 24 h. Loss of Hg from hepatocytes pulsed with 30 μM -Hg was also observed upon the addition of fresh medium and continued for the duration of the experiment. No time-dependent increase in Hg binding to metallothionein was observed. A maximum of about 10% of the intracellular Hg was found associated with metallothionein in hepatocytes exposed to 30 μM -Hg. Studies utilizing [^{35}S]cysteine incorporation indicated significant increases in the amount of metallothionein synthesized in hepatocytes exposed to 3 and 10 μM -Cd (300% of control value) and 30 μM -Hg (150% of control value) 24 h after metal pulsing. Time-course studies revealed a 6–12 h lag in metallothionein synthesis, followed by a significant elevation in [^{35}S]cysteine incorporation into metallothionein between 12 and 24 h. These studies suggest that (a) isolated hepatocytes differentiate between Cd and Hg and preferentially accumulate the former, and (b) Cd strongly stimulates the induction of, and preferentially binds to, metallothionein, whereas Hg induces weakly, and does not preferentially bind to, metallothionein.

The administration of inorganic Cd and Hg *in vivo* has been shown to result in markedly different tissue distributions of these metals (Piotrowski & Szymanska, 1976). Whereas a large proportion of the Cd body burden has been shown to accumulate in rat liver (Friberg *et al.*, 1974), exposure of rats to Hg results in its preferential accumulation in the kidney (Piotrowski *et al.*, 1974; Cherian &

Clarkson, 1976). Both metals are known to induce and bind a cytosolic protein known as metallothionein (Shaikh & Lucis, 1970; Webb, 1972; Shaikh *et al.*, 1973; Piotrowski *et al.*, 1974; Winge *et al.*, 1975; Shaikh & Smith, 1975, 1976; Cherian & Clarkson, 1976; Sato & Nagai, 1980; Zelazowski & Piotrowski, 1980). Previous investigations have not determined why these chemically similar metals have such different tissue distributions *in vivo*.

There are several investigations in which workers have reported the induction of metallothionein in rat liver after Hg exposure (Suzuki, 1972; Martin & Brady, 1977). Durnan & Palmiter (1981) found Hg to be a weak inducer of mouse hepatic metallothionein *in vivo* and reported corresponding increases in mouse hepatic metallothionein mRNA

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; HBS solution, Hanks balanced salt solution; DME medium, Dulbecco modified Eagle medium.

* Present address and address for reprint requests: Department of Pharmacology and Toxicology, College of Pharmacy, University of Rhode Island, Kingston, RI 02881, U.S.A.

after exposure to Hg *in vivo*. Although these studies seem to suggest that Hg may induce hepatic metallothionein, one cannot ascertain whether Hg directly stimulated metallothionein induction, or did so indirectly, via stress-mediated elevations in glucocorticoids (Failla & Cousins, 1978a; Karin *et al.*, 1980; Klaassen, 1981; Mayo & Palmiter, 1981; Sato *et al.*, 1981).

In recent years a good deal of research has examined the disposition of Cd in hepatocyte cell cultures. Cd has been shown to induce the synthesis of metallothionein in exposed primary cultures (Hidalgo *et al.*, 1978; Bryan *et al.*, 1979; Failla *et al.*, 1979) and in a liver cell line (Daniel *et al.*, 1977; Rudd & Herschman, 1978). No studies have been reported on the disposition of Hg in primary hepatocyte cultures. The present study was carried out to compare the uptake of Cd and Hg and subsequent induction of metallothionein in primary cultures of rat hepatocytes.

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Materials and methods

Chemicals

Calcium-free HBS solution, DME medium, MEM non-essential amino acids and heat-inactivated calf serum were purchased from GIBCO (Grand Island, NY, U.S.A.). Type IV collagenase, insulin, gentamycin sulphate and Hepes buffer were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HgCl₂ was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). CdCl₂ was purchased from Alfa Products (Beverly, MA, U.S.A.). ¹⁰⁹CdCl₂ (378 mCi/mg) and ²⁰³HgCl₂ (2.56 mCi/mg) as well as [³⁵S]cysteine (1240 mCi/μmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.).

