Profiles of ^{67}Cu in blood, bile, urine and faeces from ^{67}Cu-primed lambs: effect of ^{99}Mo-labelled tetrathiomolybdate on the metabolism of ^{67}Cu after long-term storage

BY S. R. GOONERATNE[1]*, B. LAARVELD[1], R. K. CHAPLIN[2] AND D. A. CHRISTENSEN[1]

[1] *Department of Animal and Poultry Science and* [2] *Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada*

(*Received 24 May 1988 – Accepted 4 November 1988*)

1. The effectiveness of tetrathiomolybdate (TTM) in the removal of ^{67}Cu from the long-term storage compartment in liver was studied. Lambs receiving 5 mg Cu/kg dry matter (DM) or 35 mg Cu/kg DM were primed intravenously (iv) with ^{67}Cu and challenged 10 d later with ^{99}Mo-labelled TTM given either iv or intraduodenally (id). The profiles of ^{67}Cu and ^{99}Mo and of Cu and Mo with time were measured in blood, bile, urine and faeces.

2. The level of dietary Cu affected the amplitude of profiles of ^{67}Cu and Cu in blood, bile and urine after administration of ^{99}Mo-labelled TTM. TTM administration increased liver Cu removal and this was most marked in sheep given TTM iv. The liver Cu removal from the long-term storage Cu compartment was low and was not affected by the route of administration of TTM. Endogenous Cu excretion was higher in lambs given TTM id.

3. Excretion of ^{67}Cu in bile through the transhepatocellular pathway after TTM administration appeared absent, while the transbiliary and hepatolysosomal pathways were operative. The potential reasons for this change are discussed.

4. TTM predominantly enhances the removal of Cu from the short-term storage compartment, but effects on the long-term storage compartment may still be of significance.

There is increasing evidence that thiomolybdates (TM) play a key role in the pathogenesis of molybdenum- and suphur-induced hypocuprosis and molybdenosis in ruminants (Dick *et al.* 1975; Suttle, 1980; Gooneratne, 1986; Mason, 1986). The TM not only reduce absorption of dietary copper from the gut (Mills *et al.* 1978), but also affect the Cu metabolism systemically.

Liver is the major storage organ for Cu (Underwood, 1977; Weber *et al.* 1980). Sheep accumulate excessive amounts of Cu in liver because their capacity to excrete excess Cu via bile is limited (Gooneratne *et al.* 1988). Cu is present in liver in possibly two storage compartments (Weber *et al.* 1983; Fig. 1): a temporary storage compartment destined for excretion in bile or incorporation into a caeruloplasmin (Cp), and a longer-term storage compartment. The fate of Cu from this long-term storage compartment is obscure. However, Weber *et al.* (1983) described an increase in magnitude of an undefined kinetic variable K_{20} (Fig. 1), representing a loss of Cu from the long-term storage compartment during Mo supplementation. We have previously shown that tetrathiomolybdate (TTM) efficiently removes recently stored (within 27 h) tissue Cu (possibly of hepatic origin), by increasing its excretion via bile and endogenous secretions into the gastrointestinal tract, and via urine (Gooneratne *et al.* 1989). The greatest effect of TTM on bile Cu excretion occurred within 24 h following its administration when systemic effects were maximal. Systemic effects on Cu metabolism due to TTM decline after repeated injections (100 mg) (Gooneratne *et al.* 1981*a*). This suggests that TTM acts primarily on the short-term liver Cu storage compartment and that the effects of TTM on the long-term Cu storage compartment may be limited. However, the latter effect has not been studied. The present investigation was

* Present address: Animal and Veterinary Sciences Group, Lincoln College, University of Canterbury, Canterbury, New Zealand.

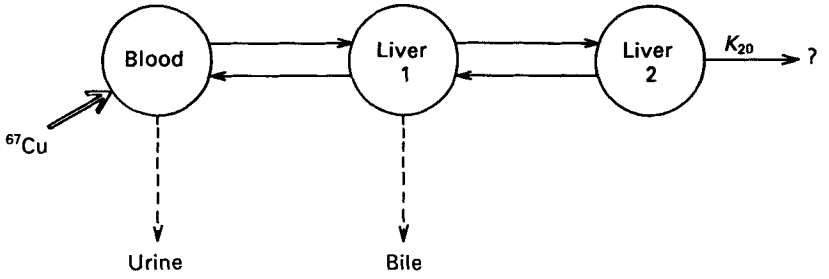

Fig. 1. Three-compartmental model describing the kinetics of intravenously administered radiolabelled copper (^{67}Cu) from Weber *et al.* (1983). The first compartment represents blood, and compartments 1 and 2 short- and long-term Cu storage compartments in the liver respectively. K_{20} represents an undefined rate constant which increased with molybdenum supplementation. Compartment 0 referred to by Weber *et al.* (1983) was undefined, but the present study illustrates that this constitutes both bile and blood.

carried out to evaluate the fate of Cu in the long-term Cu storage compartment, and to determine the effectiveness of TTM in the removal of Cu from this compartment. Systemic effects of TTM and relative efficiencies of body Cu removal via the different excretory pathways were examined, with special emphasis on deviations in mechanisms of biliary Cu excretion compared with the mechanisms proposed for bile Cu excretion from the short-term Cu storage compartment (Gooneratne *et al.* 1989).

MATERIALS AND METHODS

Experimental animals

Four female lambs drawn from a pool of eight cross-bred female lambs (17–22 kg) were used in the present study. The procedures for housing, feeding, bile duct and duodenal cannulation techniques, period of time allowed for recovery from surgery, bile flow stabilization period and sampling schedule to determine baseline values of Cu in plasma, bile, urine and faeces before radiotracer studies, have been reported (Gooneratne *et al.* 1989). The lambs received either 5·1 mg Cu/kg (lamb nos. 3 and 4) or 35 mg Cu/kg (lamb nos. 7 and 8) in the diet dry matter (DM).

Radiotracer studies

Each lamb was fitted with a Foley catheter and harness for the collection of urine and faeces respectively, before receiving the isotope. Lambs were injected intravenously (iv) with 1·5 mCi ^{67}Cu (0·6 mg Cu as Cu acetate; Oakridge National Laboratory, Oakridge, TN, USA) in 5 ml physiological saline (9 g sodium chloride/l). One animal from each dietary group was then challenged 10 d later with a 5 ml iv dose of ^{99}Mo-labelled TTM (0·2 mCi in 5·9 mg Mo; lamb nos. 3 and 7) or with a 7·5 ml intraduodenal (id) dose of ^{99}Mo-labelled TTM (0·3 mCi in 8·85 mg Mo; lamb nos. 4 and 8). The procedures for the preparation of TTM, administration and analysis of radioactivity in blood, bile, urine and faeces have been described (Gooneratne *et al.* 1989).

