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

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1. The relative importance of excretory routes in the removal of recently stored ^{67}Cu following tetrathiomolybdate (TTM) administration was studied. Lambs fed on either 5 mg Cu/kg dry matter (DM) or 35 mg Cu/kg DM, were primed intravenously (iv) with ^{67}Cu and challenged 27 h 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 in blood, bile, urine and faeces were measured.

2. Level of dietary Cu and route of administration of ^{99}Mo-TTM affected the amplitude of blood, bile and urine profiles of ^{67}Cu and stable Cu, but not the pattern of the responses observed.

3. The present study describes for the first time increased excretion of endogenous ^{67}Cu through gastrointestinal secretions other than bile due to TTM administration.

4. Administration of TTM resulted in the immediate release of ^{67}Cu from storage compartments in the body into the blood circulation. Changes in stable Cu levels in blood, bile, urine and faeces, and gut and systemic effects were evident. Biliary and urinary Cu excretion due to TTM was rapid and maximal within 24 h of injection.

5. Administration of ^{67}Cu iv resulted in the immediate excretion of ^{67}Cu in bile in a pulsatile, constant pattern. A similar pattern of ^{67}Cu excretion into bile in synchrony with that of ^{99}Mo was observed after ^{99}Mo-labelled TTM administration.

6. The similar pattern of biliary ^{67}Cu excretion observed after injection of ^{67}Cu and after injection of ^{99}Mo-labelled TTM 27 h later is discussed in relation to the times required to process the Cu through different hepatic pathways for excretion in bile.

Molybdenum and sulphur-induced copper deficiency in ruminants (Mills, 1985) is probably mediated through the thiomolybdates (TM) (MoO_nS_{4-n} where n is 0–3). Di- and trithiomolybdates predominate in plasma of sheep given either normal or slightly elevated levels of Mo and S in the diet (Price *et al.* 1987) or molybdate as a single dose into the rumen (Mason *et al.* 1982*a*). However, tetrathiomolybdate (TTM) appears to predominate in rumen digesta at moderate to high Mo and S intakes (Dick *et al.* 1975). At high rumen TTM concentrations the capacity of rumen solids to bind TTM becomes saturated resulting in appearance of TTM in the rumen liquid phase (Price *et al.* 1987). Recent studies (N. H. Osman, A. S. Familton and A. R. Sykes, personal communication) suggest that TTM is absorbed into the bloodstream although Price *et al.* (1987) were able to detect only traces of it in the blood.

Thiomolybdates reduce the absorption of dietary Cu in sheep (Suttle & Field, 1983). They also affect systemic Cu metabolism (Gooneratne *et al.* 1981*a*; Mason *et al.* 1982*a*) by changing markedly the distribution of Cu in plasma and by reducing the availability of Cu to metabolic sites within the body. Intravenous (iv) administration of TTM results in elevated whole blood Cu and plasma Cu levels and direct reacting Cu (copper bound to amino acids and peptides) in plasma (Gooneratne *et al.* 1981*a*). Most of the increased Cu in plasma is present in an unidentified trichloroacetic acid (TCA)-insoluble form, the source

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of which is unknown. Intravenous TTM decreases liver Cu levels and this has been used successfully in sheep to treat and prevent Cu toxicity (Gooneratne *et al.* 1981*b*; Humphries *et al.* 1986). TTM has a high affinity for Cu-thioneins (Clarke *et al.* 1987) and alters the distribution of Cu in the liver (Kelleher & Ivan, 1985; Wang *et al.* 1987; S. R. Gooneratne, B. Laarveld and D. A. Christensen, unpublished observation). Administration of TTM (Gooneratne *et al.* 1981*a*) and trithiomolybdates (Mason *et al.* 1988) to sheep also increases Cu excretion in urine and faeces respectively. The origin of faecal Cu and the mechanisms of liver Cu excretion are not clear, but biliary secretion is a possible route of Cu removal (Gooneratne & Christensen, 1984).

In the present study we evaluated TTM in the removal of recently stored liver Cu in lambs. Lambs were primed with ^{67}Cu iv and challenged 27 h later with ^{99}Mo-labelled TTM given either iv or intraduodenally (id). Oral administration of TTM was not used because of modifications that may take place in the rumen. We also examined the systemic effects of TTM and the relative importance of the excretory routes for administered ^{67}Cu following TTM administration with special emphasis on mechanisms of biliary Cu excretion. Preliminary communications of some of these results have been reported elsewhere (Gooneratne, 1986; Gooneratne *et al.* 1987). Evaluation of TTM in the removal of liver Cu after long-term storage is reported elsewhere (Gooneratne *et al.* 1989*a*).

MATERIALS AND METHODS

Experimental animals

Experiments were carried out using ^{67}Cu and ^{99}Mo to evaluate Cu excretion patterns after TTM administration. Four lambs used in the present experiment were drawn from a group of eight 10-week-old female lambs (Suffolk × Finn × Rambouillet cross-breds) weighing 17–22 kg. They were housed in individual metabolism cages with free access to water. The basic ration was (g/kg) a barley (600)–lucerne meal (320)–soya-bean meal (64) mixture containing (mg/kg dry matter (DM)) 42 zinc, 63 iron, 0·12 Mo and 2·13 g S. Lamb nos. 1–4 received this ration which contained 5·1 mg Cu/kg diet DM. Lamb nos. 5–8 received the same ration but containing 35 mg Cu/kg diet DM. The lambs were given 1 kg/d as a single meal in the morning. After 4 weeks all lambs were fitted with permanent rumen fistulas (for use in subsequent experiments). After 2 weeks bile duct and duodenal cannulation was carried out as described by Caple & Heath (1972). Bile flow was allowed to stabilize for 10 d before data collection. The flow and Cu concentrations of bile and urine were measured over 2 d to obtain baseline values. After this, three 3 h bile collections (9 h/d) continued for another week. Only half the bile collected was returned to the duodenum. The remainder was stored frozen to be used to replace the bile collected during radiotracer experiments. Animals were allowed to recover for 5 d for bile flow to return to baseline levels.

