Turnover of Metallothioneins in Rat Liver

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Two electrophoretically distinguishable metallothioneins were isolated from the livers of Cd^{2+} -treated rats and had thiol group/metal ratios of 3:1, a total metal content, in each of these proteins, of 3.6 atoms of Cd^{2+} + 2.4 atoms of Zn^{2+} /molecule and 4.2 atoms of Cd^{2+} + 2.8 atoms of Zn^{2+} /molecule and respective apoprotein mol.wts. of 5844 and 6251. Studies with 1 h pulse labels of [^3H]cysteine, given after a single injection of ZnCl_2 or CdCl_2 , showed that these metals stimulated radioactive isotope incorporation into the metallothioneins over the control value by 10- and 15-fold respectively. This stimulation was maximal at 4 h after a single CdCl_2 injection and decreased to control values by 16 h, suggesting that either a translational event is responding to free intracellular Cd^{2+} or a short-lived mRNA is being produced or stabilized in response to the metal treatment. In rats chronically exposed to CdCl_2 , the metallothioneins increased to 0.2% of the liver wet weight from a control value of 2–4 $\mu\text{mol/kg}$ of liver, with a maximum rate of accumulation of 2–3 $\mu\text{mol/h}$ per kg of liver. The turnover of these proteins in control animals was 0.3–0.6 $\mu\text{mol/h}$ per kg of liver, measured by the rate of disappearance of $^{203}\text{Hg}^{2+}$, which binds irreversibly to the metallothioneins. Pretreatment with CdCl_2 completely stopped the rapid ^{203}Hg turnover observed in untreated animals. Unlike CdCl_2 , treatment with ZnCl_2 increased the concentration of metallothioneins to a new steady-state pool, 11 $\mu\text{mol/kg}$ of liver, after 10 h. The increase in the zinc-thionein pool by exposure to ZnCl_2 *in vivo* was determined to be primarily due to a stimulation of metallothionein biosynthesis.

Metallothioneins, first isolated by Margoshes & Vallee (1957), have subsequently been extensively studied in a number of mammalian systems. These low-molecular-weight cysteine-rich proteins bind the essential metal ions Zn^{2+} and Cu^{2+} , and are capable of scavenging Cd^{2+} , Hg^{2+} and Ag^{+} ions on exposure of animals to salts of these metals (Winge *et al.*, 1975). The capacity of the mammalian liver to sequester in the form of metallothioneins a large portion of a toxic dose of Cd^{2+} (Friberg *et al.*, 1971) has led us to consider the hypothesis, first proposed by Piscator (1964) and Piscator & Axelsson (1970), that one function of these proteins, whose biosynthesis is triggered in response to exposure to heavy metals, is to detoxify ions of heavy metals. [Numerous references exist on the role of metallothionein in Cd^{2+} toxicity: for reviews see Nordberg (1972) and Webb (1975).] We have isolated and characterized the two metallothioneins reported to be found in rat liver (Shaikh & Lucis, 1971; Winge *et al.*, 1975). By studies of half-life, we have determined the relative stabilities

in vivo in rat liver of zinc-thioneins that are present normally in the liver and of the $(\text{Cd}^{2+} + \text{Zn}^{2+})$ -thioneins that result on injection of CdCl_2 . We present evidence that exposure to Cd^{2+} leads to a stabilization of these proteins. We interpret data on turnover of protein and on the incorporation of [^3H]cysteine as showing that both Zn^{2+} and Cd^{2+} produce a transient stimulation of the biosynthesis of these proteins in rat liver.

Materials and Methods

Animals

Female rats (CFN strain) weighing 180–220 g were used. The animals were kept in stainless-steel cages and supplied with Ralston Purina rat chow and tap water *ad libitum* during the course of all radioactive isotope experiments.

Chemicals

All chemicals were of analytical grade. CdCl_2 was purchased from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A.; ZnCl_2 was prepared by neutralization of ZnO with 6M-HCl; CdCl_2 and

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ZnCl₂ were kept as stock solutions (0.01 M) at 4°C; *p*-chloromercuribenzoate, 5,5'-dithiobis-(2-nitrobenzoic acid) and Tris buffer were from Sigma Chemical Co., St. Louis, MO, U.S.A.; acrylamide (Eastman, Rochester, NY, U.S.A.) was recrystallized in two steps from ethyl acetate (4°C) and chloroform (-20°C), and bisacrylamide (Eastman) was used as supplied. For preparative discontinuous-gel electrophoresis stock acrylamide solutions were freshly prepared shortly before use. [³H]Cystine (sp. radioactivity 2.5 Ci/mmol) was supplied by Schwarz/Mann, Orangeburg, NY, U.S.A., or Amersham-Searle, Arlington Heights, IL, U.S.A., and [³⁵S]cystine (sp. radioactivity 40 Ci/mmol), ¹⁰⁹CdCl₂ (carrier-free) and ²⁰³Hg(NO₃)₂ (99% radiochemical purity) were from New England Nuclear Corp., Boston, MA, U.S.A. Deionized glass-distilled water was used for Sephadex G-75 chromatography. Double-glass-distilled water was used in all subsequent purification steps.

Equipment

Glass columns for Sephadex chromatography, Sephadex G-25 (coarse grade), Sephadex G-75 (40–120 μm particle size) and DEAE-Sephadex A-25 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrafiltration was carried out with a Diaflo cell (model 202) and UM-2 filters (62 mm diam.) obtained from Amicon Corp., Lexington, MA, U.S.A. Centrifugation was carried out in a Sorvall RC-2 centrifuge with an SS-34 rotor. Ultracentrifugation was carried out in a Beckman model L centrifuge with type 60 or SW-30 rotors.

Spectrophotometry

Atomic-absorption spectrophotometry was carried out with a Perkin-Elmer model 403 atomic-absorption spectrophotometer. Metal concentration was kept in the range 0–50 μM by dilution with water when necessary. U.v. spectrophotometry was carried out with either a Beckman DB double-beam recording spectrophotometer or a Zeiss PMQ single-beam spectrophotometer.