Collection of blood, bile, urine and faeces

The basic protocol for the collection of blood, bile, urine and faeces was similar to that described by Gooneratne *et al* (1989), except for the following. Sampling of blood, bile and urine continued at 3 h intervals for an additional 24 h for a total of 48 h following ^{67}Cu administration. Samples of blood, bile and urine were also collected at 3 h intervals for

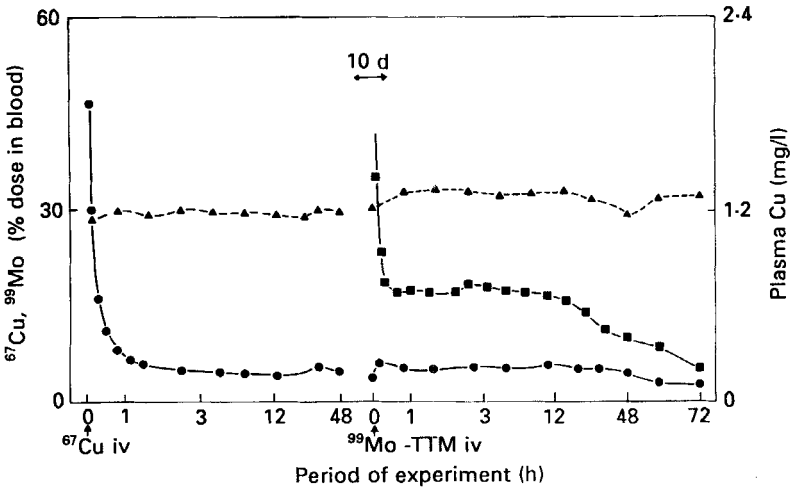

Fig. 2. Changes in ^{67}Cu (●——●) and ^{99}Mo (■——■) in blood, and plasma Cu (▲---▲) in lamb no. 7 infused intravenously (iv) with ^{67}Cu (1·5 mCi; 0·6 mg Cu) and challenged after 10 d with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·2 mCi, 5·9 mg Mo) infused iv.

24 h before TTM administration, as well as a single 24 h faecal sample to determine baseline ^{67}Cu and ^{99}Mo levels. The total radioactivity in blood was estimated assuming a blood volume of 7% of body-weight.

Cu and Mo analyses

Cu content in feed, plasma, bile, urine and faeces, and Mo in plasma and bile were determined as described previously (Gooneratne *et al.* 1989).

RESULTS

Effect of dietary Cu on ^{67}Cu and ^{99}Mo profiles

Before TTM administration the profiles of ^{67}Cu in blood, bile, urine and faeces were similar in lambs from both dietary groups. After TTM the peak heights and areas for ^{67}Cu and ^{99}Mo were most marked in lambs fed on the high-Cu diet (lamb nos. 7 and 8).

Blood ^{67}Cu before and ^{67}Cu and ^{99}Mo after iv or id challenge with ^{99}Mo-labelled TTM

Clearance of ^{67}Cu from blood was rapid in all lambs. At 1 h after injection of ^{67}Cu about 6–8% of the injected dose remained in the circulation (Figs. 2 and 3). Blood ^{67}Cu continued to decline steadily and by 24 h the levels had stabilized in all animals at a level of approximately 3–5% of the injected dose. A slight elevation of blood ^{67}Cu was observed in three lambs (nos. 3, 4 and 7) between 33 and 38 h. Samples taken during 24 h before TTM administration showed that ^{67}Cu had stabilized in all animals to levels ranging from 0·8 to 1·7% of the injected dose.

Administration of TTM resulted in an immediate increase in blood ^{67}Cu levels reaching a peak of approximately 1·1–2·1% of the injected dose at 30 min. Thereafter ^{67}Cu declined but slight elevations were observed at approximately 2·5 and 11 h. A steady-state of 0·6–1% of the injected dose was reached at 65 h. In lamb no. 7 given TTM iv (Fig. 2), four phases of ^{99}Mo clearance were apparent: an initial rapid disappearance of ^{99}Mo reaching a plateau in 45 min, a slight elevation at 2·5 h, then a gradual decline over the next 9 h, followed by a rapid decline to reach a steady equilibrium at approximately 3% of the injected dose at

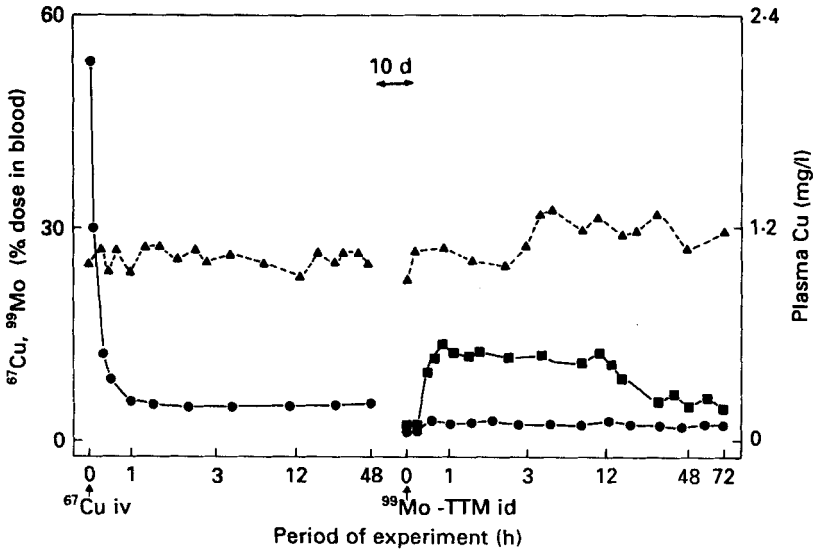

Fig. 3. Changes in ^{67}Cu (●——●) and ^{99}Mo (■——■) in blood, and plasma Cu concentration (▲---▲) in lamb no. 8 infused intravenously (iv) with ^{67}Cu (1·5 mCi; 0·6 mg Cu) and challenged after 10 d with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·3 mCi, 8·85 mg Mo) infused intraduodenally (id).

