Induction of Cadmium-Thionein in Isolated Rat Liver Cells

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The uptake of cadmium by isolated liver cells was linearly related to the cadmium concentration to which the cells were exposed in the medium. Cadmium-treated cells synthesized proteins *de novo* with the characteristics of cadmium-thionein induced in the liver of cadmium-treated animals. Thionein from liver cells incorporated cadmium and [³⁵S]cysteine, had a V_e/V_0 (Sephadex G-50) of 1.8–1.9, and was separated into two subfractions by DEAE-cellulose ion-exchange chromatography. Cycloheximide and actinomycin D when added after a cadmium exposure prevented the synthesis of thionein. However, addition of actinomycin D after synthesis had started only decreased the total amount of thionein synthesized. The concentration of cadmium to which the cells were exposed affected the amount of cadmium-thionein synthesized in 6 h. The maximum response occurred when cells were exposed to 0.5 µg of cadmium/ml; at higher metal concentrations the total amount of cadmium-thionein synthesized declined. The system described in the present paper can be used to study the mode of metal toxicity and the mechanism of cadmium-thionein synthesis.

Sulphur-rich metal-binding proteins (metallothioneins), first reported by Margoshes & Vallee (1957) in horse renal cortex, have been identified in a variety of other organisms (Bouqueneau *et al.*, 1975; Bremner & Marshall, 1974; Bush *et al.*, 1973; Kagi *et al.*, 1974; Kagi & Vallee, 1960; Olafson & Thompson, 1974; Pulido *et al.*, 1966). These proteins are induced in the liver and kidneys of animals in response to sublethal doses of certain heavy metals (Bremner & Young, 1976; Nordberg *et al.*, 1972; Shaikh & Lucis, 1971; Weser *et al.*, 1973; Winge & Rajagopalan, 1972; Wisniewska *et al.*, 1970), and their appearance is accompanied by enhanced incorporation of radioactively labelled amino acids into metallothionein fractions (Bremner & Davies, 1974; Cherian & Clarkson, 1976; Piotrowski *et al.*, 1974; Richards & Cousins, 1975a,b).

Information on the effects of cadmium and other metals has been largely obtained by studying the response of a given tissue after whole-body exposure to the metals. Although this approach is useful in establishing dose-response relationships, it has the disadvantage of not allowing for careful control of the metal concentrations that finally reach the target organ; further, this method provides no control over synergistic or antagonistic effects between different tissues. Hence there are few studies on the effect of cadmium on cells that synthesize cadmium-thionein, in the absence of other variables. To our knowledge the only work in this area has been

carried out by Webb and colleagues (Daniel & Webb, 1975; Daniel *et al.*, 1977) who, by using cells derived from pig kidney and liver, showed that the metal was incorporated into a protein fraction that behaved as metallothionein when fractionated by gel filtration. Those studies, however, did not include experiments (which we now report) on the time course of thionein synthesis, correlation of uptake of cadmium to thionein synthesis, incorporation of radioactively labelled amino acids into the cadmium-thionein fraction, and effects of protein-synthesis inhibitors on thionein synthesis. Results of studies such as these, and their comparison with what is known about cadmium-thionein synthesis in the whole animal, would provide necessary information to establish the viability of the use of liver and kidney single-cell systems for determining the effects of cadmium and other metals on these tissues.

Experimental

Materials

Male Sprague-Dawley rats weighing 250–300 g were used in all experiments. The animals, which were housed in galvanized-steel cages, had free access to Purina Rat Chow (Ralston Purina Co., St. Louis, MO, U.S.A.) and glass-distilled water. Analytical-grade cadmium chloride and other metal salts were from Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Sephadex G-50 was from Pharmacia (Piscataway, NJ, U.S.A.), Ultrapure Tris/HCl from Schwarz-Mann (Orangeburg, NY, U.S.A.). DEAE-cellulose

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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(Cellex-D) with an exchange capacity of 0.85 mequiv./g was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Tissue-culture-grade foetal calf serum was from North Carolina Biological Supply Co. (Burlington, NC, U.S.A.). ^{115}Cd (0.132 mCi/mg) and [^{35}S]cysteine (192 mCi/mg) were from New England Nuclear Corp. (Boston, MA, U.S.A.). Collagenase type IV, actinomycin D, cycloheximide, bovine serum albumin, Hepes, amino acids and vitamins were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Tissue solubilizer was from Eastman-Kodak Co. (Rochester, NY, U.S.A.). Buffers were prepared in resin-deionized water, filtered through filters (pore diam. 0.45 μm), and saturated with O_2 before use.