Radioactive isotope use and radioactivity counting

[³H]- and [³⁵S]-Cystine were supplied at 1 mCi/ml concentration and were diluted, before injection, with 0.2 ml of 0.1 M-Tris/HCl, pH 8.6, for a total injected volume of 0.22–0.30 ml. Reduction of cystine to cysteine occurred *in vivo*. Identical results were obtained when cystine was reduced with dithiothreitol before injection. ²⁰³Hg(NO₃)₂ was injected in 0.5 ml of water, and ¹⁰⁹CdCl₂, in a 0.1 M-CdCl₂ solution, was injected in a volume of 1.0 ml/kg body wt. Counting of radioactivity in liquid samples was carried out in PCS (Amersham-Searle). Counting of radioactivity in discontinuous-gel slices was carried out in Perma Blend in toluene (Packard Instrument Co., Downers Grove, IL,

U.S.A.) after solubilizing with Protosol or NCS (New England Nuclear). Scintillation counting of all radioactive isotopes (³H, ³⁵S, ¹⁰⁹Cd and ²⁰³Hg) was carried out in a Packard Tri-Carb scintillation counter. Corrections were made for background and, where double-label studies were carried out, for channel overlaps (2% for ³H overlap on the ³⁵S-channel; 18% for ³⁵S overlap on the ³H-channel).

Preparative discontinuous-gel electrophoresis

This was carried out at 4°C in a system consisting of 7.5% acrylamide + 0.2% bisacrylamide + 0.15% (NH₄)₂S₂O₈ in 0.38 M-Tris/HCl, pH 8.9 (resolving gel, 5 cm high and 80 ml volume) and 3.5% acrylamide + 0.6% bisacrylamide + 10% sucrose in 0.06 M-Tris/phosphate, pH 6.8 (concentrating gel, polymerized with riboflavin-light, 1 cm high). A Buchler preparative polyacrylamide-gel-electrophoresis apparatus was used. A sample (20 mg) of metallothionein 1 or 2 in 0.006 M-Tris/HCl, pH 8.1, and a lower buffer and elution buffer of 0.1 M-Tris/HCl, pH 8.1, were used. Bromophenol Blue was used as tracking dye. Electrophoresis was begun at 30 mA current and 120 V. After the tracking dye was two-thirds of the way through the concentrating gel, the current was increased to 50 mA and 200 V and maintained at 40–50 mA. Elution buffer was pumped at 65 ml/h and 10 ml fractions were collected. In a typical run, the tracking dye was eluted at 7.4 h and the protein peak (metallothionein 1) at 14.1 h. Under similar conditions metallothionein 2 was eluted in 8.5 h.

Amino acid analysis

Amino acid analyses were carried out with the Beckman 120 C automatic amino acid analyser equipped with an Infotronics 110A automatic integrator and a 5× range expander. Samples were sealed in acid-washed Pyrex test tubes (16 mm × 150 mm) under vacuum in 1 ml of constant-boiling (5.7 M) HCl and hydrolysed in a thermostatically controlled block at 110°C for the indicated times. Samples were hydrolysed in duplicate for 24, 48 and 72 h. The 72 h values were used in the calculation of valine and isoleucine. The 24 h values were used for threonine and serine. Others were averaged over all six values. Cysteine was converted into cysteic acid before hydrolysis by performic acid oxidation (Hirs, 1967). A 10-fold molar excess of reagent over thiol groups was allowed to react for 16 h at 0°C and the reaction stopped with a 20-fold excess of cold water. The mixture was freeze-dried, desalted by ultrafiltration and redissolved for analysis.

Results

Isolation, purification and composition of metallothioneins

Ten rats were injected once daily for a period of 2 weeks with 10 μmol of CdCl₂/kg body wt. During

the first week, injections were administered subdermally in the neck and back region to lessen animal mortality. In the following week they were administered intraperitoneally. Animals were killed by decapitation and were exsanguinated. The livers were excised, washed in cold unbuffered 0.25M-sucrose and minced in 4vol. (v/w) of the buffer. The tissue was homogenized in an ice-cold Teflon/glass homogenizer (eight to ten strokes). All operations after death were carried out at 4°C. Metallothioneins were isolated by a modification of the procedure developed by Shaikh & Lucis (1971) as shown in Scheme 1. In a typical preparation of these proteins, 79g of rat liver from Cd²⁺-treated animals yielded a metallothionein peak eluted from Sephadex G-75 at $V_e/V_0 = 1.9$ ($V_0 = 850$ ml), and containing 57.4 μ mol of Cd²⁺ and 42.8 μ mol of Zn²⁺ (combined fractions from two runs). This fraction was adsorbed directly on DEAE-Sephadex A-25, washed with 500ml of 0.001M-Tris/HCl, pH8.6, and eluted with a 2-litre linear gradient from 0.001 to 0.25M-Tris/HCl, pH8.6, followed by a further wash with 500ml of end buffer. Four peaks were found, with the following metal recoveries (in fractions I-IV respectively): 0.7, 26.8, 26.4 and 1.5 μ mol of Cd²⁺; 0.3, 19.4, 18.2 and 0.8 μ mol of Zn²⁺. Further

purification of these proteins after the ion-exchange-chromatographic step generally led to a small and variable loss of Zn²⁺. Preparative discontinuous-gel electrophoresis gave homogeneous metallothionein 1 and eliminated a small contaminating peak, migrating just ahead of this protein on analytical discontinuous-gel electrophoresis (Fig. 1). Metallothionein 2 always gave a minor band on analytical discontinuous gels that, on the basis of the homogeneity of this protein shown by amino acid analysis (Table 1), was judged to be a polymer formed during the electrophoretic run, possibly by dissociation of Zn²⁺. Purified metallothionein 2 was eluted as a single peak from a column (2.5cm $\times$ 100cm) of Sephadex G-75 or a column (2.5cm $\times$ 60cm) of DEAE-Sephadex A-25. The latter column was washed with 0.1M- and eluted with 0.15M-Tris/HCl, pH8.6.

A purification step, which was an alternative to preparative discontinuous-gel electrophoresis, involved decreasing the pH of the protein solution (2mg/ml) to 4.5 with 1M-HCl, centrifuging for 30min at 50000g and recovering the purified protein in the supernatant solution. The recovery of electrophoretically homogeneous metallothionein necessitated a further treatment of the protein in which the pH of a 2mg/ml solution was increased to 8.0 with

Scheme 1. Isolation of metallothioneins from rat liver
For details see the text.

