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# A study of the role of metallothionein in the inherited copper toxicosis of dogs

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The role of metallothionein (MT) was assessed in the copper-loading disease prevalent in Bedlington terriers. Fractionation of tissue supernatants over Sephadex G-75 showed that most of the additional cytosolic copper present in liver tissue of these dogs was bound to MT, and that substantially more MT-bound copper could be solubilized by detergent plus mercaptoethanol. Zinc contents were only slightly raised, although most of the extra zinc was associated with a 4000-$M_r$ ligand. Ion-exchange chromatography revealed two isoproteins, MT1 and MT2, in all the dog liver samples examined. In Bedlington terrier liver, copper associated with both isoproteins was increased, although the increase for MT2 was greater than for MT1. The content of MT protein was also raised, although cell-free translations and RNA blots of total liver RNA showed that this increase was not associated with a rise in MT mRNA. The significance of these results to the mechanism of copper accumulation in the Bedlington terrier disorder is discussed.

## INTRODUCTION

Wilson's disease in man is an inherited disorder in which copper contents are raised in liver, brain, kidney and urine. There is a lower ability to incorporate labelled copper into caeruloplasmin and, in most patients, serum caeruloplasmin contents are depressed. As the disease advances, characteristic copper deposits are seen in the cornea, the Kayser–Fleischer rings, and cirrhotic changes in the liver and haemolytic crisis may become apparent. Neurological symptoms may also be present. Patients generally respond favourably to treatment with the copper chelator penicillamine. The defect in copper homoeostasis is attributed to defective biliary excretion of copper.

Many of the above symptoms are present in a fatal inherited liver disease in Bedlington terriers first described by Padula (1974) and present in about two-thirds of the highly inbred population of Bedlington terriers in the U.S.A. Caeruloplasmin concentrations in diseased dogs are slightly elevated rather than depressed (Su *et al.*, 1982*a*), Kayser–Fleischer rings are not seen, and organs other than the liver are not generally involved, although behavioural changes have been noted in older affected dogs, and elevated contents of copper in brain, kidney and corneal tissue have been reported (Hardy *et al.*, 1975; Su *et al.*, 1982*a*). Defective biliary excretion of copper in these dogs has been demonstrated by Su *et al.* (1982*b*), indicating that this disease in the dog is very similar and may be homologous to the Wilson's disease mutation in man.

In both diseases, most of the additional hepatic copper is contained in lysosomal granules which appear as dark bodies in light- and electron-microscopic sections. Isolation of lysosomes from diseased dog liver and characterization of contents has shown that they contain considerable quantities of metallothionein (MT), a low-$M_r$, cysteine-rich, metal-binding protein (Johnson *et al.*, 1981). The defective biliary excretion of copper seen in these dogs may be the result of increased binding of copper to MT, arising from a change either in the structure of MT or in the regulation of its synthesis. The present paper examines the role of MT in the aetiology of this disorder.

## MATERIALS AND METHODS

### Tissues

Liver specimens from diseased Bedlington terriers and from normal dogs of mixed pedigree were obtained at surgery and stored at −70 °C. Two dogs of mixed pedigree were injected subcutaneously with either 0.9% NaCl or $CuCl_2$ (3 mg of $Cu^{2+}$/kg). The dogs were killed 5 h later with an overdose of Fluorothane, and the livers were excised, cooled on solid $CO_2$ and stored at −70 °C.

### Metal analysis

Metal concentrations were determined by atomic absorption spectrophotometry with a Perkin–Elmer model 5000 spectrophotometer. Tissue samples were dried in a vacuum oven to constant weight, followed by wet digestion in conc. $HNO_3$, and column fractions were acidified prior to analysis.

### Metal content of metallothionein

The metal content of MT was determined after column chromatography of tissue supernatants over Sephadex G-75. Tissues were homogenized with a Polytron homogenizer in 10 mM-Tris/HCl buffer, pH 7.4, in the same buffer containing 0.4% SDS and 1% mercaptoethanol, or in buffer containing 1% Triton X-100 and 1% mercaptoethanol, and centrifuged in a Beckman model L8-80 ultracentrifuge at 80000 *g* for 75 min. Samples of supernatant were applied to a calibrated column (1.5 cm × 65 cm) and eluted at a flow rate of 10 ml/h; 2.5 ml fractions were collected.

Ion-exchange chromatography was used to examine

Abbreviation used: MT, metallothionein.