Preparation of ^{99}Mo-labelled TTM

The ^{99}Mo was obtained from ^{99m}Tc generators (Health Sciences Centre, Winnipeg, Manitoba). ^{99}Mo-labelled TTM was prepared by passing hydrogen sulphide gas through a solution of sodium molybdate (75 mg Mo) containing 3 mCi ^{99}Mo in 3 ml 0·2 M-sodium phosphate buffer, pH 6, for 45 min. After removing excess H_2S, labelled TTM was purified by immediate passage through a Sephadex G25 column (25 × 150 mm) using Tris-hydrochloride buffer (10 mM, pH 7·6) as eluent. Six 5 ml fractions of TTM of characteristic dark reddish brown colour were pooled. The purity and recovery of ^{99}Mo as TTM was verified spectrophotometrically (Clarke & Laurie, 1979). The amounts administered were calculated from the ^{99}Mo-TTM pooled solution.

Radiotracer studies

Each lamb (nos. 1, 2, 5 and 6) was fitted with a Foley catheter and harness for the collection of urine and faeces respectively, before receiving the isotope. Lambs were injected iv with 1·5 mCi ^{67}Cu (0·6 mg Cu as acetate; Oakridge National Laboratory, Oakridge, TN) in 5 ml physiological saline (9 g sodium chloride/l). One animal from each diet group was then challenged 27 h later with a 5 ml iv dose of ^{99}Mo (0·2 mCi in 6 mg Mo; lamb nos. 1 and 5) or with a 7·5 ml id dose of ^{99}Mo (0·3 mCi in 9 mg Mo; lamb nos. 2 and 6)-labelled TTM. Duodenal administration of TTM was performed via the duodenal re-entrant cannula as described by Mason *et al.* (1980). Analysis of the radioactivity of blood, bile, urine and faeces was carried out using a LKB 1282 Compugamma counter. Sealed reference samples of ^{67}Cu and ^{99}Mo-labelled TTM from each batch of the isotope were used to calculate the activity of the dose and the rate of decay.

Collection of blood, bile, urine and faeces

Jugular blood samples for plasma Cu analysis were collected fortnightly into heparinized tubes before the radiotracer experiments. A sample was also obtained just before each radiotracer injection. After each injection of ^{67}Cu or ^{99}Mo-labelled TTM, blood samples were taken at intervals of 5 min for the first 30 min, then at intervals of 10 min for the next 2·5 h, every 0·5 h for the next 3 h, and then at 3 h intervals to a maximum of 5 d. Each sample of blood collected was replaced with an equal volume of saline. The total radioactivity in blood was estimated assuming a blood volume of 7% of body-weight.

Bile and urine samples were collected and volumes measured at every second sampling of blood during the first 3 h, and then at the same frequency as for blood sampling. The bile removed was replaced with non-radioactive bile collected before radiotracer studies. Radioactive bile collected from this experiment was saved for use during subsequent experiments.

Faeces were collected at intervals of 12 h for a maximum of 5 d. Faeces were weighed, homogenized and sampled for ^{67}Cu and ^{99}Mo measurements and freeze-dried for Cu analysis.

Cu and Mo analysis

Cu concentration in plasma, bile and urine was determined directly by flame atomic absorption spectrometry (AAS) (Model no. 5000; Perkin Elmer) after appropriate dilution. Plasma was diluted with 4 vol. deionized water (plasma (1:4)). Cu in the TCA-soluble fraction of plasma was determined by the method of Smith & Wright (1975). Plasma and bile Mo were determined by graphite furnace AAS (model no. 4000 with HGA 500 programmer; Perkin Elmer) after appropriate dilution. Recoveries of Mo after serial additions varied from 82 to 105% for plasma and from 85 to 107% for bile. Feed and faecal samples were dried and digested in a nitric–perchloric (3·5:1, by vol.) acid mixture before Cu analysis by flame AAS.

RESULTS

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

The dietary Cu content did not appear to affect the profile of ^{67}Cu excretion in blood, bile, urine or faeces before TTM administration. But after TTM the peak heights of ^{67}Cu and ^{99}Mo and areas under those peaks were most marked in the lambs fed on the high-Cu diet (lamb nos. 5 and 6).

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

In all lambs ^{67}Cu was cleared rapidly from blood and only about 6–8% of the injected dose remained in the blood after 1 h (Figs 1 and 2). ^{67}Cu in blood continued to decrease slowly, and by 24 h the level had stabilized to approximately 3–5% of the injected dose.

Administration of TTM (Figs 1 and 2) immediately increased ^{67}Cu in blood, reaching a peak 6–8% of dose by 45 min. Then ^{67}Cu subsided to approximately 5% of the injected dose at 1·5 h, but an elevation was observed between 6 and 12 h. In lamb no. 5 another slight elevation was observed at approximately 54 h after TTM injection. The levels then declined to about 4% of the injected dose. In lambs given TTM iv three phases of ^{99}Mo clearance could be observed: an initial rapid disappearance of ^{99}Mo reaching a plateau in approximately 1 h; then a gradual decline over the next 6 h, followed by a rapid decline to a basal equilibrium at about 6–7% of injected dose in 48 h. In those given TTM id (Fig. 2), ^{99}Mo absorption was rapid with a maximum peak ^{99}Mo in blood of 14–15% of the injected dose at 40 min, followed by a plateau for 6 h. The levels then declined and the pattern of ^{99}Mo appeared similar to that observed in lambs given TTM iv.

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

Basal plasma Cu concentration of lamb no. 6 (Fig. 2) was 0·6 mg/l higher than that of lamb no. 5 (Fig. 1). A slight increase in plasma Cu and plasma TCA-soluble Cu concentrations occurred in all lambs following ^{67}Cu administration. Both iv and id TTM administration immediately increased plasma Cu concentration. After 12–24 h Cu concentration gradually declined to basal levels at approximately 48–72 h. In contrast the plasma TCA-soluble Cu level declined immediately after injection of TTM. At 1 h about 30% of the plasma Cu in lamb no. 5 was TCA-insoluble whereas in lamb no. 6 only about 7% was TCA-insoluble. In both animals TCA-soluble Cu increased from then onwards and by 48–72 h the level was comparable to the plasma Cu concentration.