Hepatocyte cultures

Hepatocytes were prepared from livers of 175–200 g male Sprague–Dawley rats by using the two-step perfusion technique as described by Seglen (1976). The hepatocytes were separated from liver fragments, cellular debris and other non-parenchymal cells by filtration through cheesecloth followed by three 2-min centrifugations at 10 g and 0°C. Viability after these purification steps, as determined by Trypan Blue dye exclusion, was between 80 and 95%.

The cells were pipetted into 75 cm² flasks [approx. (4–6) × 10⁶ cells per flask] containing DME medium supplemented with: insulin (1 μg/ml), non-essential amino acids (0.1 mM), gentamycin (50 μg/ml), 10% (v/v) calf serum, 25 mM-Hepes buffer and glucose

(4500 mg/litre). The flasks were then placed in an incubator (37°C; air/CO₂, 19:1) and the cells were allowed to attach for 1.5 h. At the end of this time the medium and unattached cells were decanted and replaced with fresh medium. The cells were then allowed to incubate overnight before the start of the experiment.

Pulse exposure to Cd or Hg

The medium from the flask was decanted and replaced with fresh medium (same composition as above) containing either 3, 10 or 30 μM-CdCl₂ with 0.3 μCi of ¹⁰⁹Cd/flask or 3, 10 or 30 μM-HgCl₂ with 5 μCi of ²⁰³Hg/flask. The flasks were then incubated at 37°C in air/CO₂ (19:1) for 30 min. At the end of the 30 min pulse the radioactive medium was decanted and the cells were washed three times with 3 ml of Hepes-buffered HBS solution. Fresh, metal-free medium was then added to the flasks and the cells were incubated until the time they were harvested. In experiments utilizing [³⁵S]cysteine, 10 μCi/flask of the radiolabelled amino acid was added with the fresh metal-free medium.

Harvest of cells

The medium was decanted from the flasks and the cells were washed three times with buffered HBS solution. Approx. 2 ml of water was then added to each flask, the cells were scraped free with the use of a rubber policeman and frozen until assayed. Cell harvest times, depending on the experimental protocol were: immediately after the 30 min incubation with metal (zero time) and at 1, 4, 6, 8, 12 and 24 h after exposure to metal.

Assay for metallothionein

Cell suspensions were thawed and then sonicated at 50 W for 10 s (model W 185 Sonifier; Heat Systems Ultrasonics, Plainview, NY, U.S.A.). The resulting homogenate was counted for radioactivity on a Packard Auto Gamma scintillation spectrometer or a Packard Tri-Carb 2450 liquid-scintillation counter and a sample was taken for protein determination by the Lowry method (Lowry *et al.*, 1951). The homogenate was then centrifuged at 100 000 g for 60 min (Beckman L5-50 ultracentrifuge) and the resulting supernatant (cytosol) was counted for radioactivity. A portion (1 ml) of cytosol from each sample was applied to a column (0.9 cm × 60 cm) of Sephadex G-75 and eluted with 0.5 mM-Tris buffer, pH 8.6, containing 0.02% NaN₃. Radioactivity associated with the metallothionein fractions (elution-volume/void-volume ratio 1.5–2.0) and with the fractions containing high-molecular-weight proteins was individually combined and the resulting radioactivity counts corrected for recovery, which ranged between 85 and 98%.

Determination of non-protein-bound-metal concentration in medium

Non-protein-bound metal was determined in metal-containing medium labelled with either ^{109}Cd or ^{203}Hg by precipitating the proteins in 250 μl of medium with 1.5 ml of ethanol/acetone (1:1, v/v) solution. The suspended proteins were pelleted by centrifuging at maximum speed in a IEC model HN centrifuge for 10 min. The resulting supernatant was then counted for radioactivity in a Packard Auto Gamma scintillation spectrometer.

Results

Effect of metal dose

The concentrations of total as well as non-protein-bound Cd and Hg in the cell-culture medium are shown in Table 1. At 3 and 10 μM -Cd and -Hg there was more non-protein-bound Cd than Hg. At 30 μM there was twice as much non-protein-bound Hg as Cd.