Assays

Protein concentrations were determined by a modification of the method of Lowry (Schacterle & Pollack, 1973) with bovine serum albumin as the standard. Zinc and cadmium were measured directly by flame atomic absorption spectrometry with a Perkin-Elmer model 360 instrument; cadmium concentrations were also calculated from the ^{115}Cd radioactivity. Samples (0.2 ml) containing ^{115}Cd or ^{35}S were dissolved (1:5, v/v) in tissue solubilizer, and radioactivity was measured as previously described (Bryan & Hidalgo, 1976). Counting efficiencies for ^{115}Cd and ^{35}S were 68 and 60% respectively. Trypan Blue-negative cells were counted with a haemocytometer after dilution with appropriate volumes of a 0.5% (w/v) Trypan Blue solution in the same buffer in which cells were suspended. Results given below in the text are means $\pm$ S.E.M. (n).

Isolation of rat liver cells

Rats were anaesthetized with sodium pentobarbital (40 mg/kg), and cells were isolated by the method of Berry & Friend (1969), as described by LeCam *et al.* (1976). Additional modifications were the continuous oxygenation of the recirculating collagenase-enzyme buffer, and the filtering of the cells through eight layers of cheesecloth after mechanical disruption of the liver. The cells were cooled to 4°C, and centrifuged in a Beckman J-21B refrigerated centrifuge for 2 min at 150g. The cell pellets were gently resuspended in 100 ml of cold calcium-free Earle's balanced salt solution, pH 7.4 (6.8 g of NaCl, 0.40 g of KCl, 0.10 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.00 g of glucose, 0.05 g of Phenol Red, 2.20 g of NaHCO_3 /litre of water) and the suspension was centrifuged once more as described above for 1.5 min. Typically at this stage the cells were resuspended in Earle's solution to a concentration of 100 mg cell wet wt./ml. Recoveries of hepatocytes ranged from 250 million to 400 million cells. Cells were diluted to 50 mg wet wt./ml with the

appropriate cadmium solutions prepared in Earle's solution. Portions (4 ml) were pipetted into 25 ml conical Nalgene flasks and incubated for 15 min in a water-saturated O_2/CO_2 (19:1) atmosphere at 37°C by using a Dubnoff metabolic incubator set at 60 oscillations/min. After cadmium preincubation at 37°C, cells were collected by centrifugation (150g for 1.5 min) and resuspended to one-half the volume used in the preincubation with Eagle's minimum essential medium (Eagle, 1955) prepared in Earle's solution containing 20 mM-Hepes, and supplemented with 10% (v/v) foetal calf serum (Eagle's medium+10% serum). Portions (2 ml) were incubated in 25 ml glass Erlenmeyer flasks at 30 oscillations/min as described above. At the appropriate times cells were removed from the flasks with the aid of a 'rubber policeman' for thionein isolation. The percentages of Trypan Blue-negative cells at the beginning and end of the incubation period were 78.3 ± 6.2 (17) and 49.3 ± 6.1 (9) respectively. These values are in good agreement with values reported by Hirsiger *et al.* (1976) for rat liver cells. O_2 uptake throughout the incubation period was 16.0 ± 3.4 (17) μl of O_2 /h per mg dry wt. in the presence of glucose, and was comparable to values reported by Berry & Friend (1969). Incorporation of [^{35}S]cysteine into trichloroacetic acid-precipitable protein was linear with time and proceeded at a rate of 2683 ± 435 c.p.m./h per mg dry wt. (6) under the incubation conditions described in the legend of Fig. 3(b) (below).