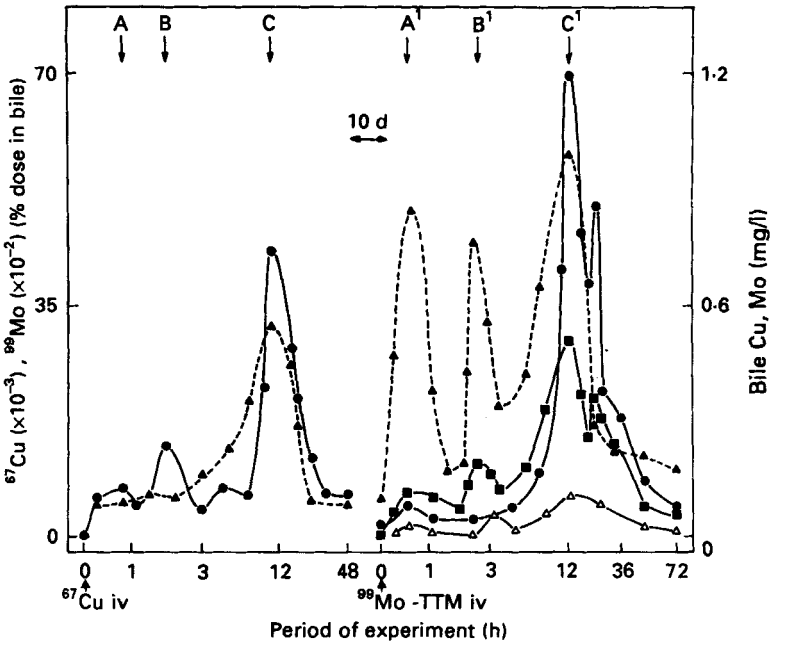

Fig. 4. Biliary changes in ^{67}Cu (●——●), ^{99}Mo (■——■), and Cu (▲---▲) and Mo (△——△) concentrations in lamb no. 7 infused intravenously (iv) with ^{67}Cu (1·5 mCi; 0·6 mg Cu), and challenged after 10 d with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·2 mCi, 5·9 mg Mo) infused iv. Peaks of ^{67}Cu excretion of increasing amplitude observed at 45 min, 2 h and 12 h after ^{67}Cu administration have been termed A, B and C respectively. Stable Cu profiles of similar pattern and frequency observed at 45 min, 2 h and 11·5 h after ^{99}Mo-labelled TTM administration have been termed A^1, B^1 and C^1. Note the absence of ^{67}Cu in peak B^1.

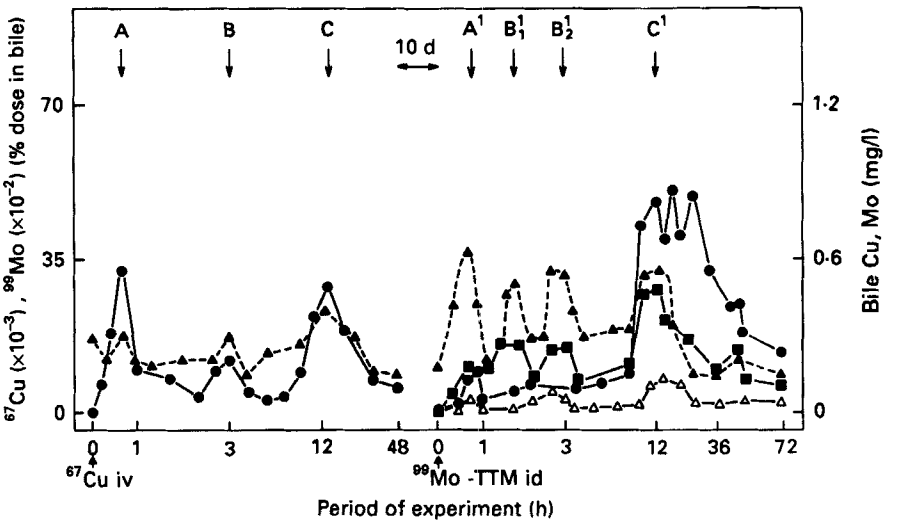

Fig. 5. Biliary changes in ^{67}Cu (●——●), ^{99}Mo (■——■), and Cu (▲---▲) and Mo (△——△) concentrations in lamb no. 8 infused intravenously (iv) with ^{67}Cu (1·5 mCi; 0·6 mg Cu), and challenged after 10 d with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·3 mCi, 8·85 mg Mo) infused intraduodenally (id). Peaks of ^{67}Cu excretion of increasing amplitude were observed at 30 min, 3 h and 12 h after ^{67}Cu administration, and have been termed A, B and C respectively. Peaks of stable Cu excretion observed at 45 min, 1·75 h, 2·75 h and 12·5 h after ^{99}Mo-labelled TTM have been termed A^1, B^1_1, B^1_2, and C^1 respectively. Note slight elevation of ^{67}Cu at 2 h after TTM administration and absence of ^{67}Cu in both B^1_1 and B^1_2 peaks.

72 h. Lamb no. 3 fed on the low-Cu diet did not exhibit the elevation of ^{99}Mo at 2·5 h. Absorption of ^{99}Mo in lambs given TTM id was rapid. The maximum concentration of ^{99}Mo in blood of 14–16% of the injected dose was observed at 45 min. A prolonged steady-state was then observed for the following 9 h. Following this, ^{99}Mo increased at approximately 12 h and then rapidly declined until 36 h. The levels fluctuated thereafter until the end of the sampling period.

Stable Cu concentration in plasma before and after iv or id challenge with ^{99}Mo-labelled TTM

Basal plasma Cu concentrations in all animals before the injection of ^{67}Cu varied from 0·8 to 1·2 mg/l. Administration of ^{67}Cu increased stable Cu concentrations slightly in all but one sheep; the levels then ranged between 0·95 and 1·3 mg/l until TTM administration. Both iv and id TTM administration resulted in an immediate increase in plasma Cu concentration in all lambs and this was most marked in animals fed on the high-Cu diet. A sustained elevation of plasma Cu concentration was observed for 12 h in lamb nos. 3 and 7 (Fig. 2) given TTM iv. The plasma Cu concentration in lamb no. 8 after the initial elevation, declined during the next 2 h, but again increased markedly starting at 3 h to peak at 7 h. Levels declined thereafter, but a series of fluctuations was observed until the end of the sampling period.