Fig. 1. *Electrophoresis of metallothioneins*

Electrophoresis was carried out at 4°C in 7% polyacrylamide gels, pH 8.3 (see the Materials and Methods section). (1) Metallothionein 1 after ion-exchange chromatography, 50 µg; (2) metallothionein 1 after preparative discontinuous-gel electrophoresis, 50 µg; (3) metallothionein 2 after ion-exchange chromatography or preparative discontinuous-gel electrophoresis, 70 µg. Mobilities relative to the tracking dye, Bromophenol Blue (position indicated by arrow), are: metallothionein 1, 0.48; metallothionein 2, 0.65.

1M-Tris base, incubated with 1mM-dithiothreitol, a 5-fold excess of ZnCl₂ added and the protein desalted.

The u.v.-absorption spectra of the metallo-

thioneins were quite similar, showing an A_{250}/A_{280} value as high as 50 in highly purified preparations and giving, with both proteins, molar absorption coefficients (based on Cd²⁺) at 250nm of 1.4×10^4 litre·mol⁻¹·cm⁻¹.

The compositions of metallothioneins 1 and 2 are shown in Table 1. No traces of histidine, arginine, leucine, tyrosine or phenylalanine were detected in the analyses. With an A_{250}/A_{280} value of 50, tryptophan was calculated to be 0.2 residue (or less) per molecule and was therefore assumed to be absent. The minimum molecular weights of the apoproteins, on the basis of the best whole-residue numbers found, were calculated to be 5844 for metallothionein 1 and 6251 for metallothionein 2. For comparison, the amino acid compositions of human liver metallothioneins 1 and 2 (Bühler & Kägi, 1974) are given in Table 1.

The molar ratio of Cd²⁺ to Zn²⁺, after DEAE-Sephadex A-25 chromatography, was 1.38 (metallothionein 1) and 1.45 (metallothionein 2). Analyses with *p*-chloromercuribenzoate and 5,5'-dithiobis-(2-nitrobenzoic acid) were carried out to determine the thiol group/metal ratio in these proteins by displacement reaction. By the method of Boyer (1954) *p*-chloromercuribenzoate was found to react quite slowly with the metallothioneins at pH 4.5, and reproducible results were often difficult to obtain. 5,5'-Dithiobis-(2-nitrobenzoic acid) reacted rapidly in 0.1M-potassium phosphate buffer at pH 7.6 and, with either reduced glutathione or cysteine as thiol standards, gave molar ratios of thiol groups to cadmium+zinc in these proteins of 3.01 ± 0.06 (metallothionein 1) and 3.00 ± 0.06 (metallothionein 2). On the basis of a cysteine content from amino acid analysis of 18 and 21 residues/molecule in metallothioneins 1 and 2 respectively, the metal content of these proteins was found to be 3.6 Cd²⁺ + 2.4 Zn²⁺ atoms/molecule (metallothionein 1) and 4.2 Cd²⁺ + 2.8 Zn²⁺ atoms/molecule (metallothionein 2), indicating a variable molar ratio of these metals bound to individual molecules and a total metal-binding capacity of 6 and 7 atoms/molecule in metallothioneins 1 and 2 respectively.

Comparable yields of both metallothioneins were found in numerous preparations of these proteins from rat liver. The Cd²⁺/Zn²⁺ ratios in both metallothioneins from a given preparation were found to be the same. However, in rats receiving various degrees of continuous exposure to Cd²⁺, the Cd²⁺/Zn²⁺ ratio was 1:2 after a few hours to 1 day and changed to 1:1 after 1 week and to 1.4:1 after 2 weeks, indicating a gradual enrichment of Cd²⁺ in these proteins.

The concentration of metallothionein in controls was determined by saturation of all liver supernatant proteins with ¹⁰⁹Cd (sp. radioactivity 706 c.p.m./nmol) followed by a separation on Sephadex G-75

Table 1. Amino acid composition of metallothioneins 1 and 2

Amino acid residue	Metallothionein 1*		Metallothionein 2†		Whole residues (nearest integer)		Data of Bühler & Kägi (1974)	
	(Residues/ molecule)‡	(Residues/ 100 residues)	(Residues/ molecule)‡	(Residues/ 100 residues)	Metallo- thionein 1	Metallo- thionein 2	Metallo- thionein 1	Metallo- thionein 2
Lysine	8.0	13.4	8.1	12.9	8	8	8	8
Cysteic acid	18.6	31.1	20.9	33.3	18-19	21	18	21
Aspartic acid	4.0	6.7	4.1	6.6	4	4	3	4
Methionine sulphone	0.9	1.5	0.9	1.5	1	1	1	1
Threonine	3.6	6.0	2.0	3.2	4	2	2	2
Serine	10.3	17.2	10.3	16.4	10	10	8	8
Glutamic acid	1.2	2.0	3.1	4.9	1	3	3	2
Proline	2.2	3.7	2.2	3.4	2	2	2	2
Glycine	6.0	10.0	4.2	6.6	6	4	5	5
Alanine	3.0	5.0	5.0	8.0	3	5	6	7
Valine	2.0	3.3	1.2	1.9	2	1	2	1
Isoleucine	—	—	0.8	1.3	—	1	1	1
Leucine	—	—	—	—	—	—	1	—
Total residues					59-60	62	60	62

* Corrected for 10% serine and 5% threonine losses per 24h.

† Corrected for 4% serine loss per 24h.

‡ Based on integral values for alanine of 3 (metallothionein 1) and 5 (metallothionein 2).

Fig. 2. Binding of ^{109}Cd to rat liver supernatant proteins

The S^{105} subcellular fraction was isolated from three 250 g rats as shown in Scheme 1. Samples (1 ml) from Sephadex G-75 chromatography ($2.5\text{ cm} \times 100\text{ cm}$ column) were dissolved in 10 ml of PCS scintillation fluid and counted for radioactivity (b). Fractions 26-31 (indicated by the horizontal arrow) were pooled (10 ml/fraction), freeze-dried and desalted, and 1000 c.p.m. (^{109}Cd) was electrophoresed in 7% polyacrylamide gels and sliced with a gel-slicing apparatus, starting at the tracking dye Bromophenol Blue. The top of the gel is at slice no. 84, indicated by the vertical arrow. Each slice was incubated overnight in NCS toluene (1:1, v/v) at 37°C with shaking, 5 ml

(Fig. 2b). From these experiments a concentration of $24\text{ }\mu\text{mol}$ of bound Cd^{2+} /kg of liver was found in the low-molecular-weight Sephadex G-75 fraction. Similar results were obtained ($24\text{ }\mu\text{mol/kg}$ of liver) when the pH of the supernatant was decreased to pH 4.0 with 1M-HCl and $^{109}\text{CdCl}_2$ added, centrifuged at $50000g$ for 30 min to remove a precipitate, readjusted to pH 8.6 with 1M-Tris base and then chromatographed on Sephadex G-75 as before. From electrophoretic analysis the concentration of metallothioneins in the liver was estimated (Fig. 2a) to be between 2 and $4\text{ }\mu\text{mol/kg}$ of liver.