Mo could not be detected in plasma before TTM administration. TTM (iv) resulted in extremely high concentrations of plasma Mo, with levels in excess of 2 mg/l in the first sample collected after 5 min (Fig. 1). The levels declined rapidly to approximately 0·6 mg/l at 2 h and remained steady for the following 2 h before declining slowly to 0·1 mg/l at 36 h. In lamb no. 6, which received TTM id, the plasma Mo level increased rapidly following administration (Fig. 2), reaching a maximum of 0·5 mg/l at 30 min. From then onwards the profile was similar to that of lamb no. 5.

Profiles of ^{67}Cu and ^{99}Mo, and stable Cu and Mo concentration in bile

^{67}Cu (iv) resulted in an immediate increase in ^{67}Cu in bile (Figs 3 and 4). Peaks of increasing amplitude were observed at 45 min–1 h, at 2–4·5 h and at 11·5–13 h before declining to baseline levels at 20–24 h. These peaks were named A, B and C for descriptive purposes. When more than one peak was observed between peaks A and C, they were termed B_1 and B_2. Intravenous TTM (Fig. 3) resulted in a rapid increase in both bile ^{67}Cu and ^{99}Mo with appearance of peaks similar in pattern and frequency to those observed following ^{67}Cu injection, but of a larger magnitude; these peaks were A^1, B^1 and C^1. Two additional peaks were observed at 54 and 60 h after TTM. Intraduodenal TTM (Fig. 4) resulted in a similar profile of ^{67}Cu and ^{99}Mo in bile.

Changes in stable Cu and Mo concentration in bile following iv or id TTM were similar to the respective bile ^{67}Cu and ^{99}Mo profiles (Figs 3 and 4) in most instances, except for the following. The maximum peak concentrations of Cu and Mo were lower in lambs given TTM id. The stable Cu profile in bile from lamb no. 6 following TTM id consisted of only

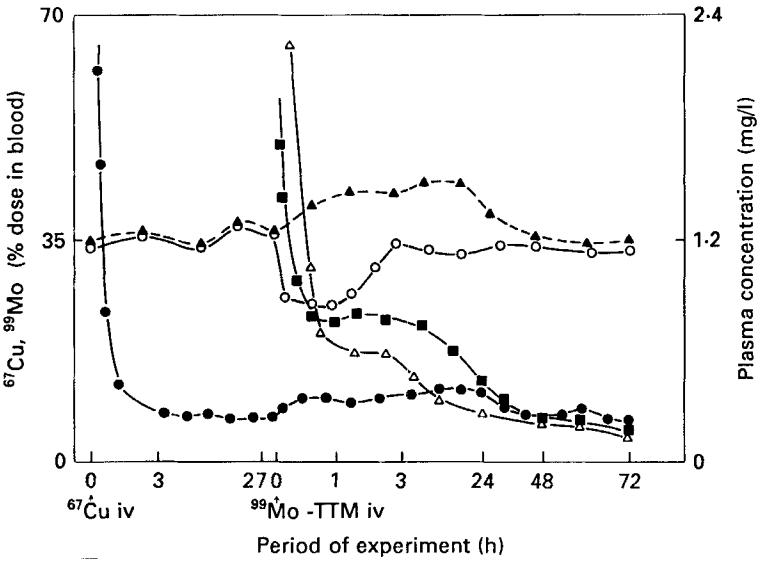

Fig. 1. Changes in ^{67}Cu (●——●) and ^{99}Mo (■——■) in blood, and Cu (▲---▲), TCA-soluble Cu (○——○), and Mo (△——△) in plasma of lamb no. 5 infused intravenously (iv) with ^{67}Cu (1·5 mCi, 0·6 mg Cu) and challenged after 27 h with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·2 mCi, 6 mg Mo) infused iv.

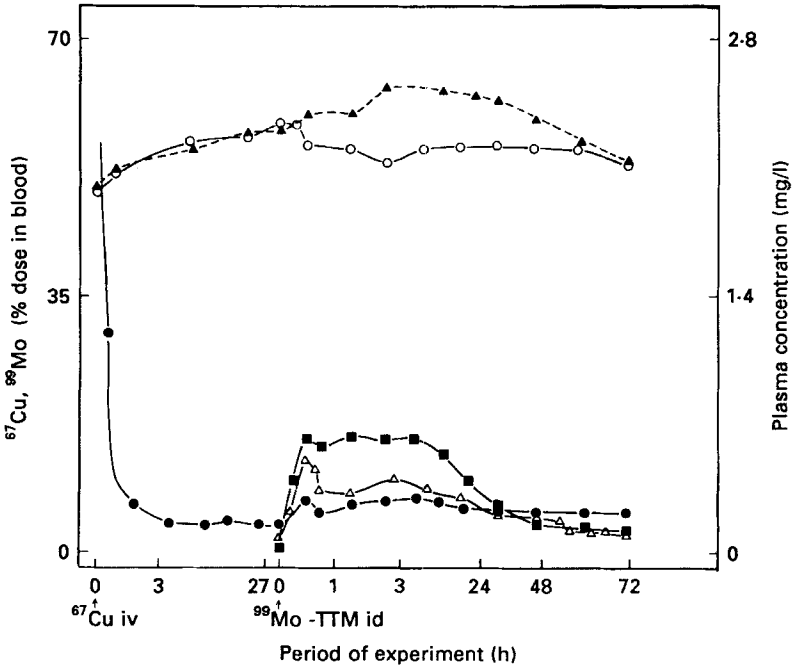

Fig. 2. Changes in ^{67}Cu (●——●) and ^{99}Mo (■——■) in blood, and Cu (▲---▲), TCA-soluble Cu (○——○), and Mo (△——△) in plasma of lamb no. 6 infused intravenously (iv) with ^{67}Cu (1·5 mCi, 0·6 mg Cu) and challenged after 27 h with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·3 mCi, 9 mg Mo) infused intraduodenally (id).