Isolation of cadmium-thionein

Cells from three flasks (6 ml total volume) were pooled and brought to 4°C before centrifugation at 150g for 1.5 min. The supernatants were decanted, the cells resuspended in 6 ml of 0.154 M-NaCl, and centrifuged as above. The cell pellets were resuspended in 3 ml of 0.154 M-NaCl and sonicated at 4 A for 5 s with a Branson sonifier. Thionein was isolated from the cell lysates by modification of the procedure of Cherian (1974). The cell lysates were centrifuged at 35000g for 15 min at 4°C; the resulting supernatants were heated to 70°C at a rate of 1.5°C/min, held at that temperature for 1 min, and were immediately cooled in an ice bath before centrifugation at 35000g for 10 min. A portion (2 ml) of the resulting supernatants was fractionated by using a water-jacketed column (1.65 cm $\times$ 65 cm) of Sephadex G-50, maintained at 4°C and equilibrated with 0.02 M-Tris/HCl (pH 8.6)/0.02% NaN_3 . Fractions (3 ml) were collected, and the volume at which the cadmium-thionein fraction was eluted ($V_e/V_0 = 1.8\text{--}1.9$) was predetermined by running a thionein-containing sample obtained from the liver of a rat treated *in vivo* with cadmium as previously described (Hidalgo & Bryan, 1977). In all cases the elution profile of the cadmium-thionein fraction isolated from liver cells

coincided with the fraction obtained from liver cytosols of cadmium-treated rats. The relative amounts of thionein present were determined by measuring the amounts of cadmium or [^{35}S]cysteine present in the five tubes containing the highest radioactivity count in the cadmium-thionein fraction, which represented more than 90% of the radioactivity associated with cadmium-thionein. The amounts of cadmium or [^{35}S]cysteine were normalized for the amounts of protein put on the column; the protein values from the heat-treated cytosols ranged from 0.35 to 0.50 mg of protein/ml.

The DEAE-cellulose fractionation of cadmium-thionein into two subfractions was carried out essentially as previously described (Hidalgo & Bryan, 1977). The pooled fractions containing cadmium-thionein recovered from gel filtration were used with the modification that the gradients ranged from 0.005 to 0.3M-Tris/HCl, pH 8.6. Under these conditions cadmium-thionein 1 was eluted at around 50mM-Tris/HCl and cadmium-thionein 2 around 65mM-Tris/HCl.

Results and Discussion

Cadmium uptake by liver cells

At a concentration of 50mg cell wet wt./ml in Earle's solution containing 1 μg of Cd/ml, $24.8 \pm 0.8\%$ (4) of the cadmium was absorbed by the cells. Cadmium uptake did not vary significantly with time, therefore a 15 min cadmium-exposure time was used in all subsequent experiments. During the cadmium preincubation the percentage of Trypan Blue-negative cells remained unchanged. When the cadmium concentrations in the preincubation buffer were varied, the metal uptake increased linearly with increasing cadmium concentration (see Fig. 1). These observations suggest that the cells absorbed the metal by diffusion and that an equilibrium was established fairly rapidly after exposure.

Typically after cadmium preincubation, cells were collected by centrifugation, resuspended in Eagle's medium+10% serum, and incubated for various times. Cadmium content in those cells exposed to 1 μg of Cd/ml decreased with time (compared with the cadmium in the cells when they were resuspended in Eagle's medium+10% serum) from 33.2 ± 3.4 (6) to $25.9 \pm 2.9\%$ (8) at 2 and 6h of incubation respectively. The cadmium remaining at 6h also depended on the concentration of metal to which the cells were initially exposed. At concentrations of 3.0, 1.0 and 0.1 μg of Cd/ml, the cells' cadmium content decreased (after 6h of incubation) to 39.0, 25.9 and 12.2% respectively. Thus the data indicated that (1) the higher the concentration of cadmium to which the cells were exposed the more they absorbed, and

Fig. 1. Cadmium uptake by isolated liver cells

Rat liver cell suspensions (2.65×10^6 cells/ml) were incubated for 15 min with various amounts of ^{115}Cd in Earle's solution (0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 μg of Cd/ml). At the end of the preincubation period, cells were collected by centrifugation, resuspended in Earle's solution and the radioactivity of the ^{115}Cd absorbed was determined.

