Effects of dietary copper supplementation of rats on the occurrence of metallothionein-I in liver and its secretion into blood, bile and urine

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The appearance and excretion of metallothionein-I (MT-I) was studied in rats given a diet containing 1000 mg of Cu/kg for several weeks. No significant increase in MT-I concentrations in liver, plasma or bile was detected in rats with liver copper concentrations less than 600 µg of Cu/g fresh wt. Above this concentration, liver MT-I concentrations increased in proportion to the increase in hepatic copper content. Plasma and bile MT-I concentrations were directly related to those in the liver and were about 10 times those in normal rats. Urinary MT-I concentration also increased 10-fold within 1 week. Fractionation of bile and urine on Sephadex G-50 revealed the presence of monomeric MT-I and a range of possible degradation products of the isoprotein.

INTRODUCTION

Metallothionein (MT) has been identified as a major copper-binding protein in the liver of neonatal and copper-loaded animals (see Bremner, 1979). Its synthesis in rats and mice can be induced by injection of copper salts (Bremner *et al.*, 1978a; Winge *et al.*, 1981) by a process involving increased production of MT mRNA and increased transcription of MT genes (Durnam & Palmiter, 1981; Wake & Mercer, 1985). However, the occurrence of Cu-containing MT in liver under normal circumstances depends not only on copper status but also on factors such as species, age and zinc status (Bremner, 1979). In addition, it can be influenced by the route of administration of copper salts, as MT synthesis in rat liver is induced after parenteral administration of copper but not necessarily after dietary supplementation with copper salts, despite the fact that hepatic copper content may be greater in the latter case (Mehra & Bremner, 1984b).

The dependence of hepatic CuMT accumulation on zinc status is particularly evident in sheep, the concentration of the protein frequently being low and largely determined by liver zinc content, although it does increase significantly in copper-poisoned animals (Bremner & Marshall, 1974; Bremner *et al.*, 1976; Saylor *et al.*, 1980). This increase in copper-poisoned animals may occur because liver copper content has increased above a critical value or may result from stress-induced synthesis of MT. The onset of the 'haemolytic crisis' in copper-poisoned sheep (the terminal stage of the disease) is often induced by stress or anorexia (Todd, 1969), both of which will induce hepatic MT synthesis in the rat (Bremner & Davies, 1975; Oh *et al.*, 1978).

The haemolytic crisis in copper-poisoned sheep is associated with mobilization of liver copper and its transfer via the blood to the kidneys, where it accumulates as CuMT (Bremner and Young, 1977). The possibility was considered (Bremner & Young, 1977) that

CuMT might be transferred from the liver to the kidneys in the same way as CdMT is transported in cadmium-poisoned animals (Nordberg *et al.*, 1971), and it was shown that parenterally administered CuMT in rats is removed efficiently and preferentially by the kidneys (Bremner *et al.*, 1978b). However, it has not so far been possible with the techniques available to detect CuMT in the blood of a copper-poisoned sheep.

The development of radioimmunoassays for MT (Garvey *et al.*, 1982; Mehra & Bremner, 1983) has revealed that MT is generally present in low concentrations in the plasma of normal rats, that plasma MT concentrations are increased after induction of tissue MT synthesis and that there is often a relationship between liver and plasma MT concentrations (Mehra & Bremner, 1984a; Sato *et al.*, 1984). The aims of the work reported below were to determine whether hepatic MT synthesis occurs in rats when liver copper concentrations are increased above a critical threshold as a result of dietary supplementation with copper salts and to see if there is sufficient secretion of MT into the plasma, bile or urine to indicate an involvement in copper transport or excretion.

MATERIALS AND METHODS

Animals

Several groups of male Hooded Lister rats (Rowett Institute strain) aged 4–5 weeks were given a semi-synthetic diet based on albumin and sucrose (Williams & Mills, 1970) for 4–6 weeks. The copper content of the diet was increased progressively over the first week from 10 mg of Cu/kg to 1000 mg/kg by the addition of CuSO₄·5H₂O and was maintained at this level thereafter. Bile was collected under ether anaesthesia for 2 h after cannulation of the bile duct distal to the junction of the hepatic ducts. Urine was collected on ice from rats kept in plastic

Abbreviation used: MT, metallothionein.