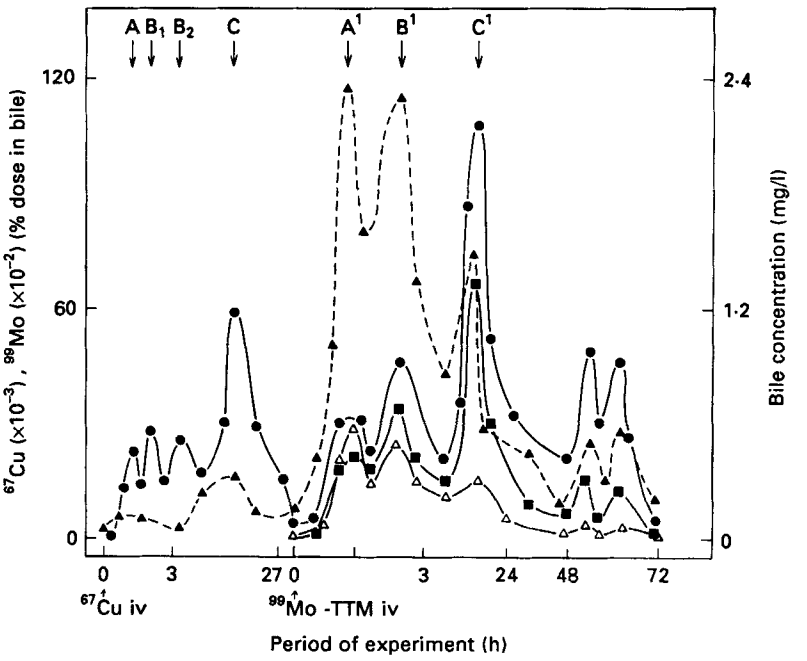

Fig. 3. Biliary changes in ^{67}Cu (●——●), ^{99}Mo (■——■), Cu (▲---▲), and Mo (△——△) in lamb no. 5 infused intravenously (iv) with ^{67}Cu (1·5 mCi, 0·6 mg Cu) and challenged after 27 h with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·2 mCi, 6 mg Mo) infused iv. Peaks of ^{67}Cu excretion observed at 1, 2, 4 and 11·5 h after ^{67}Cu administration have been termed A, B_1, B_2 and C respectively. Peaks of ^{67}Cu observed 1, 2·7 and 12·5 h after ^{99}Mo-labelled TTM administration have been termed A^1, B^1 and C^1 respectively.

three peaks, whereas four were observed during 27 h following ^{67}Cu administration (Fig. 4). Intravenous TTM was most effective in increasing bile Cu concentration with peak maxima of A^1 and B^1 as high as 2·4 μg/l (Fig. 3). The increase in bile Cu excretion in response to TTM was most marked in lambs fed on a high-Cu diet and given TTM iv (Fig. 5). Thus lamb no. 5 given TTM iv had an increase of 174% in biliary Cu excretion, whereas lamb no. 6 given TTM id showed an increase of 70%. These increases occurred notwithstanding 39 and 35% reductions in bile volumes during this period in lamb nos. 5 and 6 respectively. The promotion of biliary Cu excretion by TTM was a short-term phenomenon with maximum effects occurring within 24 h (Fig. 5).

Profiles of ^{67}Cu and ^{99}Mo and stable Cu concentration in urine

Figs 6 and 7 show the ^{67}Cu, ^{99}Mo and stable Cu concentration profiles in urine before and after TTM administration. ^{67}Cu administration resulted in fluctuations of ^{67}Cu in urine with two peaks at 1–1·5 h and at 16–20 h in three animals. In lamb no. 1, an additional ^{67}Cu peak accompanied with an increase in stable Cu was observed at 4 h post ^{67}Cu injection (not shown). Intravenous ^{67}Cu also resulted in either a gradual increase in stable Cu concentration (lamb no. 1) or in two sustained elevations (lamb no. 5) or peaks at similar times as the ^{67}Cu peaks (lamb no. 6). In lamb no. 2 fed on the low-Cu diet, ^{67}Cu administration resulted in an initial decline in stable Cu followed by a marked increase after 45 min to 1 h. The level declined slightly at approximately 2 h, but increased shortly afterwards and remained high until 13–15 h. Intravenous ^{67}Cu produced a small increase in urine Cu excretion in all lambs, whereas TTM administration resulted in a moderate to