Profiles of ^{67}Cu and ^{99}Mo and excretion of Cu and Mo in bile

Intravenous administration of ^{67}Cu immediately increased ^{67}Cu levels in bile (Figs. 4 and 5). Peaks of ^{67}Cu excretion of increasing amplitude were observed at 30 min–1 h, 2–3 h and at 12–13 h before declining to basal levels at 18–24 h, and remained stable (Fig. 4) or

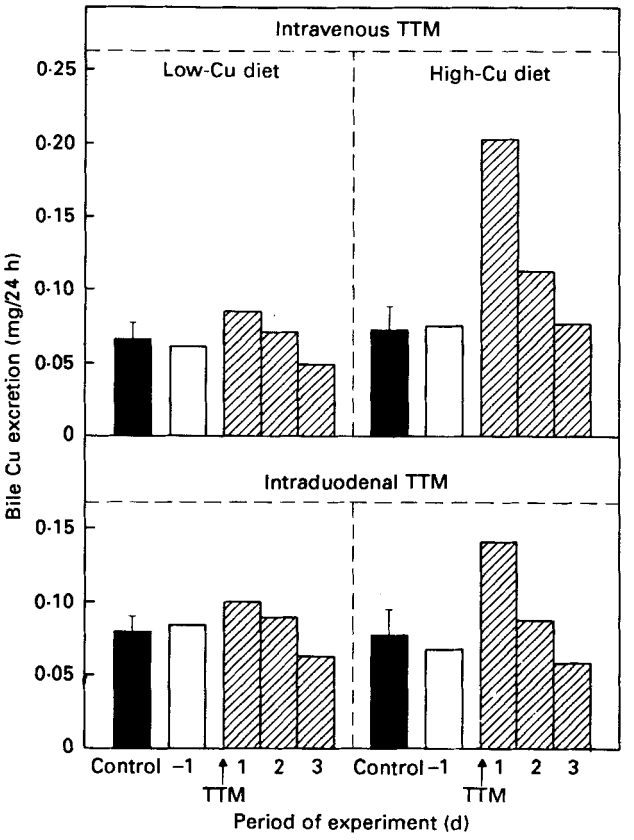

Fig. 6. Daily excretion of copper in bile before the start of the radiotracer administration (control: mean (SD represented by vertical bars) of two consecutive days sampling (■)), 24 h before tetrathiomolybdate (TTM) administration (−1) (□), and during the 3 d following either intravenous or intraduodenal administration of TTM (▨) in lambs fed on either a low-Cu (5·1 mg/kg dry matter (DM)) or a high-Cu (35 mg/kg DM) diet.

decreased only slightly (Fig. 5) during the following 24 h. For descriptive purposes these peaks were termed A, B and C. In lamb no. 3 an additional elevation of ^{67}Cu was observed at 4·5 h. Administration of ^{67}Cu also increased stable Cu concentration in bile in all lambs. Elevations of stable Cu coincided with ^{67}Cu peaks in most instances in all but one lamb (no. 7) fed on the high-Cu diet. In this animal a slight increase in stable Cu concentration was observed between peaks A and B and a marked increase was observed under peak C. There were only minor fluctuations of ^{67}Cu and stable Cu concentrations during the 24 h before TTM administration.

Challenge with ^{99}Mo-labelled TTM iv produced an immediate increase in ^{67}Cu, ^{99}Mo and stable Cu concentration in bile (Fig. 4). The profiles of these variables along with Mo concentration in bile were similar in pattern and frequency to those observed during the initial 24 h following ^{67}Cu administration and have been termed A^1, B^1 and C^1. But an important finding in this experiment was the absence of the B^1 peak of ^{67}Cu in bile from three lambs. In lamb no. 8 given TTM id, two B^1 peaks (B^1_1 and B^1_2) for ^{99}Mo and stable Cu concentration were observed (Fig. 5). However, only a slight elevation of ^{67}Cu occurred at approximately 2 h and this did not coincide with either of the B^1_1 or the B^1_2 peaks. In all lambs the levels of ^{67}Cu, ^{99}Mo, Cu and Mo were most marked in peak C^1. In lamb no. 8,

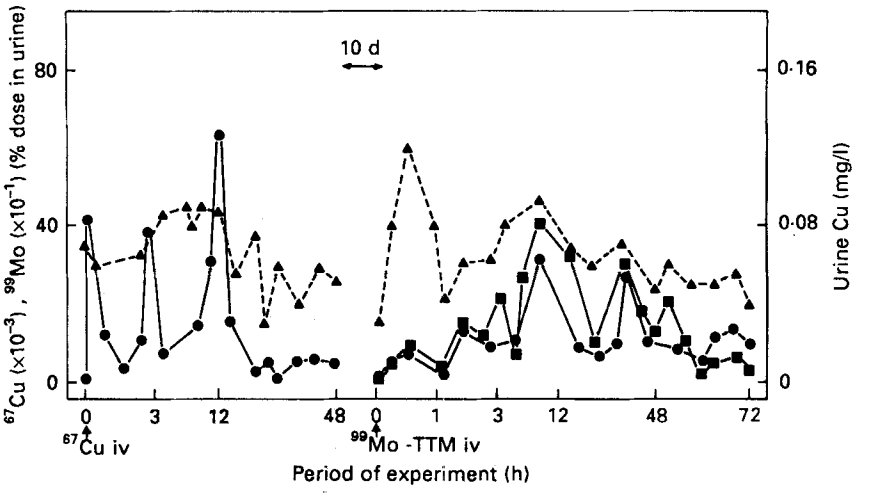

Fig. 7. Changes in ^{67}Cu (●——●), ^{99}Mo (■——■) and Cu (▲---▲) concentrations in urine of lamb no. 7 infused intravenously (iv) with ^{67}Cu (1·5 mCi; 0·6 mg Cu) and challenged 10 d later with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·2 mCi, 5·9 mg Mo) infused iv.

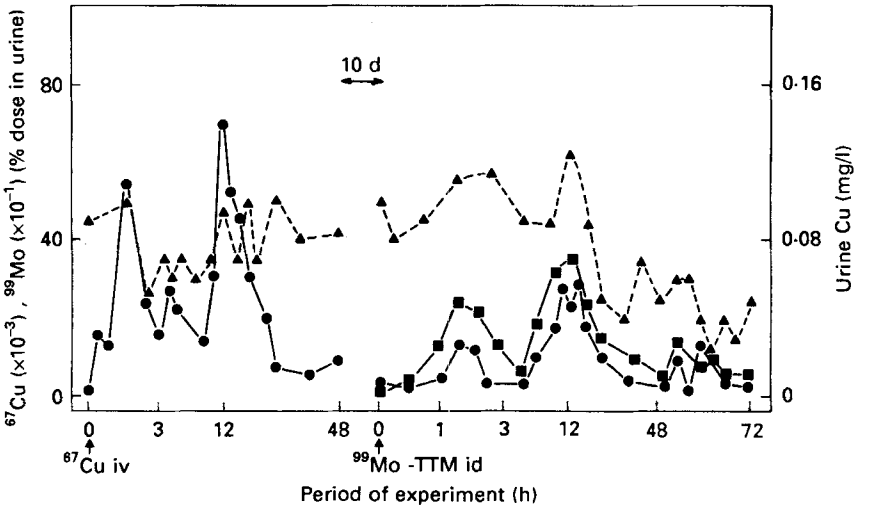

Fig. 8. Changes in ^{67}Cu (●——●), ^{99}Mo (■——■) and Cu (▲---▲) concentrations in urine of lamb no. 8 infused intravenously (iv) with ^{67}Cu (1·5 mCi; 0·6 mg Cu) and challenged 10 d later with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·3 mCi, 8·85 mg Mo) infused intraduodenally (id).

two additional peaks of ^{67}Cu not accompanied by either stable Cu or ^{99}Mo were observed.