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the tissue content of the MT isoproteins. Tissue was homogenized in 2 vol. (v/w) of nitrogen-purged 10 mM-Tris/HCl buffer, pH 7.4, containing 1% mercaptoethanol, centrifuged as described above, and applied to a calibrated Sephadex G-75 column (2.6 cm × 70 cm) made anaerobic by several column volumes of $N_2$-purged buffer. To maintain anaerobic conditions, the MT-containing fraction was run directly on to a column (1.5 cm × 20 cm) of DEAE-Sephadex A-25 and eluted with a Tris gradient formed with 100 ml of 10 mM-Tris/HCl, pH 7.4, and 100 ml of 200 mM-Tris/HCl, pH 7.4, at a flow rate of 10.5 ml/h. All buffers were continuously purged with $N_2$ during use.

### Metallothionein content of tissues

MT protein in liver tissue was measured by a method modified from that of Piotrowski *et al.* (1973) as described by Hunt & Clarke (1983). This method depends on the competitive replacement of all metals bound to the protein by mercury atoms and on the solubility of the protein at low pH. Liver was homogenized in 7 vol. of 1.15% KCl and the homogenate centrifuged at 4000 *g* for 25 min. Supernatant (1 ml) was then incubated with 0.125 ml of a $HgCl_2$ solution (12 mg of $Hg^{2+}$/ml, including 30 $\mu$g of $^{203}$Hg/ml) for 10 min at room temperature. Then 0.25 ml of 10% trichloroacetic acid was added, and the resulting precipitate pelleted in a bench centrifuge. Supernatant (1 ml) was then applied to a column (1.5 cm × 36 cm) of Sephadex G-50 and eluted with 20 mM-sodium citrate buffer, pH 2.4. Fractions (2.0 ml) were collected and the mercury content was determined by gamma counting.

### Isolation of total RNA

Total tissue RNA was isolated by a modification of the method of Liu *et al.* (1979). Tissue was homogenized in a Sorvall Omni-Mixer at 4 °C in 7.5 M-guanidinium chloride/50 mM-sodium citrate (pH 7.0)/0.1% sarcosyl/100 mM-mercaptoethanol, before CsCl centrifugation (Wake & Mercer, 1985). The yield of RNA was determined by assuming that $1A_{260}$ unit was equivalent to 40 $\mu$g of total RNA.

### RNA blots

Total RNA was denatured by heating for 5–10 min at 65 °C in 50% (w/v) formamide/6% (w/v) formaldehyde and electrophoresed in 2.2% agarose gels containing 6% formaldehyde as described by Dobner *et al.* (1981). The RNA was transferred to nitrocellulose (Thomas, 1980). The MT mRNA was detected by hybridization with a 400-base-pair mouse MT1 *Eco*RI/*Hin*dIII insert (Durnam *et al.*, 1980) previously labelled to a specific radioactivity of $> 10^8$ c.p.m./$\mu$g by nick translation. Hybridization was carried out for 18 h at 20 °C in a solution containing 50% formamide, 1 × Denhardt's reagent (polyvinylpyrrolidone, Ficoll and bovine serum albumin, each 2 mg/ml), herring sperm DNA (250 $\mu$g/ml), 5 × SSCE (0.75 M-NaCl/0.075 M-sodium citrate/25 mM-EDTA, pH 8.0) and 100 $\mu$g each of poly(U) and poly(C)/ml. The probe concentration was 10 ng/ml. Filters were washed to remove unbound probe. The final wash was with 1 × SSC/0.1% SDS at 50 °C for 1 h. MT mRNA was detected by autoradiography, by using a 24 h exposure.

**Table 1. Copper and zinc contents of liver tissue from several normal dogs of mixed pedigree, several diseased Bedlington terriers, a saline-injected and a copper-injected dog**

Values are given in $\mu$g of metal/g dry wt. of liver tissue, as means ± S.E.M. where appropriate. Numbers of animals are given in parentheses.

| Tissue from: | Content ($\mu$g/g) Copper | Zinc |
|---|---|---|
| Normal dog | 178.5 ± 32.6 (8) | 137.5 ± 10.7 (4) |
| Bedlington terrier | 4972.8 ± 596.5 (10) | 244.7 ± 10.4 (4) |
| Saline-injected dog | 83 (1) | 232 (1) |
| Copper-injected dog | 302 (1) | 132 (1) |