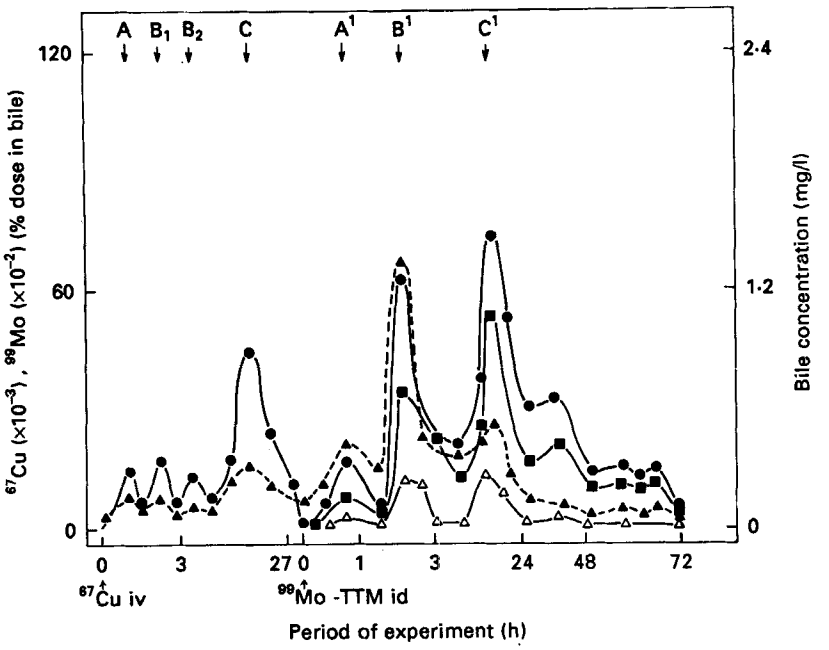

Fig. 4. Biliary changes in ^{67}Cu (●——●), ^{99}Mo (■——■), Cu (▲---▲), and Mo (△——△) in lamb no. 6 infused intravenously (iv) with ^{67}Cu (1·5 mCi, 0·6 mg Cu) and challenged after 27 h with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·3 mCi, 9 mg Mo) infused intraduodenally (id). Peaks of ^{67}Cu excretion observed at 1, 2, 4·5 and 13 h after ^{67}Cu administration have been termed A, B_1, B_2 and C respectively. Peaks of ^{67}Cu observed at 45 min, 2·2 and 12 h after ^{99}Mo-labelled TTM administration have been termed A^1, B^1 and C^1 respectively.

marked increase (Figs 6 and 7). A series of elevations of ^{67}Cu, ^{99}Mo and stable Cu concentration were observed in all animals following TTM administration with peaks occurring at 30 min, 1·5, 3, 10 and 34 h in lamb no. 5 (Fig. 6), and at 1, 3, 15, 32 and 54 h in lamb no. 6 (Fig. 7). Urine volume also increased in all lambs following TTM administration. This was partly responsible for the increase in Cu excretion which was most marked in lambs fed on the high-Cu diet (Fig. 8). In contrast, among the two lambs given TTM id, the percentage increase in Cu excretion was most marked in lamb no. 2 fed on the low-Cu diet.

Excretion of stable Cu in faeces

Faecal excretion of Cu increased in all lambs injected with TTM. The percentage increase over 72 h in daily faecal Cu excretion compared with 24 h before TTM administration was 4·9, 6·8, 3·7 and 8·3% for lamb nos. 1, 2, 5 and 6 respectively.

Cumulative excretion in bile, urine and faeces of ^{67}Cu before, and of ^{67}Cu and ^{99}Mo after ^{99}Mo-labelled TTM iv or id

The level of dietary Cu did not affect the magnitude or the profile of ^{67}Cu excretion before TTM administration. The excretion of ^{67}Cu was highest in urine, followed equally by both faeces (free of bile) and bile. Infusion of ^{99}Mo-labelled TTM iv (Fig. 9) or id (Fig. 10) increased ^{67}Cu excretion via all the major pathways, and this was most marked in lambs fed on the high-Cu diet and given TTM iv. At 24 h the increase (%) in ^{67}Cu excretion was highest in the order bile > urine > faeces. The cumulative excretory pattern of ^{99}Mo varied with the route of administration of TTM. TTM iv resulted in the recovery of most of the

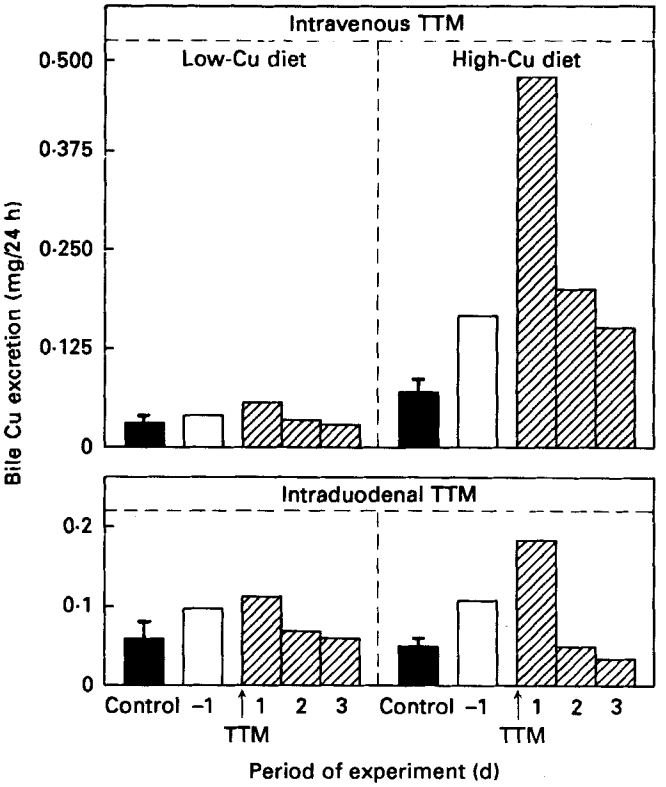

Fig. 5. Daily excretion of copper in bile before the start of radiotracer infusion (control: mean and SD of two consecutive days sampling) (■), 24 h before TTM administration (−1) (□), and during 3 d following either intravenous or intraduodenal administration of tetrathiomolybdate (TTM) (▨) in lambs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

^{99}Mo in the urine and less than 10% was in faeces or bile (Fig. 11). TTM id resulted in the recovery of 42–49% of the injected ^{99}Mo in faeces in 72 h, and 36–41% in urine. Cumulative excretion of ^{99}Mo in bile in all lambs over this period was less than 8% of the injected dose.

DISCUSSION

The present study provides the first direct evidence for the release of Cu from storage compartment(s) in the body into the blood circulation and its excretion in bile at specific times following TTM administration. TTM also induced changes in stable Cu levels in blood, bile, urine and faeces, and gut and systemic effects were evident in spite of the low dose of TTM (6 mg Mo) injected per animal. Both iv and id administration of TTM resulted in a similar sequence of events in all variables measured, but the overall effect of id administration on Cu metabolism was lower. This was probably related to the reduced absorption of TTM via the id route, since a high percentage of ^{99}Mo (42·1 and 48·7 in lamb nos. 2 and 6 respectively) appeared in the faeces.