Administration of TTM was effective in increasing the bile Cu concentration and this was most marked in lambs fed on the high-Cu diet (Fig. 6). Bile Cu excretion expressed as the percentage increase over the 24 h period before TTM administration was most marked in lambs given TTM iv. In all lambs the greatest increase in bile Cu was observed during the initial 24 h following TTM administration. Thereafter the Cu concentration declined to pre-TTM levels or lower by day 3.

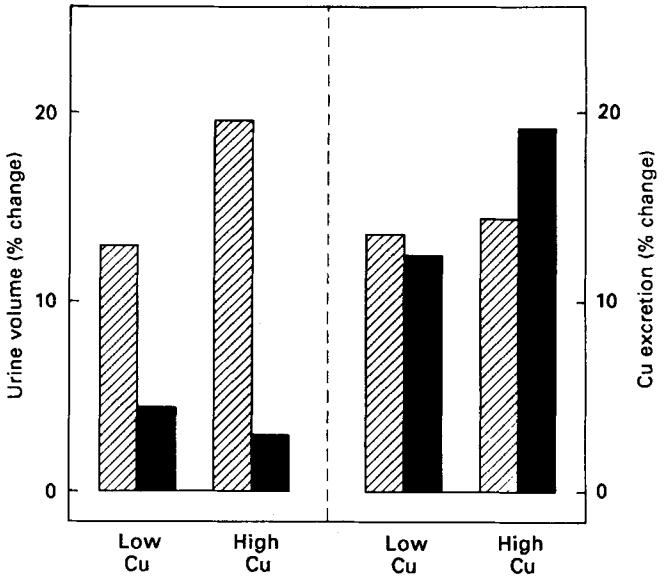

Fig. 9. The effect of administration of tetrathiomolybdate (TTM) either intravenously (iv) (▨) or intraduodenally (id) (■) on changes in the volume of urine and excretion of copper in urine within a 24 h period for lambs given low-Cu (5·1 mg/kg dry matter (DM)) or high-Cu (35 mg/kg DM) diets. The values are expressed as the percentage change from values for the 24 h period before TTM administration.

Excretion of ^{67}Cu, ^{99}Mo and stable Cu in urine

Radioactivity appeared quickly in urine after ^{67}Cu administration (Figs. 7 and 8). Levels of ^{67}Cu fluctuated during the 48 h period following ^{67}Cu administration. These fluctuations were most marked during the initial 12 h when two to three peaks were present. In all lambs except one (no. 7), stable Cu concentration in urine increased immediately following ^{67}Cu administration. In lamb no. 7 (Fig. 7) stable Cu concentration in urine initially declined but began increasing after 30 min. In all lambs the fluctuations of stable Cu concentrations in urine coincided in most instances with changes in ^{67}Cu. Samples taken during the 24 h period before TTM administration showed only minor fluctuations of urine ^{67}Cu and stable Cu concentrations, which had stabilized to $< 0{\cdot}5 \times 10^{-3}$ % of the injected dose and $< 0{\cdot}042$ mg/l respectively. TTM administration (iv) immediately increased urine ^{67}Cu, ^{99}Mo and stable Cu excretion (Fig. 7). Injection of TTM id (lamb no. 8) resulted in a gradual increase in ^{67}Cu, ^{99}Mo and stable Cu concentration after a 10 min lag period (Fig. 8). Such a lag period was not apparent in lamb no. 4, which also received TTM id but was fed on a low-Cu diet. A series of peaks of ^{67}Cu, ^{99}Mo and stable Cu concentration in urine of different amplitudes occurred in all animals thoughout the 72 h post-TTM sampling period. In all lambs TTM administration resulted in moderate increases in urine volume and in marked increases in urine Cu excretion (Fig. 9). This was most marked in lamb no. 7 fed on the high-Cu diet and given TTM iv.

Faecal Cu excretion

Faecal Cu excretion increased in all animals after TTM. The percentage increase in average daily excretion of faecal Cu over the 72 h period after TTM administration compared with the 24 h period before TTM was 2·2, 1·0 and 7·6% for lamb nos. 3, 7 and 8 respectively. The results for lamb no. 4 are not available.

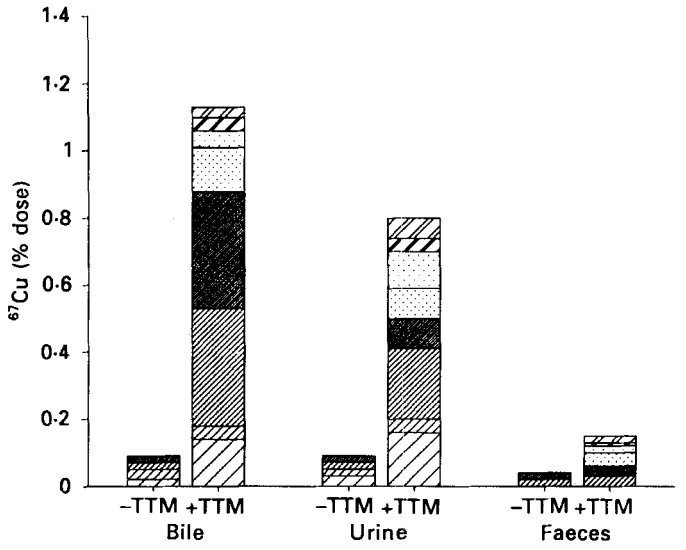

Fig. 10. Comparison of the cumulative excretion of ^{67}Cu in bile, urine and faeces from lamb no. 7, 24 h before and 72 h after challenge with intravenous ^{99}Mo-labelled tetrathiomolybdate (TTM; 0·2 mCi, 5·9 mg Mo). ( ), 3 h; ( ), 6 h; ( ), 12 h; ( ), 24 h; ( ), 36 h; ( ), 48 h; ( ), 60 h; ( ), 72 h.