The concentration of Cd or Hg (expressed as pmol of metal per mg of homogenate protein) in hepatocyte homogenate, cytosol and metallothionein, 24 h after exposure to 3, 10, and 30 μM -metal are also shown in Table 1. At concentrations of 3 and 10 μM -Cd, 87 and 77% of the intracellular Cd was found in the cytosol, with 70 and 65% of the intracellular Cd bound to metallothionein respectively. At a concentration of 30 μM -Cd, cytotoxicity was observed, as indicated by a significant elevation in the release of lactate dehydrogenase into the incubation medium and by morphological changes in cell cultures (results not shown). This produced a decrease in the amount of Cd in cytosol and metallothionein fractions to 46 and 18% of total cell Cd respectively.

In contrast with Cd, Hg demonstrated a much different intracellular distribution. At all concen-

trations of Hg, between 70 and 80% of intracellular Hg was found in the membranous fractions (100000g pellet). The concentration of Hg in the homogenate was always significantly lower than the corresponding concentrations of Cd after exposure to equimolar concentrations of metal. At 30 μM -Hg a maximum of 10% of the intracellular Hg was found associated with metallothionein. No cytotoxicity was observed at any of the Hg concentrations used in these experiments.

Corresponding experiments were conducted with [^{35}S]cysteine to monitor the synthesis of metallothionein. The uptake of [^{35}S]cysteine and its incorporation into metallothionein and other proteins was measured in hepatocytes 24 h after pulsing with 3 and 10 μM -Cd (Table 2). Since the previous study had demonstrated exposure to 30 μM -Cd to be toxic, 30 μM -Cd was not utilized in this experiment. The amount of [^{35}S]cysteine incorporated into metallothionein (as determined by Sephadex G-75 column chromatography) in hepatocytes pulsed with 3 and 10 μM -Cd was elevated 3-fold over that found in control hepatocytes.

No significant difference between the [^{35}S]cysteine incorporation into metallothionein in hepatocytes pulsed with 3 and 10 μM -Cd was observed. [^{35}S]Cysteine incorporation into high-molecular-weight proteins continued at control levels in hepatocytes pulsed with 3 or 10 μM -Cd, despite the fact that the concentration of [^{35}S]cysteine associated with the cytosolic fraction was significantly altered.

The incorporation of [^{35}S]cysteine into metallothionein in Hg-pulsed hepatocytes was significantly increased over controls after exposure to 30 μM -Hg. No significant differences with respect to controls in [^{35}S]cysteine incorporation into high-molecular-weight proteins were observed at any of the Hg concentrations used.

Table 1. *Effect of Cd and Hg concentration in the medium on metal uptake, distribution and binding in the hepatocytes* Hepatocytes were exposed to Cd and Hg as described in the Materials and methods section and harvested 24 h after metal exposure. The metal concentration in homogenate, cytosol and metallothionein was determined. The concentration of non-protein-bound metal in the medium was determined as described in the Materials and methods section. Values given are means $\pm$ S.E.M. of four separate determinations.

Metal	Metal concentration				
	In medium (μM)		As pmol/mg of homogenate protein		
	Total	Non-protein	Homogenate	Cytosol	Metallothionein
Cd	3	0.44 $\pm$ 0.02	472 $\pm$ 14	408 $\pm$ 16	336 $\pm$ 27
Hg	3	0.06 $\pm$ 0.002	59 $\pm$ 6	10 $\pm$ 0.04	0.5 $\pm$ 0.5
Cd	10	1.59 $\pm$ 0.05	993 $\pm$ 23	771 $\pm$ 20	651 $\pm$ 18
Hg	10	0.81 $\pm$ 0.01	125 $\pm$ 10	37 $\pm$ 2	6 $\pm$ 0.9
Cd	30	5.46 $\pm$ 0.42	1233 $\pm$ 55	573 $\pm$ 49	225 $\pm$ 61
Hg	30	10.72 $\pm$ 0.15	886 $\pm$ 47	237 $\pm$ 4	84 $\pm$ 5*

* No significant difference from respective Cd concentration was detected as calculated by Student's *t* test, $P < 0.05$.