The systemic effects due to TTM were a prolonged increase in stable blood Cu and plasma Cu and a rapid decrease in TCA-soluble Cu in plasma. Cu levels were comparable to those following TM administration reported previously (Gooneratne *et al.* 1981*a*; Mason *et al.* 1980, 1982*b*), but the magnitude of the effects reported here is comparatively

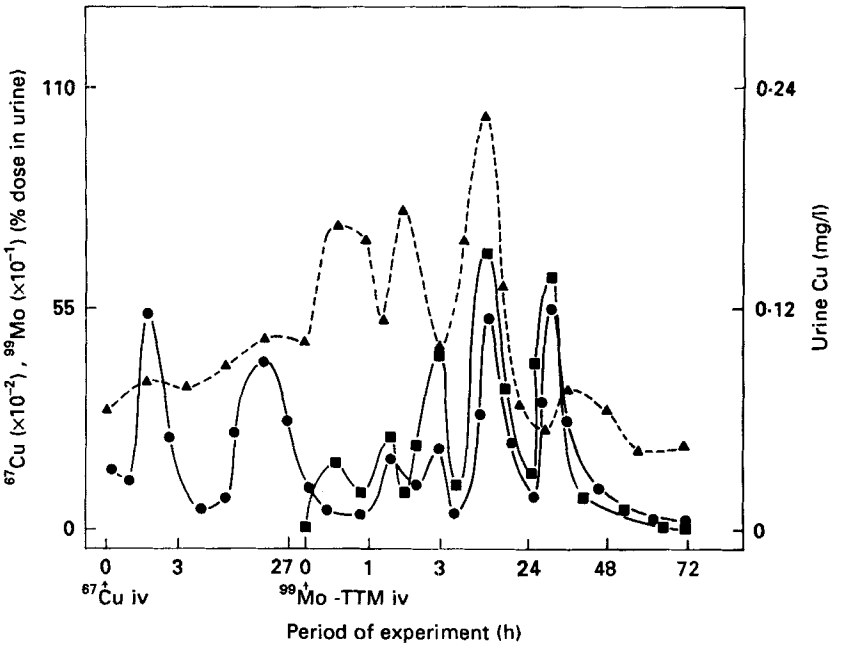

Fig. 6. Changes in ^{67}Cu (●——●), ^{99}Mo (■——■) and Cu (▲---▲) in urine of lamb no. 5 infused intravenously (iv) with ^{67}Cu (1·5 mCi, 0·6 mg Cu) and challenged 27 h later with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·2 mCi, 6 mg Mo) infused iv.

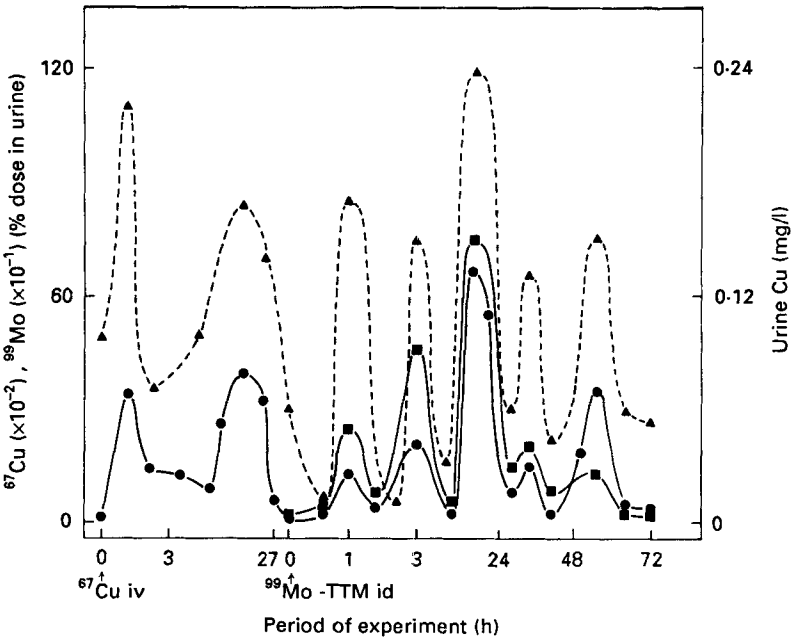

Fig. 7. Changes in ^{67}Cu (●——●), ^{99}Mo (■——■) and Cu (▲---▲) in urine of lamb no. 6 infused intravenously (iv) with ^{67}Cu (1·5 mCi, 0·6 mg Cu) and challenged 27 h later with ^{99}Mo-labelled tetrathiomolybdate (^{99}Mo-TTM; 0·3 mCi, 9 mg Mo) infused intraduodenally (id).

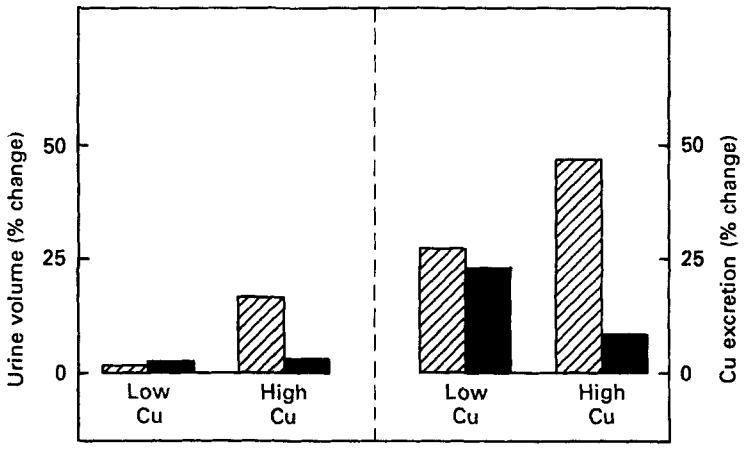

Fig. 8. The effect of administration of tetrathiomolybdate (TTM) either intravenously (iv) (▨) or intraduodenally (id) (■) on changes in the volume of urine and excretion of copper in urine within a 24 h period for lambs given a low-Cu diet (5·1 mg/kg dry matter (DM)) or a high-Cu diet (35 mg/kg DM). The values are expressed as the percentage change from the value for the 24 h period before TTM administration.