Kinetics of metallothionein formation in rat liver

Administration of 1 mM- CdCl_2 every 2 h ($1\text{ }\mu\text{mol/kg}$ body wt., injected intraperitoneally) resulted in a continuous uptake of Cd^{2+} and Zn^{2+} into each of the metallothioneins in a molar ratio of 1:2 (Fig. 3). A rate of formation of these proteins of $2\text{ }\mu\text{mol}$ of protein/h per kg of liver was found. Animals given a single injection of CdCl_2 ($10\text{ }\mu\text{mol}$ of $\text{CdCl}_2 + 2\text{ }\mu\text{Ci}$ of $^{109}\text{CdCl}_2/\text{kg}$ body wt.) showed a rapid incorporation of almost half of this metal into hepatic metallothioneins (Fig. 4). The rate of formation of the metallothioneins from the data of Fig. 4, assuming a $\text{Cd}^{2+}/\text{Zn}^{2+}$ molar ratio in these proteins of 1:2

of Perma Blend in toluene was added and shaking continued for 1 h before counting for radioactivity (a). Abbreviations: MT-1, metallothionein 1; MT-2, metallothionein 2.

Fig. 3. Cd^{2+} and Zn^{2+} continuous-dosage studies

For details see the text. Cd^{2+} and Zn^{2+} were determined by atomic-absorption spectrophotometry on fractions taken from a column (5.8 cm $\times$ 80 cm) of Sephadex G-75. Each datum point represents the results from three 200 g rats. Two Zn^{2+} -containing fractions and one Zn^{2+} (+ Cd^{2+} in Cd^{2+} -administered rats)-containing fraction were found. Total metal recovery in this latter fraction is plotted. Symbols: ■, Zn^{2+} -treated animals; ▲, Cd^{2+} -treated animals. —, Calculated curve. The first two Zn^{2+} fractions (containing traces of Cd^{2+} in the first few hours of Cd^{2+} treatment) remained constant at $156 \pm 12 \mu\text{mol/kg}$ of liver (first fraction) and $43 \pm 7 \mu\text{mol/kg}$ of liver (second fraction) in Zn^{2+} -treated animals. In Cd^{2+} -treated animals these values were 141 ± 6 and $39 \pm 7 \mu\text{mol/kg}$ of liver respectively. For the point at 75 h, animals were killed 3 h after the last daily injection. The cation(s) measured (i.e. Zn^{2+} and/or Cd^{2+}) in each curve is indicated in the Figure.

and a molar ratio of metal to protein of 6.5, was constant from 2 to 17 h, at 2–3 μmol of protein/h per kg of liver. From the results of several preparations of metallothioneins, obtained after daily injections of 10 μmol of CdCl_2/kg body wt. for up to 2 weeks, a sustained biosynthetic rate of 0.9–1.2 μmol of protein/h per kg of liver was found. This rate of biosynthesis in the liver, ignoring possible loss of these proteins into the bile fluid or the blood or through degradation, corresponds to a daily requirement for cysteine of 0.5 mmol/kg of liver. In 2 weeks these proteins can accumulate to up to 0.2% of the total wet weight of the liver.

In contrast with the studies with Cd^{2+} , exposure of rats to Zn^{2+} did not result in a continuous increase in the concentration of metallothioneins. As shown in Fig. 3, continuous administration of 2 mM- ZnCl_2 (2 $\mu\text{mol/kg}$ body wt., injected intraperitoneally) caused an increase in total Zn^{2+} present in metallothionein from 24 to 75 $\mu\text{mol/kg}$ of liver after 9 h. This 3-fold increase in hepatic metallothionein remained constant with daily Zn^{2+} injections (20 $\mu\text{mol/kg}$ body wt., injected intraperitoneally) for

Fig. 4. Chromatography of rat liver supernatant fraction from Cd^{2+} -treated rats

For details see the text. The S^{105} subcellular fraction was isolated from one 200 g rat as in Scheme 1 at 2 h (—), 5 h (····) and 17 h (---) after injection of $\text{CdCl}_2 + ^{109}\text{Cd}$, chromatographed on Sephadex G-75 (2.5 cm $\times$ 100 cm column), 10 ml fractions being collected and 1 ml fractions counted for radioactivity as in Fig. 2. The A_{254} (---) is given for the 17 h exposure. The following distribution of radioactivity between high- and low-molecular-weight proteins was found:

Time (h)	Percentage of total injected counts	
	Fractions 1–20	Fractions 21–44
2	5.0	4.5
5	1.4	12.4
17	0.5	45.8

75 h. The calculated curve for continuous Zn^{2+} treatment is discussed below.

Radioactive incorporation into metallothioneins

The observed accumulation of metallothioneins in rat liver during exposure to Cd^{2+} can be explained by either a decreased rate of degradation of these proteins from a pool that is being continually renewed or a stimulation of protein biosynthesis. Labelling of metallothionein *in vivo* by using [^3H]cysteine as the tracer has been reported in several laboratories to be stimulated by exposure to Cd^{2+} (Shaikh & Lucis, 1970; Squibb & Cousins, 1974; Chen *et al.*, 1975; Shaikh & Smith, 1975). At 4 h after a single injection of CdCl_2 , we have found the total label incorporated into the ($\text{Cd}^{2+} + \text{Zn}^{2+}$)-containing fraction obtained by chromatography on Sephadex G-75 to be 15-fold greater than in controls (Table 2). In contrast, a maximum stimulation of the incorporation of label into high-molecular-weight supernatant proteins of only 1.5-fold was observed 2 h after injection of CdCl_2 . Discontinuous-gel electrophoresis of controls and of samples taken 4 h after injection from the ($\text{Cd}^{2+} + \text{Zn}^{2+}$)-containing fractions from Sephadex G-75

Table 2. Amino acid incorporation into metallothioneins after Cd²⁺ injection

Rats were injected subdermally at zero time with 10 μmol of CdCl₂/kg body wt. 1 h before death; [³H]cysteine was administered intraperitoneally at 25–100 μCi/animal at the times indicated. Metallothioneins were isolated as shown in Scheme 1. Total radioactivity was monitored in fractions from Sephadex G-75 as in Fig. 2. Each value represents the results from one 200 g rat. The value at 4 h is from two independent experiments. Results are given as total recovered c.p.m./μCi of injected label.