**Table 2. Copper and zinc contents of liver tissue supernatants prepared by homogenization in either 10 mM-Tris/HCl, pH 7.4, or 10 mM-Tris/HCl, pH 7.4, plus 0.4% SDS and 1% mercaptoethanol (MSH), followed by centrifugation at 80000 *g* for 75 min**

(*a*) Metal content of supernatants. Values are given in $\mu$g of metal/g wet wt. of liver tissue. (*b*) Percentage of total tissue copper.

| | Homogenization buffer... | Copper Tris | Copper SDS + MSH | Zinc Tris | Zinc SDS + MSH |
|---|---|---|---|---|---|
| (*a*) | Saline-injected dog | 17.8 | 19.8 | 37.6 | 41.6 |
| | Copper-injected dog | 65.4 | 52.8 | 28.3 | 26.2 |
| | Bedlington terrier | 195.8 | 711.7 | 37.8 | 37.1 |
| (*b*) | Saline-injected dog | 85.2 | 94.8 | 64.4 | 71.2 |
| | Copper-injected dog | 85.9 | 69.4 | 85.0 | 78.7 |
| | Bedlington terrier | 10.1 | 36.6 | 61.4 | 60.2 |

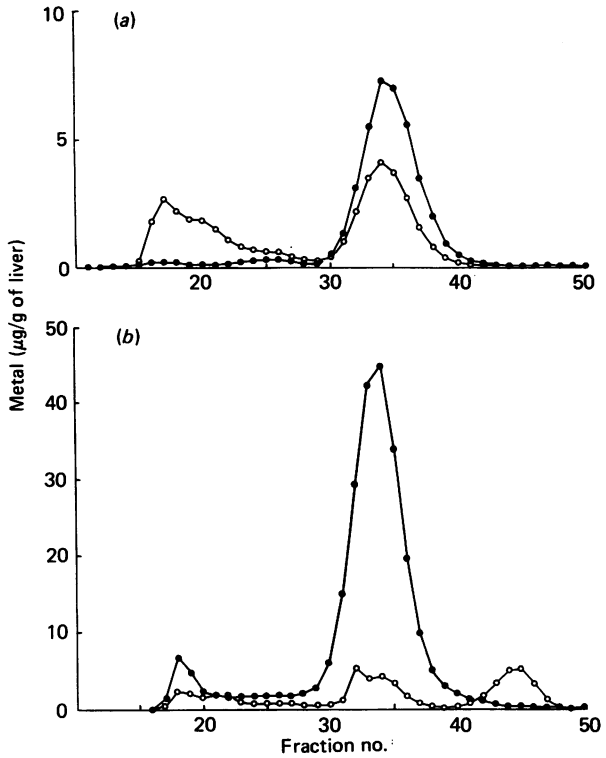


**Fig. 1. Cytosol copper- and zinc-binding proteins in liver tissue from (*a*) normal dog and (*b*) a Bedlington terrier, fractionated over Sephadex G-75**

Copper (●) and zinc (○) concentrations per fraction are given in μg/g wet wt. of tissue applied to the column. Note different scales on the metal axis.

### Cell-free translation of RNA

Wheat germ was obtained from Adelaide Mills (Adelaide, S. Australia) and an S-30 fraction prepared by the method of Marcu & Dudock (1974), except that the buffer used throughout was 20 mM-Hepes/3 mM-magnesium acetate/50 mM-potassium acetate/1 mM-dithiothreitol pH 7.6. The S-30 fraction was stored in batches in liquid $N_2$.

For the cell-free translations, each 50 μl reaction mixture contained 1 mM-ATP, 20 μM-GTP, 8 mM-phosphocreatine, 40 μg of creatine kinase/ml, 110 mM-potassium acetate, 2 mM-magnesium acetate, 2.2 mM-dithiothreitol, 19 unlabelled amino acids (25 μM each), 4 μM-spermine, 24 mM-Hepes, pH 7.6, and 3 μCi of [$^3$H]leucine or 7.5 μCi of [$^{35}$S]cysteine; 10 μl of wheat-germ S-30 fraction was used for each assay. Total RNA concentration ranged from 100 to 160 μg/ml. Incubation was for 90 min at 26 °C. Incorporation of radioactivity into polypeptides was measured as described by Marcu & Dudock (1974). The translation products were carboxamidomethylated as described by Andersen & Weser (1978) (except that iodoacetamide was used in place of iodoacetic acid), separated by polyacrylamide-gel electrophoresis and detected in the dried gels by autoradiography.