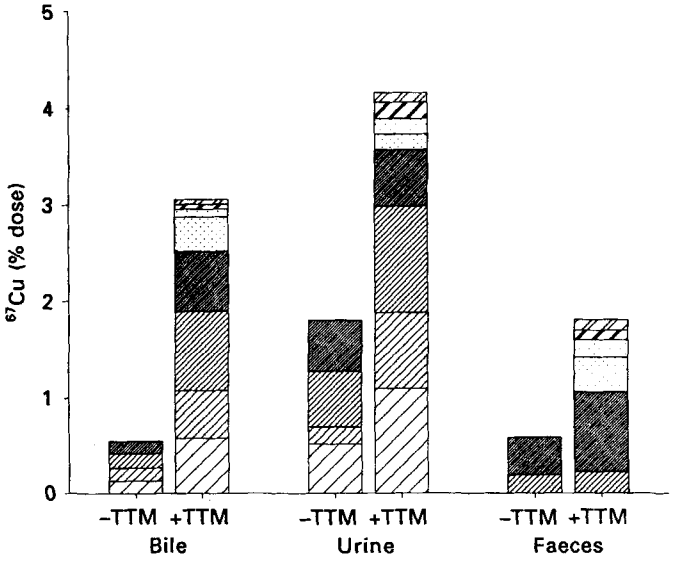

Fig. 9. Comparison of the cumulative excretion of ^{67}Cu in bile, urine and faeces from lamb no. 5, 24 h before and 72 h after challenge with intravenous ^{99}Mo-labelled tetrathiomolybdate (TTM; 0·2 mCi, 6 mg Mo). (▨), 3 h; (▨), 6 h; (▨), 12 h; (■), 24 h; (▨), 36 h; (▨), 48 h; (▨), 60 h; (▨), 72 h.

lower. This is probably related to the low dose of TTM used, since the systemic effects are dose-dependent (Gooneratne *et al.* 1981 *a*, *b*). The absorption of ^{99}Mo-labelled TTM id was rapid, with ^{99}Mo appearing in blood in the first sample collected at 5 min. This agrees with findings of Mason *et al.* (1980) who showed that blood ^{99}Mo may appear in blood within 2 min of id administration. ^{99}Mo administered either iv or id as TTM was rapidly excreted in bile along with ^{67}Cu, but the present experiment does not establish the forms in which

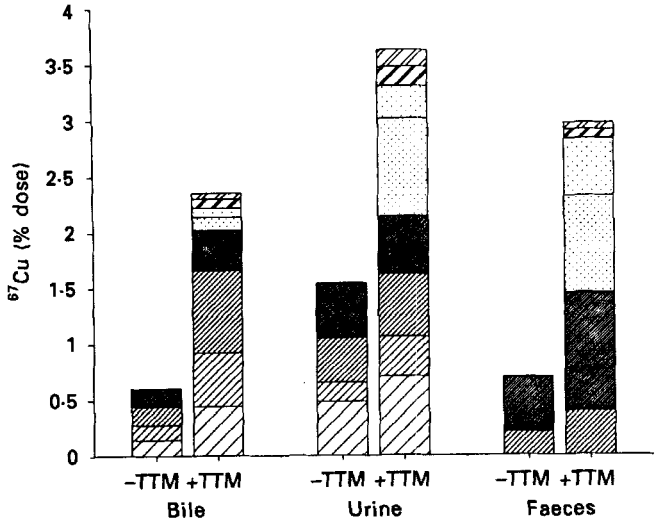

Fig. 10. Comparison of the cumulative excretion of ^{67}Cu in bile, urine and faeces from lamb no. 6, 24 h before and 72 h after challenge with intraduodenal ^{99}Mo-labelled tetrathiomolybdate (TTM; 0·3 mCi, 9 mg Mo). ( ), 3 h; ( ), 6 h; ( ), 12 h; ( ), 24 h; ( ), 36 h; ( ), 48 h; ( ), 60 h; ( ), 72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

Fig. 11. Cumulative excretion of ^{99}Mo in bile, urine and faeces, from lamb no. 5 primed intravenously with ^{67}Cu (1·5 mCi, 0·6 mg Cu) and challenged intravenously with ^{99}Mo-labelled tetrathiomolybdate (0·2 mCi; 6 mg Mo) after 27 h. ( ), 3 h; ( ), 6 h; ( ), 12 h; ( ), 24 h; ( ), 36 h; ( ), 48 h; ( ), 60 h; ( ), 72 h.

either ^{67}Cu or ^{99}Mo appeared in the bile. The major increase in bile Cu excretion observed in the lambs fed on the high-Cu diet confirms our previous observations (Gooneratne & Christensen, 1984). Biliary Cu excretion was most effective within 24 h following administration of TTM. This agrees with the findings of Ke & Symonds (1986) and thus repeated, frequent TTM treatment is required for rapid removal of liver Cu. Cu levels in