(2) the higher the cadmium uptake by the cells, the more cadmium they retained.

Frazier & Kingsley (1976) reported that in the isolated perfused rat liver, at low doses of cadmium exposure, zinc was secreted in amounts approximately equimolar with the cadmium absorbed. When we measured the zinc content of rat liver cells after a 15 min exposure to various cadmium concentrations (0.1, 0.5, 1.0, 2.5 and 5.0 μg of Cd/ml per 3.3×10^6 cells) the zinc content of the cells [0.122 ± 0.007 (5) μg of Zn/ 10^6 cells] was independent of the cadmium concentration in the medium and was similar to the zinc content of control cells not exposed to cadmium [0.115 ± 0.003 (3) μg of Zn/ 10^6 cells]. Differences between the present data and those of Frazier & Kingsley (1976) are probably due to the different systems used. Apparently the response of the isolated perfused rat liver on exposure to cadmium is different from that of isolated liver cells.

Cadmium-thionein synthesis

The cadmium-thionein fraction was isolated by differential centrifugation of cell lysates, heat treatment and gel-filtration chromatography. Typical profiles obtained from the Sephadex G-50 column (Fig. 2a) are in agreement with previously published results obtained by using whole animals to induce and label thionein in the intact liver (Bryan & Hidalgo,

Fig. 2. Isolation of cadmium-thionein from liver cells: elution profiles of cadmium-thionein from (a) Sephadex G-50 and (b) DEAE-cellulose

Rat liver cell suspensions (50 mg/ml) were exposed to ¹¹⁵Cd or unlabelled cadmium for 15 min and incubated for 6 h. At the end of the incubation period, cells were harvested for isolation of cadmium-thionein. (a) The cadmium and [³⁵S]cysteine data were obtained from cells exposed to 0.1 and 1.0 µg of Cd/ml respectively. For the [³⁵S]cysteine-incorporation experiment, cells were incubated in a modified Eagle's medium containing 10 µCi of [³⁵S]cysteine/ml, 1% (w/v) bovine serum albumin, and no added unlabelled cysteine or serum. (b) The cadmium-thionein fraction (15.0 ml) obtained from gel filtration was dialysed against 4 litres of 5 mM-Tris/HCl for 16 h, and a portion of the sample was adsorbed to a column (0.9 cm × 10 cm) of DEAE-cellulose and eluted with a Tris/HCl gradient (----). Cadmium (○) and ³⁵S (□) were measured as outlined in the Experimental section.

1976; Shaikh & Smith, 1976; Squibb & Cousins, 1974). The thionein fraction isolated by gel filtration was separated into two subfractions (cadmium-thionein 1, cadmium-thionein 2) by DEAE-cellulose ion-exchange chromatography (see Fig. 2b) similarly to thionein induced *in vivo* in rat liver (Hidalgo & Bryan, 1977; Kimura *et al.*, 1974; Shaikh & Lucis, 1971). Thus the cadmium-thionein obtained from isolated liver cells resembled in its characteristics the protein induced in the liver of rats by cadmium treatment.