## RESULTS

### Copper and zinc contents of tissues

The liver copper contents of the Bedlington terriers examined ranged from 919 to 7717 μg/g dry wt., with an overall mean value 27 times the mean value for normal dog liver (Table 1). In contrast, the zinc content, although significantly different ($P < 0.001$) from normal, was only raised 2-fold.

The copper and zinc contents of liver tissue from the copper-injected and saline-injected dogs are also shown in Table 1. The copper content of the copper-injected dog, although higher than that of the normal and saline-injected dogs, did not approach that in the Bedlington terriers.

### Subcellular distribution of metal

Although more copper was present in the supernatants of Bedlington-terrier liver (Table 2), it represented proportionately far less of the total tissue copper. The addition of SDS and mercaptoethanol substantially

**Table 3. Copper and zinc contents of the major Sephadex G-75 chromatography peaks of dog liver**

Liver was homogenized in: (*a*) 10 mM-Tris/HCl, pH 7.4; (*b*) 10 mM-Tris/HCl plus 1% Triton X-100 and 1% mercaptoethanol; (*c*) 10 mM-Tris/HCl plus 0.4% SDS and 1% mercaptoethanol. Values are given in μg of metal/g wet wt. of tissue applied to the column.

| | | | Content (μg/g) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Copper | | | Zinc | | |
| | Tissue from: | Peak... | Void-volume | MT | 4000-$M_r$ | Void-volume | MT | 4000-$M_r$ |
| (*a*) | Normal | | 0.9 | 36.8 | — | 16.2 | 19.4 | — |
| | Saline-injected | | 0.8 | 16.3 | — | 14.4 | 32.3 | 1.3 |
| | Copper-injected | | 6.8 | 52.9 | — | 14.3 | 8.6 | — |
| | Bedlington terrier | | 20.7 | 217.9 | — | 13.7 | 22.0 | 21.7 |
| (*b*) | Saline-injected | | 4.3 | 16.7 | 0.8 | 22.9 | 30.3 | 6.2 |
| | Copper-injected | | 6.2 | 53.6 | — | 17.3 | 8.1 | 0.8 |
| | Bedlington terrier | | 68.3 | 465.8 | — | 30.0 | 8.8 | 19.5 |
| (*c*) | Saline-injected | | 2.0 | 35.4 | — | 28.0 | 37.0 | 0.7 |
| | Copper-injected | | 20.2 | 121.1 | 16.0 | 21.4 | 17.2 | 33.4 |
| | Bedlington terrier | | 324.9 | 642.9 | — | 28.2 | 32.5 | — |