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Table 1. Effect of dietary supplementation with Cu²⁺ on concentrations of copper and MT-I in tissues

Groups of male rats (approx. 90 g) were given a diet containing 1000 mg of Cu²⁺/kg for 4–6 weeks. Means $\pm$ S.E.M. are given.

Time (weeks) ...	4	5	6
No. of rats	5	6	7
Liver Cu (μ g/g wet wt.)	678 $\pm$ 46	735 $\pm$ 62	850 $\pm$ 88
Plasma Cu (μ g/ml)	1.5 $\pm$ 0.4	1.7 $\pm$ 0.1	2.0 $\pm$ 0.1
Bile Cu (μ g/ml)	5.5 $\pm$ 1.3	6.3 $\pm$ 2.3	15.2 $\pm$ 3.8
Liver MT-I (μ g/g)	75 $\pm$ 36	128 $\pm$ 59	126 $\pm$ 15
Plasma MT-I (ng/ml)	51 $\pm$ 23	63 $\pm$ 40	81 $\pm$ 21
Bile MT-I (ng/ml)	324 $\pm$ 162	148 $\pm$ 70	324 $\pm$ 72

metabolism cages for several hours. Heparinized blood, liver and kidneys were removed at slaughter and stored, with bile and urine, at -20°C .

Analytical procedures

Concentrations of MT-I in liver, kidneys, blood plasma, bile and urine were measured by using the radioimmunoassay method described by Mehra & Bremner (1983). This has intra- and inter-assay coefficients of variation of 2–4% and 15% respectively, and is suitable for measurement of 100–25000 pg of MT-I in all these tissues and fluids. Volumes of plasma, urine and bile used for the analyses were 100, < 10 and < 25 μ l respectively. Copper and zinc concentrations in blood and in tissues were measured by atomic absorption spectrometry.

Livers were homogenized in 10 mM-Tris/acetate buffer, pH 7.4, centrifuged at 75000 *g* for 1.5 h and the supernatant fractions separated on Sephadex G-75 as described previously (Bremner & Davies, 1975). Bile and urine were fractionated on Sephadex G-50 (superfine grade), with 10 mM-Tris/acetate buffer, pH 7.4, as eluent.

Samples of the Cd₄- α - and Cu₆- β -fragments of rat liver MT-I were kindly supplied by Dr. D. R. Winge. Their cross-reactivity in the radioimmunoassay for MT-I, in which ¹²⁵I-labelled rat liver ZnMT-I was used as tracer, was determined as described by Mehra & Bremner (1983).

RESULTS

Growth rates in the rats given the copper-supplemented diet were relatively low, only about 3.7 g/day. This compares with normal growth rates of about 6 g/day in rats given the same diet containing 5 mg of Cu/kg. There were considerable variations among experiments in the rates of increase in tissue copper and MT-I concentrations. In the first experiment, in which rats weighing 60 g were group-housed and given the high-copper diet for 5 weeks, copper concentrations (means $\pm$ S.E.M.) in liver, kidneys and plasma were 258 $\pm$ 30 μ g of Cu/g, 99 $\pm$ 9 μ g of Cu/g and 1.15 $\pm$ 0.06 μ g of Cu/ml respectively (*n* = 5). (All tissue concentrations refer to fresh wt.) The concentrations of MT-I in liver and plasma were only 11.5 $\pm$ 2.3 μ g/g and 8.1 $\pm$ 2.4 ng/ml respectively, whereas that in kidneys was 179 $\pm$ 21 μ g/g.