Table 2. *Effect of Cd and Hg concentration in the medium on [³⁵S]cysteine uptake, distribution and incorporation into hepatocytes*

Hepatocytes were exposed to Cd or Hg as described in the Materials and methods section and then incubated in the presence of [³⁵S]cysteine for 24 h before cell harvest. Control hepatocytes were incubated in the presence of [³⁵S]cysteine for 24 h. [³⁵S]cysteine associated with the homogenate, cytosol, high-molecular-weight proteins and metallothionein was determined. Each value represents the mean $\pm$ S.E.M. of four separate determinations.

		[³⁵ S]Cysteine radioactivity (c.p.m./mg of homogenate protein)			
	Concn. (mM)	Homogenate	Cytosol	High-molecular-weight proteins	Metallothionein
Control	—	83 322 $\pm$ 5252	60 758 $\pm$ 2898	12 126 $\pm$ 1637	1785 $\pm$ 116
Cd	3	120 726 $\pm$ 7584*	89 219 $\pm$ 2825*	14 024 $\pm$ 783	5239 $\pm$ 204*
	10	47 213 $\pm$ 657*	29 562 $\pm$ 1656*	11 179 $\pm$ 433	5279 $\pm$ 167*
Hg	3	34 109 $\pm$ 1785*	21 935 $\pm$ 1126*	11 957 $\pm$ 975	1494 $\pm$ 132
	10	35 318 $\pm$ 1320*	24 214 $\pm$ 1199*	10 197 $\pm$ 295	1746 $\pm$ 43
	30	61 388 $\pm$ 2364*	48 944 $\pm$ 3185*	13 209 $\pm$ 732	2823 $\pm$ 222*

* Significantly different from control as calculated by analysis of variance, $P < 0.05$.

Table 3. *Time course of [³⁵S]cysteine uptake, distribution and incorporation in hepatocytes pulsed with 3 μ M-Cd or 30 μ M-Hg*

Hepatocytes were exposed to Cd or Hg as described in the Materials and methods section and then incubated in the presence of [³⁵S]cysteine until they were harvested. Control hepatocytes were incubated in the presence of [³⁵S]cysteine alone. The [³⁵S]cysteine was determined in homogenate, cytosol, high-molecular-weight proteins and metallothionein. Each value represents the mean $\pm$ S.E.M. of four separate determinations.

		[³⁵ S]Cysteine radioactivity (c.p.m./mg of homogenate protein)			
Time (h)		Homogenate	Cytosol	High-molecular-weight proteins	Metallothionein
1	Control	18 009 $\pm$ 1785	16 957 $\pm$ 1931	6 854 $\pm$ 739	1005 $\pm$ 115
	Cd	29 099 $\pm$ 2221*	21 896 $\pm$ 1248	5 923 $\pm$ 682	901 $\pm$ 38
	Hg	30 029 $\pm$ 1750*	17 799 $\pm$ 1491	6 940 $\pm$ 787	972 $\pm$ 84
6	Control	—	—	—	—
	Cd	35 754 $\pm$ 1276	25 577 $\pm$ 1136	6 790 $\pm$ 284	1184 $\pm$ 70
	Hg	47 523 $\pm$ 3521†	33 885 $\pm$ 3516	10 059 $\pm$ 709	1642 $\pm$ 124†
12	Control	35 199 $\pm$ 2172	22 113 $\pm$ 1478	8 709 $\pm$ 581	1307 $\pm$ 49
	Cd	53 692 $\pm$ 5934*	36 835 $\pm$ 4773*	10 786 $\pm$ 798	4111 $\pm$ 170*
	Hg	44 403 $\pm$ 1147*	32 593 $\pm$ 1414*	9 685 $\pm$ 348	1841 $\pm$ 51*
24	Control	83 322 $\pm$ 5252	60 758 $\pm$ 2898	12 126 $\pm$ 1637	1785 $\pm$ 116
	Cd	120 726 $\pm$ 7584*	89 219 $\pm$ 2825*	14 024 $\pm$ 783	5329 $\pm$ 204*
	Hg	61 388 $\pm$ 2364*	48 944 $\pm$ 3185*	13 209 $\pm$ 732	2823 $\pm$ 222*

* Significantly different from respective control as calculated by multiple-inference Student's t test, $P < 0.05$.