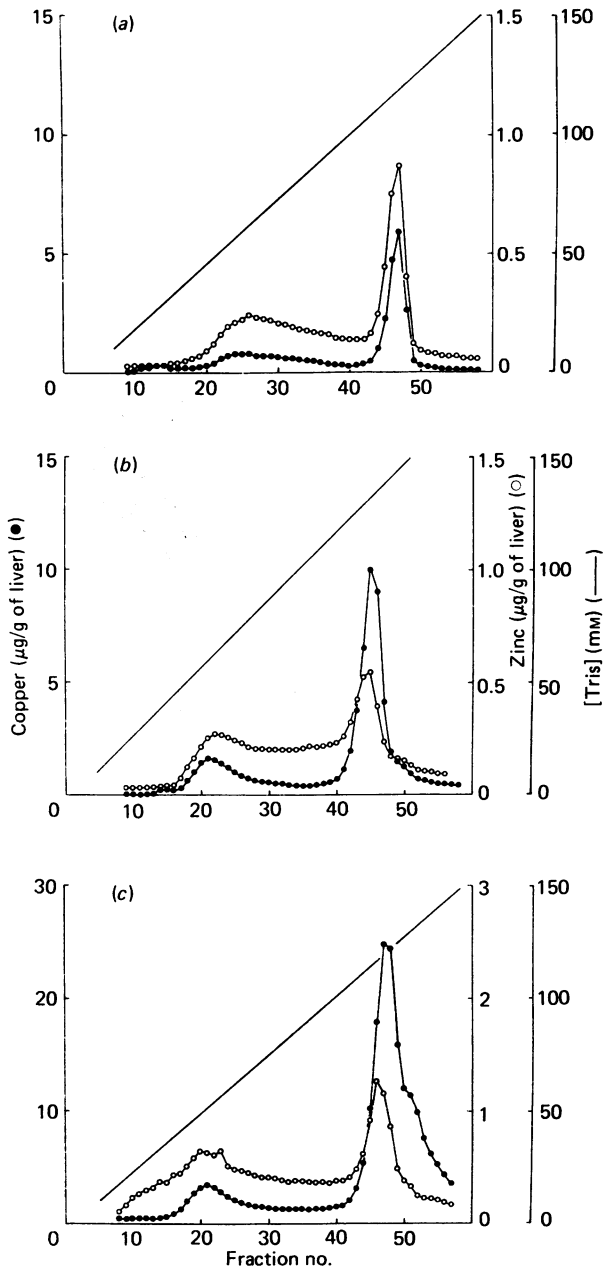


**Fig. 2. DEAE-Sephadex A-25 ion-exchange-chromatographic profiles of metallothionein from (*a*) normal dog liver tissue, (*b*) copper-injected dog liver tissue and (*c*) Bedlington-terrier liver tissue**

Tissue supernatants were prepared by homogenization in 10 mM-Tris/HCl buffer, pH 7.4, containing 1% mercaptoethanol and centrifuging at 80000 *g* for 75 min, followed by chromatography over a calibrated column of Sephadex G-75. The metallothionein-containing eluate was run directly on to a column of DEAE-Sephadex A-25 and eluted with a 10–200 mM-Tris/HCl gradient. All procedures were carried out with $N_2$-purged buffers.

increased the solubilization of the copper in the diseased dog liver, although even then over 60% remained in the pellet.

Fractionation of supernatants prepared in 10 mM-Tris/HCl, pH 7.4, over Sephadex G-75 revealed, in most cases, only two major copper-containing peaks, the first coincident with the void volume of the column and a second, much larger, MT peak eluted with an apparent $M_r$ of about 10000. In some cases, a third peak corresponding to an $M_r$ of about 4000 was also seen. Two zinc peaks were generally present, a void-volume peak and a MT peak. Typical profiles are presented in Fig. 1, with a summary of the metal contents of the major peaks in Table 3.

In the copper-injected dog, the copper contents of both the void-volume and MT peaks were raised compared with normal. In contrast, saline injection resulted in a decrease in MT-bound copper, an increase in MT-bound zinc, and the appearance of a 4000-$M_r$ zinc peak.

The copper content of MT in Bedlington-terrier liver was substantially higher than in the livers of any of the other dogs, and the copper content of the void-volume peak was also higher. In contrast, the zinc content of the void-volume and MT peaks was approximately the same as in the normal dog, although in the Bedlington terrier there was an additional zinc peak corresponding to an $M_r$ of about 4000 and accounting for 38% of the total cytosolic zinc.

As described above, additional copper is solubilized by homogenization of Bedlington-terrier liver in buffer containing 0.4% SDS and 1% mercaptoethanol. Fractionation of supernatants prepared in this way and in buffer plus 1% Triton X-100 and 1% mercaptoethanol (Table 3) resulted in a substantial increase in the copper content of both the void-volume and the MT peaks of Bedlington-terrier liver. SDS was the most effective, resulting in a 3.0-fold increase in the MT peak and a 15.7-fold increase in the void-volume peak, compared with rises of 2.1-fold and 3.3-fold respectively with Triton X-100. SDS also resulted in a substantial increase in the copper content of the void-volume and MT fractions of liver from the copper-injected dog, plus the appearance of a 4000-$M_r$ copper and zinc peak.

The MT peak was further fractionated by ion-exchange chromatography over DEAE-Sephadex A-25. The results are shown in Fig. 2. Two peaks were routinely recovered, a broad peak eluted at approx. 60 mM-Tris and a much sharper peak at 140 mM-Tris. These two peaks are referred to as MT1 and MT2, and their copper and zinc contents, determined by summing over the peak fractions, is shown in Table 4. A small shoulder is present on the MT2 copper peak in the Bedlington-terrier liver fractionation. It was not clear whether this represented a distinct molecular form of MT, and was not investigated further.

In all the dog liver samples examined, proportionately more copper was bound to MT2 than to MT1, even though the total amount of copper differed considerably in the different samples. The MT2/MT1 binding ratio for liver tissue from the untreated normal dog is 1.6, and this increases to 3.1 in liver tissue from the copper-injected dog, where most of the additional copper is bound to MT2. In Bedlington-terrier liver, there is a further increase in the amount of copper associated with MT2, together with a smaller rise in the copper content of MT1 to give a ratio of 5.1. In contrast, zinc is more evenly distributed across the two isoproteins in the different liver samples.