There are several independent studies on the kinetics of induction and appearance of cadmium-thionein in the liver (Bryan & Hidalgo, 1976; Cempel & Webb, 1976; Probst *et al.*, 1977; Shaikh & Smith, 1976). Maximum cadmium-thionein synthesis has been reported (1) to be preceded by a lag period of 2–3 h (Squibb & Cousins, 1974; Webb, 1975), and (2) to continue for approx. 8 h (Bryan & Hidalgo, 1976; Cempel & Webb, 1976). When a similar time study was made with single cells exposed

to 1 µg of Cd/ml during preincubation, the cells incorporated cadmium into thionein continuously up to the last time measured (6–8 h) (see Fig. 3a). However, 6 h after cadmium exposure (1 µg of Cd/ml) only a fraction [2.2 ± 0.6% (3)] of the intracellular cadmium was bound to thionein. If the cells were exposed to lower cadmium concentrations (0.1 µg of Cd/ml), the amount of cadmium with thionein increased to 32.2 ± 9.7% (4) of the intracellular cadmium after 6 h. These data indicate limited thionein synthesis *in vitro*, as opposed to those reported for experiments *in vivo*, where, 6 h after a comparable exposure, more than 60% of the total liver cadmium (15 µg/g of tissue) was associated with thionein (Bryan & Hidalgo, 1976; Cempel & Webb, 1976). However, that limited synthesis of the protein occurred in this system was indicated by increased cysteine incorporation into cadmium-thionein (three to four times greater in cadmium-treated than in control cells), observed at 3 and 6 h after cadmium exposure (see Fig. 3b). This enhanced cysteine incorporation

Fig. 3. Cadmium-thionein synthesis in isolated liver cells: (a) cadmium and (b) [^{35}S]cysteine incorporation into thionein. Rat liver cell suspensions (50mg/ml) were incubated for 15min in Earle's solution containing $1\mu\text{g}$ of $^{115}\text{Cd}/\text{ml}$ or in Earle's solution alone (control cells). Cells were then incubated in Eagle's medium and, at the times indicated, they were harvested for cadmium-thionein isolation. (a) Zero time is the end of the preincubation period; (b) cells were incubated in modified Eagle's medium containing $10\mu\text{Ci}$ of [^{35}S]cysteine/ml, 1% (w/v) bovine serum albumin, and no added unlabelled cysteine or serum. ■, Control; □, cadmium-treated.

has also been observed *in vivo* (Bryan & Hidalgo, 1976; Shaikh & Smith, 1976; Squibb & Cousins, 1974). Incorporation of cadmium into thionein was observed from 0 to 2h; this is in agreement with our previous report of the appearance of this protein in rat liver with 3h after cadmium exposure (Bryan & Hidalgo, 1976), but in disagreement with reports from other investigators (Cempel & Webb, 1976; Squibb & Cousins, 1974; Webb, 1975). The possibility that the increase from 0 to 2h in cadmium associated with thionein is due to thionein synthesis and not only to zinc displacement was supported by experiments where protein-synthesis inhibitors added after cadmium treatment prevented an increase in cadmium-thionein throughout the incubation period (see Table 1).

Effects of cycloheximide and actinomycin D on cadmium-thionein synthesis

Several reports are in general agreement that cycloheximide prevents thionein synthesis in rat liver (Squibb & Cousins, 1974; Webb, 1972); however, Webb (1972) has reported that actinomycin D does not inhibit thionein synthesis. Squibb & Cousins (1974) and Richards & Cousins (1975a,b, 1976) have reported that actinomycin D inhibits cadmium- and zinc-thionein synthesis respectively. In our experiments, thionein synthesis was stopped immediately

Table 1. Effects of actinomycin D and cycloheximide on cadmium-thionein synthesis

Rat liver cell suspensions (50mg/ml) were incubated for 15 min in Earle's balanced-salt solution containing $1\mu\text{g}$ of $^{115}\text{Cd}/\text{ml}$. Cells were incubated in Eagle's medium, and harvested for cadmium-thionein isolation at 6h, unless otherwise indicated. Actinomycin D or cycloheximide was added to a final concentration of $5\mu\text{g}/\text{ml}$ at the initiation (zero time), or 2h after incubation had started. Values are means $\pm$ S.E.M. (n).