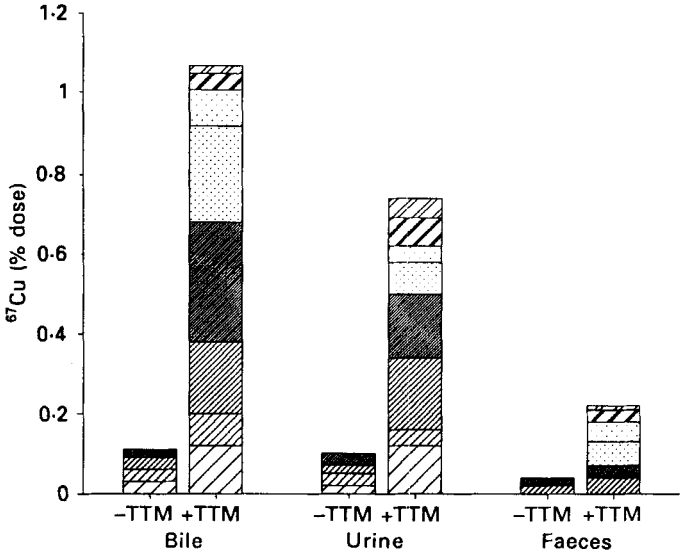

Fig. 11. Comparison of the cumulative excretion of ^{67}Cu in bile, urine and faeces from lamb no. 8, 24 h before and 72 h after challenge with intraduodenal ^{99}Mo-labelled tetrathiomolybdate (TTM; 0·3 mCi, 8·85 mg Mo). ( ), 3 h; ( ), 6 h; ( ), 12 h; ( ), 24 h; ( ), 36 h; ( ), 48 h; ( ), 60 h; ( ), 72 h.

Cumulative excretion of ^{67}Cu and ^{99}Mo in bile, urine and faeces

Administration of TTM increased ^{67}Cu excretion via all the major excretory pathways: bile, urine and faeces. Cumulative excretion of ^{67}Cu was similar in the two animals given TTM iv (Fig. 10) or id (Fig. 11). Excretion of ^{67}Cu was highest in bile > urine > faeces.

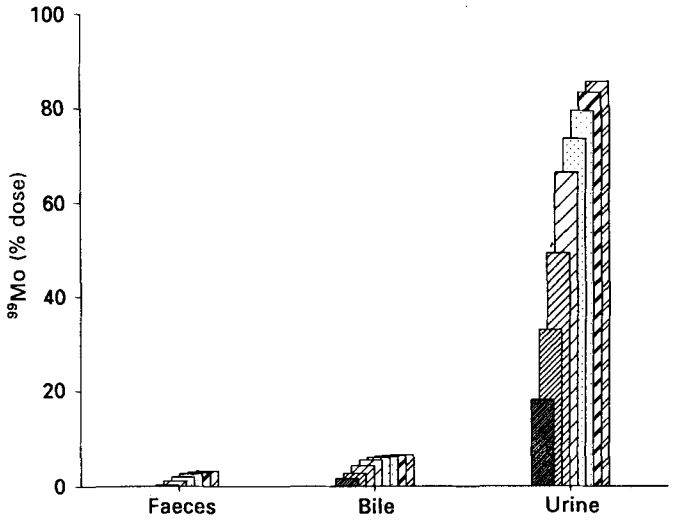

Fig. 12. Cumulative excretion of ^{99}Mo in bile, urine and faeces from lamb no. 7 primed intravenously with ^{67}Cu (1·5 mCi; 0·6 mg Cu) and challenged intravenously with ^{99}Mo-labelled tetrathiomolybdate (TTM; 0·2 mCi, 5·9 mg Mo) after 10 d. ( ), 3 h; ( ), 6 h; ( ), 12 h; ( ), 24 h; ( ), 36 h; ( ), 48 h; ( ), 60 h; ( ), 72 h.

Excretion of ^{67}Cu was higher in faeces in lambs given TTM id (Fig. 11) than in those given TTM iv.

Treatment with TTM iv resulted in the recovery of most of the ^{99}Mo in urine (Fig. 12) and less than 11% was excreted in both bile and faeces over the 72 h period. In contrast, TTM id resulted in the recovery of 34–38% of the injected dose in the faeces, 27–35% in the urine and less than 5% in bile.

DISCUSSION

The results demonstrate that TTM is not only capable of removing recently stored tissue Cu (Gooneratne *et al.* 1989), but that it also efficiently removes Cu from long-term tissue Cu storage compartments. The liver plays a central role in Cu metabolism. Therefore it is reasonable to suggest that the systemic, bile and urine Cu changes occurred through the action of TTM on liver. To accommodate the present findings we have modified and extended the three-compartment model for liver Cu metabolism by Weber *et al.* (1983; Fig. 13). The removal of Cu from liver by TTM is accomplished in two ways. First, liver Cu is returned to the blood compartment resulting in increased blood Cu. This probably accounts for the increased Cu excretion in urine and faeces and for a small percentage of the increase in bile Cu excretion. Second, the excretion of liver Cu through bile is increased.

The challenge with TTM 10 d after injection of ^{67}Cu resulted in a more pronounced increase in stable Cu than in ^{67}Cu in blood, bile and urine. In a previous study (Gooneratne *et al.* 1989) where lambs were challenged with TTM 27 h after a similar injection of ^{67}Cu, profiles of ^{67}Cu in blood, bile and urine were of higher amplitude. These findings suggest that TTM predominantly affects the short-term Cu storage compartment in the liver, although effects on long-term storage are also evident. The low level of cumulative biliary excretion of ^{67}Cu in sheep given TTM, either iv or id, emphasizes the limited effect of TTM on Cu removal from the long-term storage Cu compartment compared with its marked