Time of injection (h)	[³ H]Cysteine (c.p.m./μCi)	
	In high-molecular-weight-fraction	In metallothionein fraction
0 (control)	1000	75
0.5	1500	220
1.0	1310	340
2.0	1520	650
4.0	1200 ± 70	1100 ± 70
8.0	1180	290
16.0	1750	120

chromatography confirm the incorporation of ³H from [³H]cysteine in approximately equal amounts into metallothioneins 1 and 2 (Fig. 5). Treatment of [³H]cysteine-labelled metallothioneins with 2-mercaptoethanol or dithiothreitol to release non-specifically bound cysteine, followed by dialysis or Sephadex G-25 chromatography, did not result in the release of any ³H from the protein fraction. When ZnCl₂, at dosages of 12.5, 50 and 100 μmol/kg body wt., was injected instead of CdCl₂, followed by a 1 h exposure to [³H]cysteine at 4 h, 825, 986 and 767 c.p.m./μCi of injected radioactive isotope respectively were incorporated into the low-molecular-weight Zn²⁺ fraction, representing a 10-fold stimulation over controls. These data suggest that injection of both Cd²⁺ and Zn²⁺ produces a transient stimulation of the biosynthesis of these proteins in rat liver. In carrying out these experiments we have found it necessary to administer the metal ion subdermally and the [³H]cysteine intraperitoneally to prevent a reaction between these two substances occurring *in situ*.

Rate of degradation of metallothioneins

Double-labelling experiments. The half-life of zinc-thionein after Zn²⁺ injection was estimated by the double-labelling method of Arias *et al.* (1969). Rats were injected at zero and 48 h subdermally in the scapular region with 10 μmol of ZnCl₂/kg body wt. At 4 and 52 h, the animals were given intraperitoneal injections of 50 μCi each of [³⁵S]- and [³H]-cysteine respectively, and at 56 h total incorporated radioactivity (³⁵S and ³H) of the rat liver supernatant subcellular fractions was determined chromatographically on Sephadex G-75 and by subsequent discontinuous-gel electrophoresis of the low-molecular-weight Zn²⁺-containing fractions (Fig. 6). The choice of 4 h after the [³H]cysteine injection as the time for killing was on the basis of the observation that transient stimulation of metallothionein biosynthesis stops at 8–16 h after injection of metal ion (Table 2). The [³⁵S]cysteine was injected first, on the basis of the observation that free hepatic [³⁵S]cysteine reaches a peak concentration after 90 min and is eliminated by 200–300 min as [³⁵S]sulphate (Awwad *et al.*, 1970). Thus reutilization of radioactive isotope should be lessened by using this label first. Controls were given both radioactive isotopes simultaneously at 4 h and killed 1 h later. Discontinuous-gel electrophoresis of controls showed no significant differences in relative incorporation of [³H]- to [³⁵S]-cysteine as determined by a statistical analysis (Fig. 6a) of the deviation in the *i*th gel slice given by $D_i = [^3H]^+ - (a[^{35}S]^+ + b)$, where *a* and *b* are determined from regression analysis of the data set (Weisberg, 1974). After correction for relative incorporation of the two

Fig. 5. Polyacrylamide discontinuous-gel electrophoresis of [³H]cysteine-pulse-labelled metallothioneins 1 and 2 (MT-1 and MT-2 respectively)

Desalted (Zn²⁺+Cd²⁺)-containing fractions from [³H]cysteine-pulse-label studies (Table 2) were electrophoresed in 7% polyacrylamide gels and analysed as in Fig. 2. (a) [³H]Cysteine pulse label at 4 h after CdCl₂ injection; (b) [³H]cysteine pulse label from a control animal. In each run 1000 c.p.m. was applied to the gels. Gels were sliced starting with the tracking dye Bromophenol Blue. The top of each gel is at slice no. 89. In (a) 70% and in (b) 42% of the ³H radioactivity co-electrophoresed with purified metallothioneins (regions indicated by horizontal arrows).

Fig. 6. *Metallothionein double-label half-life studies* Three 225 g rats were injected as described in the Results section. Metallothioneins from each animal were prepared for discontinuous-gel electrophoresis as in Fig. 5. Discontinuous-gel slices were soaked in Protosol/toluene (1:1, v/v; 0.5 ml/slice) overnight and counted for radioactivity in mini-vials after addition of 5 ml of Perma Blend in toluene. In (a) a statistical analysis of the results of a control experiment are given (see discussion in the text). In (b) the half-lives of metallothioneins 1 (MT-1) and 2 (MT-2) were calculated to be 20 and 30 h respectively. The results given here were normalized to control incorporation of [³H]-+ [³⁵S]-cysteine. The half-life of all radioactively labelled proteins from the Sephadex G-75 fractions, migrating at $V_e/V_0 = 1.9$, calculated relative to control incorporation of [³H]-+ [³⁵S]-cysteine, was found, from the formula $t_{1/2} = 48 \text{ h} \times (\ln 2) / \ln (^3\text{H}/^{35}\text{S})$, to be 26 h (see the text). Gels were sliced starting with the tracking dye Bromophenol Blue. The top of the gel is at slice no. 72 (vertical arrow). —, ³⁵S radioactivity; ----, ³H radioactivity.

radioactive isotopes the ratio of ³H to ³⁵S was found to correspond to an average half-life for the metallothioneins of 26 h. A half-life of 19 h for zinc-thioneins in rat liver, as determined from studies of [³⁵S]cysteine turnover, has been reported by Feldman & Cousins (1976). Metallothioneins 1 and 2 gave individual half-life values of 20 and 30 h respectively (Fig. 6b). No explanation could be found for this difference. The interpretation of this double-labelling experiment is viewed as tentative in the light of the possible reutilization of radioactive isotope, which would contribute to the calculated half-life (see the discussion below).