Treatment	Cd associated with cadmium-thionein (ng)
None	216 $\pm$ 24 (7)
Actinomycin D	
Zero time	48 [45 $\pm$ 8 (6)]†
2h	158* [96 $\pm$ 7 (7)]‡
Cycloheximide	
Zero time	51* [45 $\pm$ 8 (6)]†
2h	87* [96 $\pm$ 7 (7)]‡

* Average for two experiments.
†, ‡ Cadmium associated with cadmium-thionein at zero time† and 2h‡ in the absence of inhibitors.

after the addition of cycloheximide (see Table 1). When actinomycin D was added at zero time (at the end of the cadmium preincubation), it also prevented any apparent increase in thionein; however, if

added 2h after thionein synthesis had started, additional synthesis of thionein was not prevented, although the amounts were lower than in cadmium-treated control (no-inhibitor) cells (see Table 1). Since actinomycin D was only partially effective in inhibiting thionein synthesis 2h after cadmium exposure, it is probable that enough RNA is synthesized between 0 and 2h to maintain a limited synthesis of thionein after inhibition of transcription. The concentration of cycloheximide used ($0.33 \mu\text{g}/10^6$ cells) was less than that used by Pariza *et al.* (1976) specifically to inhibit protein synthesis in rat liver cells. In the present experiments, $0.33 \mu\text{g}$ of actinomycin/ 10^6 cells did not prevent incorporation of [^{35}S]cysteine into proteins other than cadmium-thionein, indicating the absence of non-specific inhibition of translation.

Effects of cadmium concentration on the synthesis of cadmium-thionein

Thionein content after 6h of incubation was dependent on cadmium concentration (see Table 2), and the amounts were highest in cells exposed to $0.25\text{--}1.0 \mu\text{g}$ of Cd/ml. The amounts of thionein decreased when the cadmium concentrations were further increased, presumably because of the toxicity of the metal. It is noteworthy that even an exposure to $0.1 \mu\text{g}$ of Cd/ml triggered a significant increase in cadmium-thionein over the endogenous value (the amount of Cd associated with the cadmium-thionein fraction at zero time, the end of the cadmium preincubation).

Toxic effects of cadmium on protein synthesis observed *in vivo* in rat liver (Hidalgo *et al.*, 1976), and/or the protein-catabolic state of freshly isolated rat liver cells (Seglen, 1977) may be responsible for the low efficiency of thionein synthesis in isolated liver cells. However, cells derived from pig liver when continuously exposed to cadmium ($0.25 \mu\text{g}$ of Cd^{2+} /ml) for 8 days have been shown to synthesize large amounts of cadmium-thionein (Daniel *et al.*, 1977). In our experiments, only one-third of the intracellular cadmium binds to cadmium-thionein after 6h in cells exposed to $0.25 \mu\text{g}$ of Cd/ml.

The system described here has certain advantages for studying the effect of metals on cells that synthesize cadmium-thionein, namely: (1) the absence of secondary effects from synergistic or antagonistic relationships between the liver and other tissues; (2) the chemical environment and the amounts of cadmium to which the cells are exposed are under the control of the experimenter; and (3) the time and effort required to run the experiments are considerably decreased. A disadvantage of this system is that the efficiency of conversion of intracellular cadmium into thionein-bound cadmium is low compared with the intact liver; this problem can,

Table 2. *Effect of cadmium concentration on cadmium-thionein synthesis*

Rat liver cells ($50\text{mg}/\text{ml}$) were incubated for 15min with various concentrations of ^{115}Cd . At the end of the preincubation period the cells were resuspended in Eagle's minimum essential medium+10% serum, incubated for 6h, and harvested for isolation of cadmium-thionein. Values are means $\pm$ S.E.M. (n).

Cd in preincubation buffer ($\mu\text{g}/\text{ml}$)	Cd associated with cadmium-thionein (ng)
0.1	170*
0.25	250 ± 27.5 (4)
0.5	310 ± 75 (4)
1.0	250 ± 30 (9)
2.0	137
3.0	140

* Average for two separate experiments.

however, be partly overcome by treating the cells with lower metal concentrations. We believe that this system can be useful in studying the metabolism and toxicology of cadmium.

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