In later experiments, where the rats were housed individually in glass/Perspex cages, tissue copper and MT-I concentrations were usually greater than those cited above. This appears to result from the change in the type of accommodation used for the rats, and has been observed in other experiments also (R. K. Mehra,

Fig. 1. Relationship between liver MT-I and copper concentrations in rats

Data are from several experiments in which young male rats were given a diet containing 1000 mg of Cu/kg for 4–6 weeks. MT-I concentrations in liver homogenates were measured by radioimmunoassay.

unpublished work). In a typical experiment, copper concentrations in liver, plasma and bile after 4–6 weeks on the high-copper diet ranged over 515–1154 μ g of Cu/g, 0.7–2.8 μ g of Cu/ml and 3.1–33.4 μ g of Cu/ml respectively, whereas MT-I concentrations in liver, plasma and bile were 16–392 μ g/g, 8–253 ng/ml and 34–738 ng/ml respectively (Table 1). Liver zinc concentrations in the copper-treated rats increased slightly from 27.8 $\pm$ 0.9 μ g of Zn/g at 4 weeks to 37.2 $\pm$ 1.6 μ g of Zn/g at 6 weeks, but plasma zinc concentrations remained constant at 2.0 μ g of Zn/ml.

When liver copper and MT-I concentrations were compared in all copper-loaded rats (Fig. 1), it was apparent that liver MT-I concentrations only increased above a basal level when liver copper concentrations had increased to about 600 μ g of Cu/g and that there was a reasonable correlation between MT-I and copper concentrations above this threshold value.

The increases in liver MT-I concentrations were in turn related to increased secretion of MT-I into both plasma and bile (Figs. 2 and 3), and could be described in the following equations:

$$[\text{Plasma MT-I}] = 0.62 [\text{liver MT-I}] - 5.3$$

(S.E. of regression coefficient = 0.07, *n* = 25, *P* < 0.001) and

$$[\text{Bile MT-I}] = 2.77 [\text{liver MT-I}] + 55.7$$

(S.E. of regression coefficient = 0.59, *n* = 17, *P* < 0.01)

Fig. 2. Relationship between MT-I concentrations in the plasma and liver of copper-loaded rats

Data are from several experiments in which young male rats were given a diet containing 1000 mg of Cu/kg for 5–6 weeks. MT-I concentrations were measured by radioimmunoassay.

where bile and plasma concentrations are given as ng/ml and liver concentrations as $\mu\text{g/g}$.

Urinary MT-I concentrations also increased substantially in the copper-loaded animals and within 1 week were 563 ± 114 ng/ml (949 ± 214 ng/mg of creatinine). This compares with starting values of 77.8 ± 3.3 ng/mg of creatinine. When the rats were killed after 4 weeks on the high-copper diet, urinary MT-I concentrations were 858 ± 35 ng/mg of creatinine and had therefore not increased over the 1-week values. MT-I concentrations in bile, liver and kidneys of these rats were then 249 ± 71 ng/ml, 69 ± 14.5 $\mu\text{g/g}$ and 412 ± 21 $\mu\text{g/g}$ respectively.

Fractionation of bile and urine

Pooled bile samples from rats given the copper-supplemented diet were fractionated on Sephadex G-50 to see if there was heterogeneity in the immunoreactive components, as was found in bile from copper-injected rats (Sato & Bremner, 1984). As shown in Fig. 4, four main immunoreactive components were present in approximately equal amounts. The first was eluted at the void volume of the column (tubes 18–20), the second (tubes 24–33) corresponded in elution volume to authentic monomeric rat liver ZnMT-I, and the third (tubes 33–42) to the α - and β -fragments from rat liver MT-I (see below).

Fig. 3. Relationship between MT-I concentrations in the bile and liver of copper-loaded rats

Data are from experiments in which young male rats were given a diet containing 1000 mg of Cu/kg for 4–6 weeks. MT-I concentrations were measured by radioimmunoassay.

Fig. 4. Fractionation of Sephadex G-50 of (a) the MT-I β -fragment and (b) bile from copper-loaded rats

(a) The elution profile for the Cu_6 - β -fragment of MT-I is shown. This is similar to the profile for the Cd_4 - α -fragment of MT-I (not shown). (b) The elution profiles for copper (●) in bile and for material that cross-reacts in the MT-I immunoassay (○) are shown. The rats were given a diet containing 1000 mg of Cu/kg for 5 weeks before bile collection.