²⁰³Hg-turnover experiments. The half-life of Cd²⁺ in rat liver has been found to be several months,

compared with a half-life of 4 days for metallothionein proteins to which this metal is bound (J. Piotrowski, unpublished work; Chen *et al.*, 1975). The Cd²⁺ released by intracellular degradation of metallothioneins is thus available for further binding to newly synthesized metallothioneins in the liver. In contrast, it has been observed (Piotrowski *et al.*, 1974; J. Wiśniewska-Knypl, unpublished work) that Hg²⁺ is bound only transiently in the liver. The affinity of Hg²⁺ for metallothionein is such that a relatively non-labile cysteine-Hg²⁺ complex results from the displacement of Cd²⁺ or Zn²⁺ by the Hg²⁺ ion. The lability of the Hg²⁺ complex *in vivo* was tested by giving ²⁰³Hg²⁺ (20 μCi/animal or 0.25 μmol/kg of liver at 10 Ci/g specific radioactivity; 40 g of liver/kg body wt.) to rats that had been treated 48 h previously with CdCl₂ (10 μmol/kg body wt.) and killing the animals at intervals of up to 17 h (Fig. 7). The total radioactivity bound to soluble proteins was isolated by chromatography on Sephadex G-75 (Fig. 7a). The major Hg²⁺-binding proteins in the

Fig. 7. *Turnover of metallothioneins in rat liver* ²⁰³Hg was measured in 15 ml fractions from Sephadex G-75 chromatography of a S¹⁰⁵ supernatant sub-cellular fraction (a) as in Fig. 2. The ²⁰³Hg-containing fractions 15–20 were pooled (total pooled counts for each period of dosage given in b) and discontinuous-gel electrophoresis of desalted fractions was carried out as described in the legend of Fig. 5. The electrophoresis run for $t = 4 \text{ h}$, is shown in (c). Each data point in (b) represents the pooled livers of three 175 g rats, either control (—) or pretreated at $t = -48 \text{ h}$ with 10 μmol of CdCl₂/kg body wt. administered subdermally in the scapular region (----). In (a) the Sephadex G-75 runs are given for control ²⁰³Hg-administered animals.

low-molecular-weight fractions from Sephadex G-75 co-electrophoresed with metallothioneins 1 and 2 (Fig. 7c). Hg^{2+} incorporation into the metabolically stable (zinc+cadmium)-thionein continued to increase by 17h (Fig. 7b). The lack of turnover of $^{203}\text{Hg}^{2+}$ bound to metallothioneins in liver tissue from animals pretreated with Cd^{2+} reflects the slow turnover of these proteins in this time period. A second group of animals was given injections of $^{203}\text{Hg}^{2+}$ as before, but pretreatment with CdCl_2 was omitted. In contrast with the pretreated animals, the controls showed an initial uptake of $^{203}\text{Hg}^{2+}$ into metallothioneins, followed by a rapid disappearance of the radioactive isotope from the metallothionein protein pool in the liver (Fig. 7b). The actual recovery of $^{203}\text{Hg}^{2+}$ bound to metallothioneins at the peak of incorporation (2h in controls) represented less than 10pmol of Hg^{2+} or 1 Hg^{2+} atom bound/ 10^5 protein molecules. The absence of any alteration in the electrophoretic-migration rate of metallothionein molecules to which a single Hg^{2+} ion is bound (Fig. 7c) suggested no gross alteration in the physical properties of these species. The rapid turnover of $^{203}\text{Hg}^{2+}$ in control as compared with Cd^{2+} -pretreated animals is thus interpreted as reflecting a rapid turnover of protein in the controls. Alternatively, one can speculate that the presence of Cd^{2+} in metallothionein confers a greater stability on the Hg^{2+} -metallothionein complex than in the case of zinc-thionein. The ability of mercury to displace either cadmium or zinc from this protein to form a stable mercury-mercaptide complex and the presence of a considerable amount of Zn^{2+} in the metallothioneins in Cd^{2+} -pretreated rats (up to 2:1 for the $\text{Zn}^{2+}/\text{Cd}^{2+}$ ratio) precludes this latter speculation in our opinion.

The half-life of the two metallothioneins in control animals, estimated from the disappearance of $^{203}\text{Hg}^{2+}$ from the liver protein pool, was 4–5h. Because of the slow turnover in the Cd^{2+} -treated animals, it may be concluded that the kinetics of formation of metallothioneins during exposure to CdCl_2 (see Figs. 3 and 4) are indicative of the maximum biosynthetic rates of these proteins in the absence of significant degradation and rapid losses of these proteins from the liver through excretion into bile fluid or blood. The discrepancy between this result and the half-life determined by double labelling is discussed below.

Change in steady-state experiment. A third method for estimating the rate of degradation of metallothioneins, from the data of Fig. 3, involves the use of a steady-state equation for describing intracellular protein turnover. Assuming a first-order kinetic process for the degradation of metallothioneins in the liver, the following expression may be derived (Schimke & Doyle, 1970):

$$d[\text{P}]/dt = K_s - k_d[\text{P}] \quad (1)$$

where k_d is the first-order degradative rate constant, K_s is the rate of protein biosynthesis (assumed to remain constant) and $[\text{P}]$ is the concentration of metallothioneins in the protein pool. In the steady state (i.e. the control animals or after several hours of continuous exposure to Zn^{2+} , as in Fig. 6) we have:

$$d[\text{P}]/dt = 0 \text{ and } K_s = k_d[\text{P}]_{ss} \quad (2)$$

where $[\text{P}]_{ss}$ is the protein concentration of the pool in the steady-state. The time course of change of protein concentration in the cell resulting from a perturbation in cellular chemistry (in this case exposure to CdCl_2 or ZnCl_2), derived from eqn. (1), is:

$$[\text{P}]_t/[\text{P}]_0 = K'_s/(k'_d[\text{P}]_0) - [K'_s/(k'_d[\text{P}]_0) - 1]e^{-k'_dt} \quad (3)$$

where the steady-state condition (eqn. 2) allows the determination of K'_s , the zero-order rate of biosynthesis, from a half-life determination of k'_d , the first-order rate constant for degradation. When the rate of degradation approaches zero ($k'_d = 0$) then:

$$d[\text{P}]/dt = K'_s \text{ and } [\text{P}]_t = K'_st \quad (4)$$

This can be seen, from Fig. 3, for metallothionein biosynthesis in rat liver on continuous exposure of animals to CdCl_2 , where $k'_d = 0.007\text{h}^{-1}$ was found (Chen *et al.*, 1975). In this case, a new steady state is not attained during the course of the experiment and a ratio of $K'_s/K_s = 10$ has been estimated. In the approach to a new steady state, the ratio $K'_s/(k'_d[\text{P}]_0)$ represents the increase (or decrease) in protein concentration relative to the control value. Fig. 3 shows that, in animals exposed to Zn^{2+} , this ratio is $[\text{P}]_\infty/[\text{P}]_0 = 3.3$, where $[\text{P}]_\infty$ is the final steady-state protein concentration at $t = \infty$. The rate of approach to this new steady state is determined solely by the exponential factor, $e^{-k'_dt}$, which in Fig. 3 appears to increase with a half-life of around 4h, giving $k'_d = 0.17\text{h}^{-1}$. This value of k'_d appears unchanged from the control value of 0.15h^{-1} calculated from the $^{203}\text{Hg}^{2+}$ turnover (Fig. 7). The initial lag in the uptake curve for Zn^{2+} may represent a lag in the protein-synthetic response after exposure to this metal ion, similar to that seen after exposure to Cd^{2+} (Table 2). A plot of eqn. (3) with $k'_d = 0.15\text{h}^{-1}$, $k'_d[\text{P}]_0 = K_s$ and $K'_s/K_s = 3.3$, shifted to allow a 2h lag in response, is shown in Fig. 3. The results of this analysis, together with the stimulation of metallothionein biosynthesis by a single injection of ZnCl_2 observed in the $[\text{H}^3]\text{cysteine}$ -incorporation studies (in which $K'_s/K_s = 10$ was found), suggest that exposure to Zn^{2+} has little or no effect on the stability of zinc-thionein in rat liver and that the increase in the steady-state concentration of metallothioneins on exposure to ZnCl_2 is caused primarily by a stimulation of protein synthesis.

Discussion

The similarity in amino acid composition between metallothioneins isolated from mammalian species as widely divergent as man and rat (Table 1) suggests a conservation of function during the evolution of these proteins. The sequencing of horse renal metallothionein 1B (Kojima *et al.*, 1976) and the similarity in the positions of cysteine, serine and the basic amino acids in the horse and human metallothioneins reported by these authors is further evidence for the functional importance of these residues. In addition, we have found seven residues in metallothionein 1 to be identical in sequence with the *N*-terminus of horse metallothionein 1B, providing evidence for a close similarity between these molecules in different species. Repeated attempts at producing rabbit antibodies against rat liver metallothioneins in our laboratory failed, although the immunological technique detected a minor impurity in metallothionein 1 (Fig. 1) that was later eliminated by preparative discontinuous-gel electrophoresis. The poor antigenicity of these molecules can best be explained by the lack of aromatic residues and by the similarity in metallothionein amino acid compositions found in different species.

Several of the common amino acids are absent from the metallothioneins of rat liver (Table 1). Particularly low in number are those hydrophobic groups that normally play an important role in maintaining the globular structure of proteins. The large number of cysteine residues present as metal-mercaptide complexes (comprising one-third of the amino acid residues in the molecule), together with a high content of charged and neutral amino acids, suggests that intramolecular hydrogen-bonding and metal-ligand complex formation contribute significantly to the three-dimensional structure of these proteins. In particular, a cellular process that could effect dissociation of the metal-mercaptide complex in the metallothioneins might be expected to render these proteins more susceptible to subsequent proteolytic hydrolysis. Kägi & Vallee (1961) have studied the competition between Zn^{2+} or Cd^{2+} and H^+ for the binding sites of thionein. They found that Cd^{2+} is bound approx. 3000 times as firmly as Zn^{2+} and that the former ion is half-dissociated from cadmium-thionein at pH 3.0. The corresponding pH for half-dissociation of Zn^{2+} from zinc-thionein is then 4.2, assuming that each metal ion can displace three protons on binding. Experimentally, we have found that exposure of metallothioneins to pH 4.5 causes significant dissociation of Zn^{2+} but not Cd^{2+} from the molecule.

The principal factors influencing the catabolism of a protein in secondary lysosomes are (Gordon, 1973): (1) concentration of the protein (turnover

most likely being a first-order process); (2) native or partially denatured state of the protein; (3) conditions of pH within the secondary lysosome; (4) availability of proteinases specific for the protein as substrate; (5) absence of inhibitors of lysosomal enzymes. A mechanism has been proposed whereby the lysosome can denature a protein substrate at low pH and then expose it to a series of proteolytic digestions with different pH optima and specificity. The pH range of such a lysosomal system has been suggested to lie between 3.6 and 5.0 (Gordon, 1973). At the lower end of this range the (cadmium+zinc)-thioneins would still maintain much of their structural integrity and the zinc-thioneins would be largely dissociated, thus differentiating the susceptibility of these two protein species to lysosomal proteolysis by the conditions listed above. The gradual enrichment with Cd^{2+} found in (cadmium+zinc)-thioneins could be explained by a differential rate of degradation of metallothioneins selecting for a longer-lived Cd^{2+} -rich population of molecules over a shorter-lived Zn^{2+} -rich population. The observation that metal ions may inhibit the activity of secondary lysosomes in mouse kidney (Davidson, 1975) suggests an alternative explanation for the slow turnover of the metallothioneins during Cd^{2+} treatment.

The reutilization of amino acids in rat liver has been well documented and it has been estimated that about 50–80% of the amino acids within the intracellular pools utilized in protein synthesis are derived from protein degradation (Gan & Jeffay, 1971). Thus the half-life of catalase was found to be 4 days from turnover of ^3H leucine, 2.5 days with [*guanidino*- ^{14}C]arginine and 1.8 days when the loss of radioactivity from the haem moiety of catalase was followed by using δ -amino[^{14}C]laevulinic acid (Poole *et al.*, 1969). This last value of 1.8 days was found to be in good agreement with measurements of the rate of return of catalase activity after irreversible inhibition by aminotriazole (Price *et al.*, 1962). The ^{14}C in [*guanidino*- ^{14}C]arginine is rapidly metabolized in the rat liver, disappearing completely within 1 h of an intraperitoneal injection (Schimke, 1964). Nevertheless, reutilization of the ^{14}C , possibly from continual degradation of proteins into which it has been incorporated, requires a 1 day correction to be made to the determination of protein half-lives when this amino acid is used. Thus rapid clearance from the liver of the labelled amino acid after single injection may not be sufficient evidence to recommend the amino acid for the half-life determination of a rapidly degraded protein. For cysteins, the use of the double-label technique on a protein of known short half-life, e.g. tryptophan pyrrolase, $k_d = 0.28 \text{ h}^{-1}$ (Schimke *et al.*, 1965), or tyrosine aminotransferase, $k_d = 0.18\text{--}0.35 \text{ h}^{-1}$ (Kenney, 1962), should indicate the correction to be applied to the observed 26h

half-life calculated by this method for the metallothioneins.