† Values did not lie within the 95% confidence interval of regression line calculated for control data.

Time course of [³⁵S]cysteine incorporation into metallothionein and high-molecular-weight proteins after exposure to Cd or Hg

The amount of [³⁵S]cysteine incorporated into the metallothionein fraction with time in control hepatocytes and hepatocytes pulsed with 3 μ M-Cd or 30 μ M-Hg is shown in Table 3. In hepatocytes pulsed with 3 μ M-Cd, a significant stimulation of [³⁵S]cysteine incorporation into metallothionein occurred between 6 and 12 h after Cd pulsing as compared with controls. In hepatocytes pulsed with

30 μ M-Hg, a significant increase in [³⁵S]cysteine incorporation into metallothionein was seen between 1 and 6 h after the Hg pulse as compared with controls. No significant difference in [³⁵S]cysteine incorporation into high-molecular-weight proteins was observed in hepatocytes pulsed with 3 μ M-Cd or 30 μ M-Hg, when compared with controls, even though significant differences in the concentration of [³⁵S]cysteine associated with cytosol were observed as compared with controls. The amount of [³⁵S]cysteine associated with hepatocytes was elevated at all times after Cd pulsing as compared with

Table 4. Time-dependent association of [35 S]cysteine with the membranous fraction of control hepatocytes and hepatocytes exposed to 3 μ M-Cd or 30 μ M-Hg

Hepatocytes were exposed to Cd or Hg as described in the Materials and methods section and then incubated in the presence of [35 S]cysteine for the duration of the experiment. The membranous fraction represents the 100 000 g pellet. Values given are means $\pm$ S.E.M. of four separate determinations.

Time (h)	Proportion of homogenate [35 S]cysteine (%)		
	Control	Cd	Hg
1	6 $\pm$ 3	24 $\pm$ 2*	41 $\pm$ 2*
6	—	29 $\pm$ 2	29 $\pm$ 2
12	38 $\pm$ 1	29 $\pm$ 8	27 $\pm$ 2*
24	26 $\pm$ 7	25 $\pm$ 7	20 $\pm$ 5

* Significantly different from controls as calculated by multiple-inference Student's *t* test at $P < 0.05$.

controls. [35 S]Cysteine levels were significantly elevated in hepatocytes pulsed with 30 μ M-Hg at 1 h. These levels dropped below those found in controls by 24 h.

Determination of the fraction of [35 S]cysteine associated with the 100 000 g pellet (membranous fraction) revealed a 4-fold increase in Cd-pulsed hepatocytes over controls at 1 h (Table 4). A 7-fold increase in the [35 S]cysteine associated with the membranous fractions of Hg-pulsed hepatocytes, as compared with controls, 1 h after Hg pulsing, was also observed. The fraction of cellular cysteine associated with the membranous fraction returned to control levels within 24 h for both metals.

Time course of Cd and Hg distribution in hepatocytes

The time course of metal distribution in hepatocytes pulsed with 3 μ M-Cd was studied. After Cd exposure, an initial decrease was observed in the Cd concentration of the hepatocytes between 0 and 1 h (Fig. 1). This was due to the efflux of Cd associated with the hepatocyte into the fresh metal-free medium. After this efflux phase there was a gradual linear re-uptake of the previously released Cd. The Cd concentration in homogenate, cytosol and metallothionein fractions increased at the same rate (slopes of the lines were insignificantly different by analysis of co-variance at $P < 0.05$). By calculating the ratio of Cd bound to metallothionein versus Cd in the homogenate, a gradual redistribution of intracellular Cd, favouring binding to metallothionein with time, was observed. At zero time approx. 38% of the intracellular Cd was bound to metallothionein. This increased to approx. 64% within 8 h and to 75% within 24 h.