### MT content of liver tissue

Total cytosolic MT was estimated in liver supernatants after homogenization in detergent-free buffer by the

**Table 4. Copper and zinc contents of MT1 and MT2 isoproteins isolated by chromatography of the Sephadex G-75 MT peak over DEAE-Sephadex A-25**

Values are given in $\mu$g of metal/g wet wt. of tissue applied to the column. Numbers in parentheses refer to fractions used for summation of metal contents of isoproteins.

| Tissue | | Copper | Zinc |
|---|---|---|---|
| Normal | MT1 | 11.2 (21–38) | 3.5 (20–38) |
| | MT2 | 17.4 (43–49) | 3.0 (43–49) |
| | Ratio (MT2/MT1) | 1.6 | 0.9 |
| Copper-injected | MT1 | 13.5 (17–30) | 2.4 (17–27) |
| | MT2 | 41.2 (40–49) | 3.2 (40–49) |
| | Ratio (MT2/MT1) | 3.1 | 1.3 |
| Bedlington terrier | MT1 | 32.1 (16–30) | 8.4 (16–32) |
| | MT2 | 163.8 (42–57) | 6.9 (42–51) |
| | Ratio (MT2/MT1) | 5.1 | 0.8 |

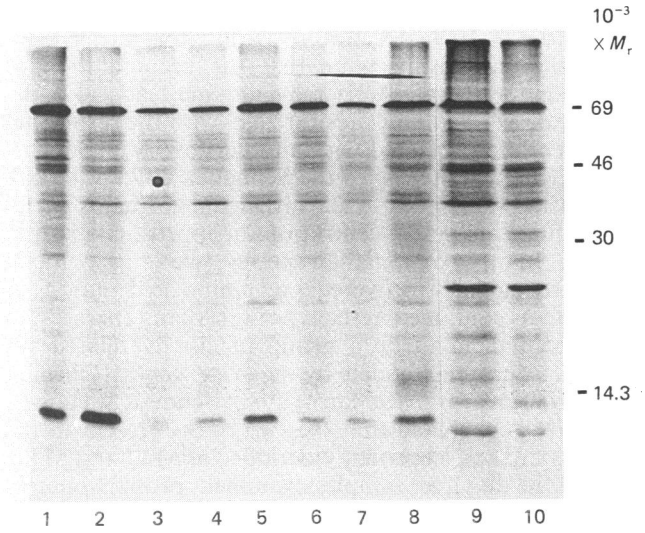


**Fig. 3. Electrophoresis of [$^{35}$S]cysteine-labelled and [$^{3}$H]leucine-labelled translation products from cell-free translations of RNA extracted from liver tissue of a saline-injected dog, a copper-injected dog, four normal dogs of mixed pedigree and two Bedlington Terriers**

Slots 1–8, translation products labelled with [$^{35}$S]cysteine; slots 9–10, translation products labelled with [$^{3}$H]leucine. RNA translated was from the following sources: slot 1, from saline-injected dog; slot 2, from copper-injected dog; slot 3, from normal dog 1; slot 4 from normal dog 2; slot 5, from normal dog 3; slot 6, from normal dog 4; slot 7, from Bedlington terrier 1; slot 8, from Bedlington terrier 2; slot 9, from normal dog 1; slot 10, from Bedlington terrier 1. The positions of the $^{14}$C-reductively-labelled $M_r$ markers (lysozyme, 14300; carbonic anhydrase, 30000; ovalbumin, 46000; bovine serum albumin, 69000) are indicated on the right-hand side.

mercury-binding assay as described in the Materials and methods section. The values of 79.6 $\mu$g of mercury/g wet wt. for normal dog liver and 478.0 $\mu$g of mercury/g wet wt. for Bedlington-terrier liver demonstrate a substantial increase in the amount of MT protein in the liver cytosol of these dogs.

## MT mRNA

To determine the liver content of MT mRNA, total RNA was translated in a cell-free wheat-germ system with either [$^{35}$S]cysteine or [$^{3}$H]leucine (Fig. 3). The MT translation product can be identified by its incorporation of [$^{35}$S]cysteine and non-incorporation of [$^{3}$H]leucine (Mercer *et al.*, 1981).

Compared with the four normal dog liver samples, the production of MT mRNA was clearly induced in the copper-injected dog. The injection of saline also induced MT mRNA synthesis, and, although such a pronounced increase was unexpected, this presumably occurred as a response to stress (Brady, 1981). In contrast, the MT mRNA content of the two Bedlington-terrier liver samples was within the range of the normal dog samples.