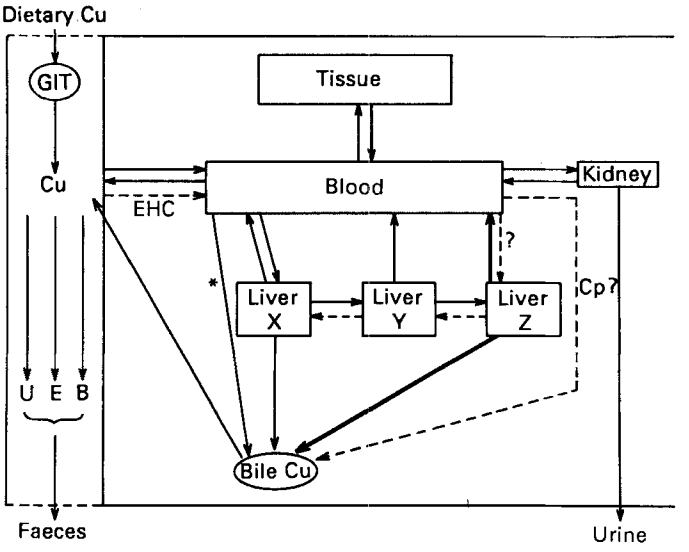

Fig. 13. Schematic diagram representing the possible movements of ingested copper in sheep given thiomolybdates (to simulate excess molybdenum and sulphur in the diet). The multifunctional role of liver in Cu metabolism is shown by: X, temporary Cu storage compartment destined for exchange with blood Cu and excretion into bile; Y, temporary storage Cu compartment for incorporation into caeruloplasmin (Cp); Z, long-term Cu storage compartment which is also capable of secretion of Cu into blood and excretion of Cu into bile. U, E and B are unabsorbed Cu, endogenous Cu excretion and unabsorbed bile Cu respectively. * Transbiliary route of Cu excretion; EHC, enterohepatic circulation; GIT, gastrointestinal tract. ➡, Removal of Cu from long-term storage Cu compartment to bile and blood (unknown pool 0 as referred to by Weber *et al.* 1983), which become active exclusively following TTM administration.

effects on stable Cu excretion in bile. Faecal ^{67}Cu excretion was most marked in lambs given TTM id (Fig. 11).

The short- and long-term liver Cu storage compartments are poorly defined. Subcellular Cu distribution (Gooneratne *et al.* 1979), electron microscopy (Gooneratne *et al.* 1980) and X-ray microanalysis (Jones *et al.* 1984) studies in normal and Cu-loaded sheep suggest that primary lysosomes capture recently absorbed Cu, and then later become secondary (dense bodies) and tertiary lysosomes (phagolysosomes) during Cu storage (Gooneratne *et al.* 1980). The latter are capable of retaining Cu for long periods of time (Barka *et al.* 1964; Goldfisher & Sternlieb, 1968; Lindquist, 1968; Sternlieb, 1972, 1980). Hence the hepatocyte secondary and tertiary lysosomes probably constitute a majority of the liver's short-term and long-term Cu storage compartments respectively.

The increases in stable Cu concentration in plasma, bile and urine due to TTM presented here are lower than those reported when TTM challenge was performed 27 h after ^{67}Cu injection (Gooneratne *et al.* 1989). Although the lambs in the present study received the same dosage of stable Cu (0·6 mg) at the time of ^{67}Cu injection, the effects of challenge with TTM 10 d later on stable Cu excretion may have been minimal since a high percentage of this Cu would have been either utilized or excreted by this time. TTM was effective in removing ^{67}Cu from the long-term storage compartment in liver. However, this effect was much less pronounced (approximately 1·71–2·18 % of ^{67}Cu injected was removed in 72 h from long-term Cu stores) compared with the effect of TTM on recently stored ^{67}Cu (approximately 7·93–9·04 % of ^{67}Cu injected was removed in 72 h) (Gooneratne *et al.* 1989). Thus TTM appears to be 4–5 times more effective in removing Cu from the short-term

storage compartment(s) than from long-term storage compartment(s). The lower effectiveness of TTM treatment after repeated injections (Gooneratne *et al.* 1981*b*) therefore, is most likely due to a progressive depletion of the short-term Cu storage compartment. But the effect of TTM on excretion of Cu from the long-term storage compartment is still substantial, and hence may still be of clinical importance in the long-term therapy of patients suffering from Wilson's disease (Walshe, 1984) and in treatment of Cu toxicosis in sheep (Gooneratne *et al.* 1981*b*). However, in view of the known toxic effect of TTM on the bone marrow (Walshe, 1986), the skeleton (Read *et al.* 1986) and the immature gut epithelium (Fell *et al.* 1979), it is not recommended for use in the young.

The possible mechanisms of excretion of recently stored liver Cu through bile were discussed previously (Gooneratne *et al.* 1989). We suggested that at least three major pathways may be involved in the transfer of Cu from liver to bile: (1) transbiliary, (2) transhepatocellular, and (3) hepatolysosomal. Furthermore, similar pathways were engaged in the removal of ^{67}Cu from the short-term storage compartment after administration of either ^{67}Cu or TTM. A similar profile of excretion of stable Cu into bile was observed in the present study which confirms the previously described observations. However, the profile of ^{67}Cu excretion in bile after TTM administration was different which suggests that a change in the pathways had occurred. In three of four animals, ^{67}Cu in peak B^1 was absent. In the other lamb (no. 8) only a slight elevation was evident which was out of sequence with the ^{99}Mo and stable Cu concentration profiles. This suggests that only the transbiliary and hepatolysosomal pathways were operative. Thus peak B^1, the trans-hepatocellular pathway, which we hypothesized to constitute Cu bound to albumin, appeared inoperative during removal of Cu from the long-term liver Cu storage compartment. The reason for this is not clear. It is not known in what form Cu is released from the long-term Cu storage compartment. This Cu may have a low affinity for albumin, or may bind to TTM readily in preference to albumin. The ^{67}Cu–TTM would then be cleared rapidly from the bloodstream via the urine and via the transbiliary (A^1) route of bile Cu excretion, or it could re-enter the liver. The large pool of serum albumin potentially acts as a buffer to increased metal levels (Woods & Mason, 1987) and also protects TM compounds against hydrolysis (Hynes *et al.* 1984). In addition bovine serum albumin contains a distinct single binding site for TM of which the affinity is increased by the presence of Cu. In the present study ^{99}Mo-labelled TTM and stable Cu were probably bound to albumin, as evidenced by the increase of both in peak B^1, while ^{67}Cu was absent. It is not known whether the forms of Cu which exist in the short- and long-term storage compartments differ. However, the relative abundance in plasma or a potentially greater affinity of stable Cu released from short-term storage may have precluded binding of ^{67}Cu from long-term storage to albumin. As reported previously (Gooneratne *et al.* 1989), the timing of the release of ^{67}Cu in peaks C and C^1 indicates that it is derived from lysosomes. This may be either from secondary lysosomes (dense bodies formed after incorporation of recently stored liver Cu) or tertiary lysosomes (phagolysosomes) which appear to be actively involved in long-term accumulation of Cu (Barka *et al.* 1964; Goldfischer & Sternlieb, 1968; Lindquist, 1968; Sternlieb, 1972, 1980). The large amount of Cu excreted via peak C^1 is in agreement with previous findings on massive liver lysosomal Cu storage in Wilson's disease, canine Cu toxicosis (Sternlieb, 1980) and in chronic Cu poisoning in sheep (Gooneratne *et al.* 1980). Collectively, these results demonstrate that in treatment and management of Cu toxicity in sheep, although iv TTM administration is of major significance in chelation of elevated blood Cu (Gooneratne *et al.* 1981*b*) and removal of recently stored Cu (Gooneratne *et al.* 1989), increases in dietary Mo and S levels may also be useful for enhancing endogenous Cu excretion.