Fig. 1. Time course of Cd and Hg distribution in hepatocytes after pulsing with (a) 3 μ M-Cd and (b) 30 μ M-Hg

Hepatocytes were exposed to 3 μ M-Cd or 30 μ M-Hg as described in the Materials and methods section and harvested at various times after metal exposure. The concentration of metal in homogenate (O), cytosol (Δ), and metallothionein ($\square$) was determined. Each point represents the mean $\pm$ S.E.M. of four separate determinations; standard errors are shown where they are larger than the symbols.

Hepatocytes pulsed with 30 μ M-Hg also showed an initial rapid decrease in Hg concentration upon the addition of fresh medium (Fig. 1). This was then

followed by a second slower phase of Hg efflux which continued for the duration of the experiment. The half-life of Hg in the slow component was 75 h for whole cells and 27 h for cell cytosol. Unlike Cd-pulsed hepatocytes, there was no re-uptake of Hg from the medium and no increase with time in the amount of Hg bound to metallothionein.

Discussion

The present data indicate significant differences in the disposition of Cd and Hg in primary cultures of rat hepatocytes. At equivalent doses of metal, there was less Hg associated with the hepatocytes than Cd. This could be for a variety of reasons: (1) Hg may bind to the serum proteins in the medium more tightly than does Cd (as indicated by the data in Table 1). This finding is in agreement with the data of Jacobson & Turner (1980), who report higher affinity constants of Hg than Cd for various amino acids. This phenomenon may result in decreased diffusible (non-protein-bound) Hg available for hepatocyte uptake. (2) Since Hg does not seem to stimulate strongly the induction of metallothionein, or preferentially bind to metallothionein, as does Cd, Hg is not sequestered in the hepatocyte bound to metallothionein, as is Cd.

After a 30 min pulse exposure to $3\mu\text{M}$ -Cd or $30\mu\text{M}$ -Hg, an initial rapid efflux of metal associated with the hepatocytes into the fresh medium was observed. This probably represented a re-equilibration of free or loosely bound metal with new metal-binding sites in the fresh medium. Whether this decrease in metal content was due to the efflux of metal from the intracellular compartment and/or a decrease in the amount of metal associated with the hepatocyte plasma membrane cannot be ascertained from the present study. After the rapid efflux phase there was a gradual, linear re-uptake of the Cd, which was directly linked to an increasing association of Cd with metallothionein. In contrast with Cd, the loss of Hg from the hepatocytes continued at a slow rate for the duration of the experiment.

The intracellular redistribution of Cd with time, to favour binding of Cd to metallothionein rather than to other proteins, was essentially complete within 8 h of Cd pulsing. This correlates well with the observed induction of metallothionein synthesis (as measured by [^{35}S]cysteine incorporation) occurring between 6 and 12 h after pulsing.

Experiments studying [^{35}S]cysteine incorporation into metallothionein, after Hg pulsing, demonstrated an increase in [^{35}S]cysteine incorporation into metallothionein between 1 and 6 h after Hg pulsing (as compared with control cultures). This increase, however, appears not to be due to the specific induction of metallothionein, since a corresponding

slight increase in [^{35}S]cysteine incorporation into high-molecular-weight proteins was also observed (Table 3). A specific increase in [^{35}S]cysteine incorporation into metallothionein (with respect to high-molecular-weight-protein synthesis) is seen first between 6 and 12 h after Hg pulsing as in the case of Cd.

Lag times between 1 and 8 h have been reported for [^{35}S]cysteine incorporation into metallothionein after various induction stimuli in different systems (Shaikh & Lucis, 1971; Chen *et al.*, 1975; Shaikh & Smith, 1977; Rudd & Herschman, 1978; Cherian, 1980; Durnam & Palmiter, 1981; Olafson, 1981). The 6 h lag in our system could be due to a combination of factors. It is thought that metallothionein induction in liver is under transcriptional control (i.e. metallothionein mRNA must first be transcribed before synthesis of the protein is carried out) (Squibb *et al.*, 1977; Shaikh & Smith, 1977; Hidalgo *et al.*, 1978; Bryan *et al.*, 1979; Durnam & Palmiter, 1981). Secondly, since the hepatocytes are placed in [^{35}S]cysteine-containing medium after the metal exposure, a certain amount of time must pass before the external [^{35}S]cysteine pool reaches equilibrium with the internal cysteine pool.