To obtain a further estimate of the relative amounts of MT mRNA, total RNA was blotted as described in the Materials and methods section and detected with a mouse MT1 probe (Durnam *et al.*, 1980). As shown in Fig. 4, the amounts of mRNA determined by this method agree fairly well with those obtained from the translation assays, except that the amount of MT mRNA in the Bedlington-terrier samples (slots 3 and 4) was slightly higher than in the normal dogs (slots 5–8), although they were clearly much lower than either the saline-injected (slot 1) or copper-injected (slot 2) animals.

## DISCUSSION

The copper content of Bedlington-terrier livers examined in this study was, on average, 27-fold higher than normal. Similar increases in the copper content of Bedlington-terrier liver tissue have been reported previously (Twedt *et al.*, 1979; Su *et al.*, 1982*a*).

Two major copper- and zinc-containing peaks were resolved by fractionation of dog liver supernatant over Sephadex G-75; a void-volume peak and a peak, identified as MT, eluted with an apparent $M_r$ of 10000. The presence of this latter peak in untreated normal dogs contrasts with the situation in adult rodents, where a discernible MT peak as determined by metal content is only found after metal challenge (Bremner *et al.*, 1978; Hunt & Clarke, 1983). It therefore appears that dogs normally contain a substantial amount of MT in their

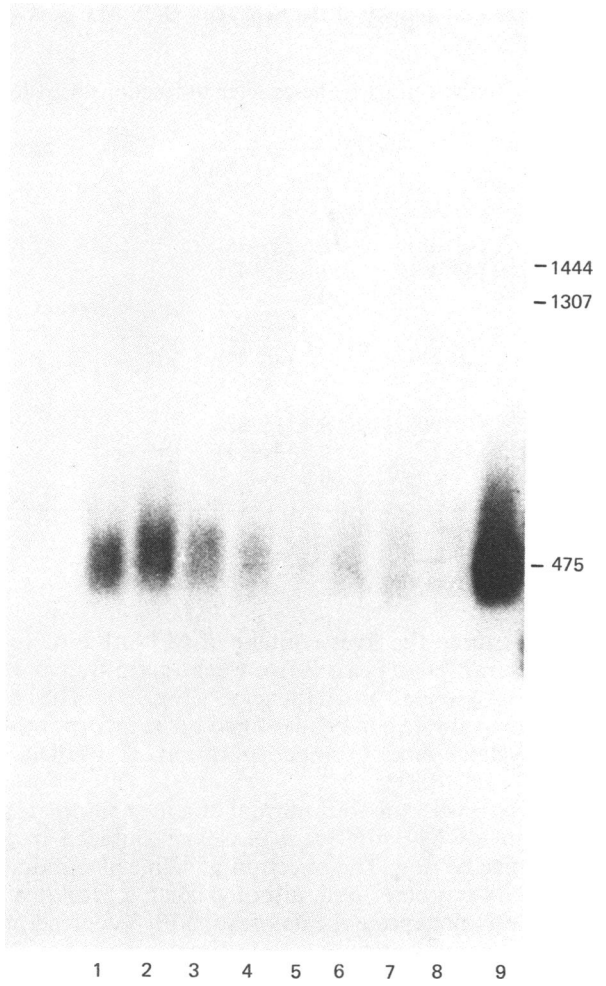


**Fig. 4. RNA blot analysis of metallothionein mRNA in liver RNA from normal dogs and Bedlington terriers**

RNA was denatured with formaldehyde and electrophoresed on formaldehyde/agarose gels as described in the Materials and methods section. The RNA was transferred to nitrocellulose filters, and MT mRNA was detected by using a mouse MT1 probe labelled with $^{32}P$ by nick translation. Slot 1, RNA from saline-injected dog; slot 2, RNA from copper-injected dog; slot 3, RNA from Bedlington terrier 1; slot 4, RNA from Bedlington terrier 2; slot 5, RNA from normal dog 1; slot 6, RNA from normal dog 2; slot 7, RNA from normal dog 3; slot 8, RNA from normal dog 4; slot 9, RNA from copper-injected rat liver. The size markers (no. of nucleotides) were derived from endonuclease-*Taq*I-digested plasmid pBR322.

livers, and the explanation for this and for the relatively much higher total copper content of normal dog liver tissue compared with that of humans (Gumbrell, 1972) or other mammals (Bremner & Young, 1976; Hunt & Port, 1979; Camakaris *et al.*, 1979) may reside in the inability of dog serum albumin to bind copper (Appleton & Sarkar, 1971; Dixon & Sarkar, 1972). Dog plasma shows a decreased retention of ionic copper, so it is possible that this leads to a more rapid and extensive passage of the metal through the liver, with the consequent induction of MT synthesis. Alternatively, these high hepatic metal contents may reflect their high contents in commercially available dog foods (Su *et al.*, 1982*b*).