In pulse-labelling experiments, it is important that the labelling time should be short relative to the half-life of the protein under study (Schimke & Doyle, 1970). Otherwise effects of inducers of protein synthesis on degradation may contribute substantially to an observed increase in radioactive isotope incorporation. For this reason 1 h pulse-labelling with [^3H]cysteine was used. Under these conditions, assuming $k_d = 0.15\text{ h}^{-1}$ for zinc-thioneins, the control [^3H]cysteine incorporation could be underestimated by as much as 14% (i.e. $1 - e^{-0.15}$). The total radioactivity incorporated into metallothioneins relative to that incorporated into soluble proteins other than metallothioneins was stimulated approx. 15-fold by Cd^{2+} (Table 2) and 10-fold by Zn^{2+} . Assuming that both metallothioneins and rat liver high-molecular-weight soluble proteins are synthesized from the same intracellular amino acid pool, this stimulation of radioactive isotope incorporation into metallothioneins by either Cd^{2+} or Zn^{2+} is evidence for a transient stimulation of protein synthesis by these metals.

Independent evidence for the enhancement of metallothionein biosynthesis found from the [^3H]cysteine-incorporation studies comes from an analysis of hepatic protein turnover. Taking $[\text{P}]_{ss} = 2\text{--}4\text{ }\mu\text{mol/kg}$ of liver as the basal concentration of metallothioneins in the liver and $k_d = 0.15\text{ h}^{-1}$, we find a basal metallothionein biosynthesis of $K_s = 0.3\text{--}0.6\text{ }\mu\text{mol/h}$ per kg of liver. From the observed maximum rate of biosynthesis of these proteins during Cd^{2+} exposure of $2\text{--}3\text{ }\mu\text{mol/h}$ per kg of liver this gives a net stimulation of metallothionein biosynthesis by Cd^{2+} of 3–10-fold.

In the ^{203}Hg -turnover and change-in-steady-state experiments two important assumptions are (a) the equivalence between ^{203}Hg and protein turnover and (b) the attainment of a new steady-state pool of metallothioneins, 3.3-fold increased in concentration over the basal value, during exposure to ZnCl_2 . In (a), the premature release of $^{203}\text{Hg}^{2+}$ may lead either to an erroneously high value of k_d or, if this radioactive isotope is re-bound to newly synthesized protein, to an erroneously low value of k_d . The validity of determining k_d in (b) rests on the assumptions inherent in eqn. (1) of zero-order biosynthetic rate and first-order degradative rate in the presence of continuous Zn^{2+} exposure. The lack of change in rate of degradation of the metallothioneins during continuous Zn^{2+} exposure could be checked by following ^{203}Hg turnover during ZnCl_2 treatment. It may be necessary to provide conclusive evidence that the turnover of $^{203}\text{Hg}^{2+}$ reflects true protein degradation and is not an artifact arising from preferential dissociation of $^{203}\text{Hg}^{2+}$ from zinc-thionein before degradation.

Finally, Webb & Magos (1976) have shown that Cd^{2+} , displaced from presynthesized metallothionein on administration of Hg^{2+} to Cd^{2+} -pretreated rats, causes further synthesis of thionein. Such an effect is unlikely to occur with $^{203}\text{Hg}^{2+}$ treatment because of the negligible concentration of this ion accumulated in the liver in these experiments.

A rapid turnover rate has also been reported for copper bound to metallothioneins in rat liver after intravenous administration of carrier-free $^{67}\text{Cu}^{2+}$ (Terao & Owens, 1973). At 0.5, 1, 6 and 24 h after $^{67}\text{Cu}^{2+}$ injection (approx. 2 pmol injected intravenously) the relative amounts of this radioactive isotope in an 11000-mol.wt. fraction isolated by Sephadex G-100 chromatography of a liver supernatant (identified as metallothionein) were (% of administered dose) 0.52, 0.42, 0.14 and 0.03 respectively, giving a half-life of 6 h. Considering the tight binding of copper to metallothionein (H^+ titration shows a release of Cu^{2+} ion from copper-thionein at $\text{pH} < 1$; H. Rupp & U. Weser, unpublished work), the most likely mechanism for such rapid copper turnover would involve the degradation of the metallothioneins with concomitant release of copper, either as free Cu^{2+} , which could be re-bound to other proteins, or as copper peptides. The appearance in bile of ^{67}Cu , bound mainly to components with lower molecular weight than the metallothioneins, suggests that these proteins are degraded before or during transit from the liver (Terao & Owens, 1973).

An interpretation of the nature of Cd^{2+} - or Zn^{2+} -induced biosynthesis of the metallothioneins is not yet possible. The transient nature of this response would suggest either that a translational event (increase in polyribosome number or in rate of ribosome translational velocity) is responding to intracellular Cd^{2+} not as yet bound to metallothioneins or that species of short-lived mRNA coding for the metallothioneins are being produced (transcriptional regulation) or stabilized in response to the metal exposure. The use of actinomycin D to distinguish between transcriptional and translational control of metallothionein biosynthesis with exposure to metal ions has been reported frequently in the literature (Shaikh & Lucis, 1970; Webb, 1972; Squibb & Cousins, 1974; Richards & Cousins, 1975; Shaikh & Smith, 1975). In the studies carried out in these laboratories the possibility of a rapidly degradable mRNA species coding for metallothioneins, which would be expected to disappear on treatment with actinomycin D, was not considered (the half-life of such a messenger, from Table 2, would have to be less than 8 h). To distinguish between transcriptional and post-transcriptional regulatory mechanisms a system for metallothionein biosynthesis *in vitro* will have to be developed (Squibb & Cousins, 1977).

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