Fig. 5. Fractionation on Sephadex G-50 of urine from copper-loaded rats

The elution profiles for copper (●) and for material that cross-reacts in the MT-I immunoassay (○) are shown. Urine was collected from rats given a diet containing 1000 mg of Cu/kg for 4 weeks.

Fig. 6. Cross-reactivity of ZnMT-I (●) and the Cu₆-β-fragment (Δ) and Cd₄-α-fragment (○) of MT-I in the immunoassay

b and b_0 refer to the ^{125}I -labelled MT-I precipitated in the presence and in the absence of competing immunoreactive material. Both are corrected for non-specific binding. Details of the Cu₆-β- and Cd₄-α-fragments are given in the text.

The fourth fraction (tubes 42–49) was eluted near the total volume of the column. Small amounts of the biliary copper were associated with the first three immunoreactive fractions, but most of the copper was eluted near the total volume of the column.

Similar fractionation of urine also revealed marked heterogeneity with regard to the immunoreactive material (Fig. 5). The 'MT-I' fraction appeared to contain at least three components, which peaked at tubes 28, 31 and 33.

The first of these corresponded in elution volume to monomeric rat liver MT-I and the third to the α- or β-fragments of MT-I. Although most of the urinary copper was eluted near the total volume of the column, sufficient copper was present in the other fractions to account for the immunoreactive material that was detected.

Cross-reactivity of Cd₄-α- and Cu₆-β-fragments of MT-I with rat liver ZnMT-I

Competitive binding assays were carried out with Cd₄-α- and Cu₆-β-fragments of rat liver MT-I (Nielson & Winge, 1983, 1984) as described by Mehra & Bremner (1983). Various amounts of these fragments, and of authentic rat liver ZnMT-I, were added to the assay mixture containing ^{125}I -ZnMT-I and the antibody to MT-I. As shown in Fig. 6, the Cu₆-β-fragment gave an inhibition curve that was parallel to that for ZnMT-I and almost overlapped with it. In contrast, the Cd₄-α-fragment showed only weak cross-reaction, although the curve was still parallel with the others. The amounts of Cu₆-β-fragment, ZnMT-I and Cd₄-α-fragment required to produce 50% inhibition of binding of the tracer were 800, 1100 and 18000 pg respectively, equivalent to the proportions 0.7:1.0:16.

DISCUSSION

It is well established that copper can induce synthesis of hepatic MT in rats and that this process is independent of any associated increase in hepatic zinc uptake (Bremner *et al.*, 1978a; Wake & Mercer, 1985). Despite this, CuMT is generally absent from the liver of zinc-deficient animals, even at relatively high liver copper contents (Bremner, 1979). This apparently anomalous situation was attributed to the increased turnover rate of liver CuMT in zinc-deficient rats (Bremner *et al.*, 1978a), but the present results suggest that it may also reflect the fact that copper is a relatively poor inducer of MT synthesis under physiological conditions.

It was demonstrated previously (Mehra & Bremner, 1984b) that only traces of hepatic CuMT could be detected in rats that were copper-loaded by supplementation of their diet with large amounts of copper salts and had liver copper contents of up to 300 μg of Cu/g. However, in the present experiment MT synthesis did occur after liver copper concentrations increased to about 600 μg of Cu/g, concentrations of the isoprotein then increasing in proportion with the increase in liver copper content. Assuming that MT-I accounted for 40% of the total liver MT, about 10% of the copper that accumulated above this threshold value was bound to MT.

Induction of MT synthesis by copper in rat liver may therefore depend on the attainment of a critical concentration of 'free' or 'labile' forms of copper. This would be readily attained in acute experiments involving injection of pharmacological amounts of Cu²⁺, but not when dietary copper is absorbed slowly from the intestine. It is perhaps significant that even in acute experiments the minimum dose of copper required to increase the rate of MT-I gene transcription in mouse liver is greater for Cu²⁺ than for Zn²⁺, although the metal ions are equally effective as MT inducers at higher dosages (Durnam & Palmiter, 1981). Under physiological conditions the binding of hepatic copper to MT probably

depends on prior induction of synthesis of the protein by zinc or some other agent.