In Bedlington-terrier liver, much of the additional cytosolic copper was recovered bound to MT, and the cytosolic content of MT (determined by a mercury-binding assay) was also elevated. Johnson *et al.* (1981) have previously established that MT accumulates along with copper in Bedlington-terrier liver lysosomes, and in the present experiments a substantial amount of additional MT-bound copper was extractable from the tissue with detergent (SDS or Triton X-100) and mercaptoethanol. There was also an increase in the amount of copper recovered in the void volume of the column.

Zinc contents were also elevated in Bedlington terrier liver, although the 2-fold increase was considerably less than for copper. An interesting finding, however, was that this additional zinc was recovered in the cytosol (detergent extraction had little effect on zinc recovery from Bedlington terrier liver), and most was associated with a low-$M_r$ (about 4000) peak. This additional zinc peak was not seen in column fractionations of liver from any of the other dogs, but was recovered after both SDS and Triton X-100 extraction of Bedlington terrier liver. It is tempting to speculate that the accumulation of copper in Bedlington terrier liver tissue leads to a deficiency of MT-binding sites for zinc, with the result that zinc becomes associated with another low-$M_r$ ligand.

Further fractionation of the Sephadex G-75 MT peak over DEAE-Sephadex A-25 revealed two major metal peaks eluted at Tris concentrations of approx. 60 mM and 140 mM. In other mammalian species, two or more metal peaks are regularly recovered, and amino acid analysis of the proteins contained in these peaks (Huang *et al.*, 1979) has shown them to be a group of closely related yet distinct isoproteins coded by separate genes (Durnam *et al.*, 1980; Karin & Richards, 1982; Schmidt & Hamer, 1983). It is concluded that the two peaks present in the dog liver are MT isoproteins identified as MT1 and MT2.

In all the dog liver samples examined, proportionately more copper was associated with MT2 than with MT1. Cain & Griffiths (1984) have reported that the two isoproteins in rat liver have markedly different half-lives, resulting in different tissue contents. In the present case, however, the approximately equivalent zinc contents of MT1 and MT2 suggests that the two isoproteins are present in similar quantities, and the failure of MT1 from the copper-injected dog liver to show a rise in copper content argues for the preferential binding of copper to MT2. In the Bedlington terrier, more copper is again associated with MT2. However, since the copper content of MT1 is also increased, although to a lesser extent than that of MT2, it would appear that both isoproteins are involved in the disorder. Since a mutation in one or other of the MT genes (it is extremely unlikely that there is a mutation in both genes) would affect the copper-binding affinity of only one of the two isoproteins, it is unlikely that the mutation in the Bedlington terrier results in a mutant MT protein.

The determination of MT mRNA content of normal and Bedlington-terrier liver tissue by cell-free translation and blotting clearly demonstrates that the amount of MT mRNA was only slightly above the range of normal dogs and well below the value in either the saline-injected or the copper-injected dogs. Such a small increase would appear insufficient to account for the increase in cytosolic MT protein or for the excessive accumulation of copper.

It is therefore unlikely that the defect in Bedlington-terrier liver arises from an elevated or unregulated synthesis of MT.

Despite the near-normal MT mRNA contents, amounts of MT protein are nevertheless raised, not only in the cytosol but also sequestered in lysosomes (Johnson *et al.*, 1981). In the absence of evidence for an altered MT protein or for defective regulation of MT synthesis, it would appear that these changes in MT content are the result rather than the cause of the changes in copper content. Two further mechanisms that would account for the decreased biliary excretion of copper in affected dogs are a defective efflux of copper from liver cells and a decrease in a MT degradation system specific for copper-thionein. In the first case, however, the resulting high intracellular copper concentrations would be expected to lead to the induction of MT synthesis. A more probable explanation, therefore, is that the defect in this disorder specifically affects the turnover of copper-thionein and, as the concentrations of the protein–metal complex build up, the excess is sequestered into lysosomes.

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