Despite the 6 h lag in [^{35}S]cysteine incorporation into metallothionein, the re-uptake of Cd was observed to be linear from 1 to 24 h. Low levels of metallothionein are present in non-exposed tissues (Olafson, 1981), and it is possible that the initial linear portion of Cd uptake represents Cd binding to endogenous metallothionein. Our observation of detectable levels of [^{35}S]cysteine incorporation into the metallothionein fractions of control hepatocytes is in agreement with previous findings (Chen *et al.*, 1975; Squibb *et al.*, 1977; Ridlington *et al.*, 1981) and probably represents the normal turnover of endogenous metallothionein and other low-molecular-weight proteins.

Whereas most of the Cd was found in the cytosol associated with metallothionein, most of the Hg burden was always found associated with the membranous fraction of the cells. Komsta-Szumaska & Chmielnicka (1977) and Chmielnicka *et al.* (1979) reported similar findings in livers of rats given inorganic Hg *in vivo*; significant amounts of Hg were found associated with the nuclear, mitochondrial and microsomal pellets.

Even though the synthesis *de novo* of metallothionein was occurring, no increase in Hg binding to metallothionein was observed. Most of the Hg, 24 h after metal pulsing, was still found in the membranous fraction, despite the fact that Hg has a higher affinity for metallothionein *in vitro* than has Cd or Zn (Holt *et al.*, 1980). This suggests that the relative affinity of Hg for metallothionein, as compared with its affinity for the proteins in the membranous fraction, may not be as high as those of Cd or Zn

(Webb *et al.*, 1980). Therefore, as more metallothionein is synthesized, Cd or Zn would tend to bind in increasing quantity to the newly synthesized metallothionein, whereas Hg may partition in a distribution favouring its concentration in the membranous fraction. Whether Hg induced metallothionein directly, or did so indirectly by displacing Zn from endogenous binding sites, cannot be ascertained from our data. It is also possible that Hg may not have induced metallothionein synthesis. The increase in [35 S]cysteine incorporation into metallothionein may be due to an increased turnover of metallothionein after Hg administration (Durnam & Palmiter, 1981).

Cd and Hg exposures also had effects on net [35 S]cysteine associated with the hepatocytes. Elevations in the amount of [35 S]cysteine associated with the hepatocytes were observed within 1 h after pulsing with Cd or Hg. This early elevation probably does not represent true uptake or incorporation of cysteine, but an enhanced association of cysteine with the hepatocyte plasma membrane. This may be due to the formation of a cysteine-metal-membrane complex and is supported by the relative increase seen in [35 S]cysteine associated with the membranous fraction at 1 h in metal-exposed hepatocytes. Despite observed decreases in the amount of [35 S]cysteine associated with hepatocyte homogenate and cytosol, no differences were observed in [35 S]cysteine incorporation into high-molecular-weight proteins. This would suggest that protein synthesis was not inhibited in these experiments.

The present results have shown that Cd and Hg behave quite differently in primary cultures of hepatocytes. Cd strongly induces the synthesis of metallothionein, whereas Hg does not. Hg is found in greatest abundance associated with membranous fractions, whereas the intracellular distribution of Cd favours binding to the cytosolic protein, metallothionein. For equimolar concentrations of metal, Cd is accumulated to a much greater extent than Hg. These findings support previous experiments *in vivo* showing that Hg appears not to accumulate in rat liver as does Cd. The explanation for this phenomenon remains unknown.