As was found in neonatal rats (Mehra & Bremner, 1984a), a good indication of hepatic MT-I content can be obtained by assay of the isoprotein concentrations in plasma, and it is possible that this could be of diagnostic value in monitoring conditions associated with excessive hepatic accumulation of copper, such as primary biliary cirrhosis, Indian childhood cirrhosis or Wilson's disease.

Although major increases in plasma MT-I concentrations occurred in some of the copper-loaded rats, the maximum amount of copper that was associated with MT-I and MT-II was probably about 60 ng of Cu/ml, or 3% of the total plasma copper. This could imply that MT contributes little to the transport of copper in plasma. However, the half-life of intravenously injected CuMT seems to be only a few minutes (Bremner *et al.*, 1978b), whereas that of copper bound to albumin is up to 10 min and that of caeruloplasmin, the main form of copper in plasma, is 13 h (Marceau & Aspin, 1973). The flux of copper through the MT pool could therefore be relatively high and of some biological significance, provided that the half-life of the endogenous plasma protein is as low as that of the parenterally administered CuMT.

An indication of the possible contribution of secretion of MT-I into plasma to the turnover of hepatic MT-I can be obtained from the data in Table 1 and the reported half-life of hepatic CuMT-I of 15.8 h (Mehra & Bremner, 1985). If the half-life of CuMT in plasma is taken to be 5 min, a value quoted for rabbit CdMT (Johnson & Foulkes, 1980) and in accord with studies involving CuMT injection in rats (Bremner *et al.*, 1978b), the flux of MT-I through the plasma after 4 weeks on the high-copper diet (3.6 µg/h) is about 12% of the turnover of liver MT-I (30 µg/h).

Biliary excretion of MT-I was also increased in the copper-loaded rats, in proportion to the hepatic MT-I content. However, the excretion rate of 0.4 µg of MT-I/h (assuming a flow rate of 1.3 ml/h) in rats after 4 weeks (Table 1) was only equivalent to 1–2% of the estimated turnover of the hepatic isoprotein. As was found in rats injected with copper salts (Sato & Bremner, 1984), several immunoreactive components were present in the bile. The elution profiles from Sephadex G-50 for rats given a high-copper diet (Fig. 4) and those injected with copper salts (results not shown) were almost identical. By analogy with earlier results (Sato & Bremner, 1984) the first and second peaks consisted of polymeric and monomeric forms of CuMT-I. The third peak corresponded in elution volume to the α - and β -fragments of MT-I, originating from residues 30–61 and 1–29 of the isoprotein (Nielson & Winge, 1983, 1984). It was speculated previously (Sato & Bremner, 1984) that this fraction in bile from copper-injected rats may have been the α -fragment, since this was believed to cross-react completely with MT-I. However, the present results show that the cross-reactivity of the authentic Cd₂- α -fragment is relatively weak, and that the antigenic determinants of MT-I are probably situated, as shown by Winge & Garvey (1983), in the N-terminal or β -fragment, which did cross-react completely. It may be significant that copper binds preferentially in this domain (Nielson & Winge, 1984). One important consequence of these findings is that the estimates of immunoreactive material in bile, and urine, will be too low if the predominant degradation fraction is the α -fragment.

Urinary concentrations of MT-I were also greatly increased in the copper-loaded rats, and fractionation on Sephadex G-50 revealed considerable heterogeneity of immunoreactive material. This was similar in some respects to that in the bile and indicated that degradation products of MT-I were being excreted into the urine. Chang *et al.* (1980) have also noted the presence of MT fragments in urine of cadmium-exposed rats. It seems unlikely that these are artifacts since the urine from the copper-loaded rats was collected on ice and fractionated immediately at 1 °C. Moreover, CuMT has been found to be remarkably resistant to hydrolysis *in vitro* with lysosomal enzymes (Mehra & Bremner, 1985).

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