The authors thank Mr T. Berryere for care of the animals and for technical assistance. This work was supported by the Saskatchewan Agriculture Development Fund and by the Burford Hooke Hantelman Trust Fund.

REFERENCES

Barka, T., Scheuer, P. J., Schaffner, F. & Popper, H. (1964). Structural changes of liver cells in copper intoxication. *Archives of Pathology* **78**, 331–349.

Dick, A. T., Dewey, D. W. & Gawthorne, J. M. (1975). Thiomolybdates and the copper–molybdenum–sulphur interaction in ruminant nutrition. *Journal of Agricultural Science, Cambridge* **85**, 567–580.

Fell, B. F., Dinsdale, D. & El-Gallad, T. T. (1979). Gut pathology of rats dosed with tetrathiomolybdate. *Journal of Comparative Pathology* **89**, 495–514.

Goldfischer, S. & Sternlieb, I. (1968). Changes in the distribution of hepatic copper in relation to the progression of Wilson's disease (hepatolenticular degeneration). *American Journal of Pathology* **53**, 883–901.

Gooneratne, S. R. (1986). Potential use of tetrathiomolybdate in copper storage diseases. *Acta Pharmacologica et Toxicologica* **59**, Suppl. VII, 518–523.

Gooneratne, S. R., Chaplin, R. K., Trent, A. M., & Christensen, D. A. (1988). Effect of tetrathiomolybdate administration on the excretion of copper, zinc, iron and molybdenum in sheep bile. *British Veterinary Journal* (In the Press.)

Gooneratne, S. R., Howell, J. McC. & Cook, R. D. (1980). An ultrastructural and morphometric study of the liver of normal and copper-poisoned sheep. *American Journal of Pathology* **99**, 429–450.

Gooneratne, S. R., Howell, J. McC. & Gawthorne, J. M. (1979). Intracellular distribution of copper in the liver of normal and copper loaded sheep. *Research in Veterinary Science* **16**, 57–64.

Gooneratne, S. R., Howell, J. McC. & Gawthorne, J. M. (1981*a*). An investigation of the effects of thiomolybdate on copper metabolism in chronic Cu-poisoned sheep. *British Journal of Nutrition* **46**, 469–480.

Gooneratne, S. R., Howell, J. McC. & Gawthorne, J. M. (1981*b*). Intravenous administration of thiomolybdate for the treatment and prevention of chronic copper poisoning in sheep. *British Journal of Nutrition* **46**, 457–468.

Gooneratne, S. R., Laarveld, B., Chaplin, R. K. & Christensen, D. A. (1989). Profiles of ^{67}Cu in blood, bile, urine and faeces from ^{67}Cu-primed lambs: effect of ^{99}Mo-labelled tetrathiomolybdate on the metabolism of recently stored tissue ^{67}Cu. *British Journal of Nutrition* **61**, 355–371.

Hynes, M., Lamand, M., Montel, G. & Mason, J. (1984). Some studies on the metabolism and the effects of ^{99}Mo- and ^{35}S-labelled thiomolybdates after intravenous infusion in sheep. *Journal of Inorganic Biochemistry* **24**, 279–288.

Jones, H. B., Gooneratne, S. R. & Howell, J. McC. (1984). X-ray microanalysis of liver and kidney in copper loaded sheep with and without thiomolybdate administration. *Research in Veterinary Science* **37**, 273–279.

Lindquist, R. R. (1968). Studies on the pathogenesis of hepatolenticular degeneration. III. The effect of copper on rat liver lysosomes. *American Journal of Pathology* **53**, 903–927.

Mason, J. (1986). Thiomolybdates: mediators of molybdenum toxicity and enzyme inhibitors. *Toxicology* **42**, 99–109.

Mills, C. F., Bremner, I., El-Gallad, T. T., Dalgarno, A. C. & Young, B. W. (1978). Mechanisms of molybdenum/sulphur antagonism of copper utilization by ruminants. In *Trace Element Metabolism in Man and Animals (TEMA-3)*, pp. 150–158 [M. Kirchgessner, editor]. Weihenstephan: Arbeitskreis für Tierernährungsforschung.

Read, R., Sutherland, J. & Ghosh, P. (1986). The matrix components of the epiphyseal growth plate and articular cartilage from dogs treated with ammonium tetrathiomolybdate, a copper antagonist. *Australian Journal of Experimental Biology and Medical Science* **64**, 545–562.

Sternlieb, I. (1972). Evolution of the hepatic lesion in Wilson's disease (hepatolenticular degeneration). In *Progress in Liver Diseases*, vol IV, pp. 511–525. [H. Popper and F. Schaffner, editors]. New York, London: Grune and Stratton.

Sternlieb, I. (1980). Copper and the liver. *Gastroenterology* **78**, 1615–1628.

Suttle, N. F. (1980). The role of thiomolybdate in the nutritional interactions of copper, molybdenum and sulfur: fact or fantasy? *Annals of the New York Academy of Science* **355**, 195–204.

Underwood, E. J. (1977). *Trace Elements in Human and Animal Nutrition*, 4th ed. New York and London: Academic Press.

Walshe, J. M. (1984). Copper: its role in the pathogenesis of liver disease. *Seminars in Liver Disease* **4**, 252–263.

Walshe, J. M. (1986). Tetrathiomolybdate (MoS_4) as an 'anticopper' agent in man. In *Orphan Diseases/Orphan Drugs*, pp. 76–85 [I. H. Scheinberg and J. M. Walshe, editors]. Manchester: Manchester University Press.

Weber, K. M., Boston, R. C. & Leaver, D. D. (1980). A kinetic model for copper metabolism in sheep. *Australian Journal of Agricultural Research* **31**, 773–790.

Weber, K. M., Boston, R. C. & Leaver, D. D. (1983). The effect of molybdenum and sulphur on the kinetics of copper metabolism in sheep. *Australian Journal of Agricultural Research* **34**, 295–306.

Woods, M. & Mason, J. (1987). Spectral and kinetic studies on the binding of trithiomolybdate to bovine and canine serum albumin in vitro: the interaction with copper. *Journal of Inorganic Biochemistry* **30**, 261–273.