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References

- Bryan, S. E., Hidalgo, H. A., Koppa, V. & Smith, H. A. (1979) *Environ. Health Perspect.* **28**, 281–285
- Chen, R. W., Whanger, P. D. & Weswig, P. H. (1975) *Biochem. Med.* **12**, 95–105
- Cherian, M. G. (1980) *Toxicology* **17**, 225–231
- Cherian, M. G. & Clarkson, T. W. (1976) *Chem.-Biol. Interact.* **12**, 109–120
- Chmielnicka, J., Komsta-Szumaska, E. & Jedrychowski, R. (1979) *Environ. Res.* **20**, 80–86
- Daniel, M. R., Webb, M. & Cempel, M. (1977) *Chem.-Biol. Interact.* **16**, 101–106
- Durnam, D. M. & Palmiter, R. D. (1981) *J. Biol. Chem.* **256**, 5712–5716
- Failla, M. L. & Cousins, R. J. (1978) *Biochim. Biophys. Acta* **543**, 293–304
- Failla, M. L., Cousins, R. J. & Mascenik, M. J. (1979) *Biochim. Biophys. Acta* **583**, 63–72
- Friberg, L., Piscator, M., Nordberg, G. F. & Kjellstrom, T. (1974) *Cadmium in the Environment*, 2nd edn., pp. 35–40, CRC Press, Cleveland, OH
- Hidalgo, H. A., Koppa, V. & Bryan, S. E. (1978) *Biochem. J.* **170**, 219–225
- Holt, D., Magos, L. & Webb, M. (1980) *Chem.-Biol. Interact.* **32**, 125–135
- Jacobson, B. K. & Turner, J. E. (1980) *Toxicology* **16**, 1–37
- Karin, M., Herschman, H. R. & Weinstein, D. (1980) *Biochem. Biophys. Res. Commun.* **92**, 1052–1059
- Klaasen, C. D. (1981) *Toxicology* **20**, 275–279
- Komsta-Szumaska, E. & Chmielnicka, J. (1977) *Arch. Toxicol.* **38**, 217–228
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, M. & Brady, F. O. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 72–76
- Mayo, K. E. & Palmiter, R. D. (1981) *J. Biol. Chem.* **256**, 2621–2624
- Olafson, R. W. (1981) *J. Biol. Chem.* **256**, 1263–1268
- Piotrowski, J. K. & Szymanska, J. A. (1976) *J. Toxicol. Environ. Health* **1**, 991–1002
- Piotrowski, J. K., Trojanowska, B., Wisniewska-Knypl, J. M. & Bolanowska, W. (1974) *Toxicol. Appl. Pharmacol.* **27**, 11–19
- Ridlington, J. W., Winge, D. R. & Fowler, B. A. (1981) *Biochim. Biophys. Acta* **673**, 177–183
- Rudd, C. J. & Herschman, H. R. (1978) *Toxicol. Appl. Pharmacol.* **44**, 511–521
- Sato, M. & Nagai, Y. (1980) *Toxicol. Appl. Pharmacol.* **54**, 90–99
- Sato, M., Sugano, H. & Takizawa, Y. (1981) *Arch. Toxicol.* **47**, 125–133
- Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
- Shaikh, Z. A. & Lucis, O. J. (1970) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **29**, 298
- Shaikh, Z. A. & Lucis, O. J. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 238
- Shaikh, Z. A. & Smith, J. C. (1975) *Int. Conf. Heavy Met. Environ.* B108–B110
- Shaikh, Z. A. & Smith, J. C. (1976) *Chem.-Biol. Interact.* **15**, 327–336
- Shaikh, Z. A. & Smith, J. C. (1977) *Chem.-Biol. Interact.* **19**, 161–171
- Shaikh, Z. A., Coleman, R. L. & Lucis, O. J. (1973) *Trace Subst. Environ. Health* **7**, 313–321
- Squibb, K. S., Cousins, R. J. & Feldman, S. L. (1977) *Biochem. J.* **164**, 223–228

Suzuki, Y. (1972) *Ind. Health* **10**, 56–58

Webb, M. (1972) *Biochem. Pharmacol.* **21**, 2751–2765

Webb, M., Magos, L. & Holt, D. (1980) *Chem.–Biol. Interact.* **32**, 137–149

Winge, D. R., Premakumar, R. & Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys.* **170**, 242–252

Zelazowski, A. J. & Piotrowski, J. K. (1980) *Biochim. Biophys. Acta* **625**, 89–99