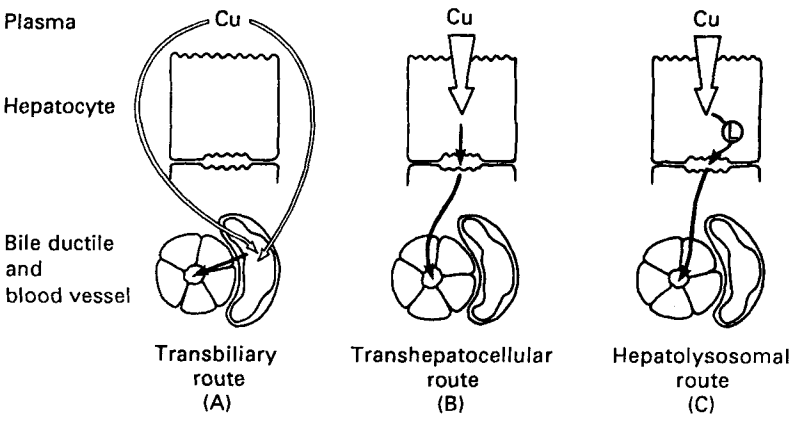

Fig. 12. Models of pathways of movement of copper from plasma to bile (adapted from Kressner *et al.* 1984). Transbiliary route (A) represents passage of Cu directly from sinusoids to the bile ductule in portal triad (PT) via blood vessels in PT area (⇓) without any passage through the hepatocytes. Transhepatocellular route (B) represents passage of Cu from sinusoids to hepatocyte cytoplasm (⇩), then to bile canaliculi and finally to the bile ductule in PT (↓). Hepatolysosomal route (C) is the classical pathway of bile Cu excretion. Cu first enters hepatocyte cytoplasm from the sinusoids (⇩), and here it is taken up by the lysosomes (L). After lysosomal processing, Cu is discharged into bile canaliculi which finally drain into the bile ductule in the PT (↓).

liver biopsies from these Cu-supplemented lambs before TTM were more than twice those in lambs fed on the low-Cu diet (S. R. Gooneratne, B. Laarveld and D. A. Christensen, unpublished results). This is a distinct advantage when TTM is used to treat Cu storage diseases. Biliary Cu excretion is dose-related up to 200 mg TTM given iv (Ke & Symonds, 1986). However, frequent use of TTM at dosages above 100 mg is not recommended as it may induce Mo toxicosis (Gooneratne *et al.* 1981*b*).

The mechanisms by which administered Cu is excreted in bile are largely unknown, particularly in ruminants. Cu is present in bile associated with bile salts (Lewis, 1973), bound to an azo dye-binding protein (Samuels *et al.* 1983), as metallothionein (MT) (Sato & Bremner, 1984) and as caeruloplasmin (Cp) (Kressner *et al.* 1984). Our observations relating to specific peak excretory periods of ^{67}Cu in bile (Figs 3 and 4) suggest that administered Cu is processed via different pathways. Hence the molecular form in which ^{67}Cu is present in bile may vary with time of sampling. A shift in radiolabelled Cu from a low to a high molecular weight fraction has been shown to occur in later samplings of bile in the rat (Terao & Owen, 1973). This probably accounts for the reduced absorption of Cu in 'late' bile (24 h sample) compared with 'early' bile (Mistilis & Farrer, 1968). Our studies (Gooneratne *et al.* 1989*b*) have confirmed and extend the previously described findings to include the effects of TTM administration. TTM not only increases bile Cu excretion, but also increases the percentage of Cu in the macromolecular fraction of bile (Gooneratne *et al.* 1989*b*) and limits enterohepatic circulation of Cu (S. R. Gooneratne, B. Laarveld and D. A. Christensen, unpublished results). The route of administration of TTM did not appear to affect the profile of biliary Cu excretion, but the magnitude was higher with iv administration.

Biliary Cu excretion may involve at least three and possibly four pathways (Fig. 12): (1) transbiliary, (2) transhepatocellular, (3) hepatolysosomal (Kressner *et al.* 1984). A paracellular short pathway has also been described (Layden *et al.* 1978). The times taken for the processing of molecules differ for the different pathways. The pulsatile pattern of excretion of ^{67}Cu and the synchrony with excretion of ^{99}Mo in bile suggests that both Cu

and TTM are processed via similar pathways and are secreted into bile at similar times following administration of either ^{67}Cu or ^{99}Mo-labelled TTM. We suggest therefore that bile Cu peaks A and A^1 (Figs 3 and 4) observed after ^{67}Cu and ^{99}Mo-labelled TTM administration respectively, represent a rapid transfer of increased levels of direct reacting Cu (DRCu) from blood to bile in the portal triad areas via the transbiliary route (Fig. 12(*a*)). This mechanism has been described for relatively small molecules such as Cu bound to amino acids and peptides (DRCu) (Sternlieb & Quintana, 1985). Micromolecules in blood arriving at the hepatocyte can also be rapidly transferred directly from hepatocyte to bile via 'blister formation' through leaky tight junctions (Layden *et al.* 1978). Some of the biliary Cu processed through these two pathways probably represents the low-molecular-weight fraction of Cu which is present in bile (Farrer & Mistilis, 1968). Relatively larger molecules such as Cp do not interact significantly with hepatocytes, and their presence in rat bile suggests transcytosis via biliary epithelium (i.e. transbiliary pathway) (Kressner *et al.* 1984). Whether Cp appears in sheep bile via this route is not known, but administration of TTM decreases Cp in sheep plasma almost immediately (Mason *et al.* 1980; S. R. Gooneratne and D. A. Christensen, unpublished results) and maximum effects last only for 45–60 min (Mason *et al.* 1980). Since the effect on Cp is so dramatic, it is unlikely that it is due to a reduction in synthesis. Hence it is possible that at least some of the decrease in Cp Cu could be accounted for by its excretion in bile. Excretion of Cp in rat bile has been reported (Jeunet *et al.* 1962) although Terao & Owen (1973) were unable to confirm these findings.

Dietary Cu after absorption is bound to albumin, which appears to have a direct role in the uptake of Cu by hepatocytes (Van den Berg & Van den Hamer, 1984). A small fraction of the Cu in albumin on reaching the liver passes directly from the sinusoids to the bile canaliculi without undergoing modification or degradation within the lysosomes (Kressner *et al.* 1984). Direct transfer of secretory proteins such as albumin and transferrin into bile has been reported (Kloppel *et al.* 1986). TTM administration results in an increase in the proportion of Cu associated with albumin (Hynes *et al.* 1984). Cu and TM bind at different sites on the albumin molecule (Woods & Mason, 1987), but the effect of such a change in configuration of albumin on subsequent distribution of Cu is not clear. Mills (1985) reported a reduction in ^{64}Cu capture by the liver as an explanation for the increased percentage of ^{64}Cu in blood of rats given TTM orally. Our observations show that tissue Cu (probably of hepatic origin) re-enters the systemic circulation following TTM administration. At least some of this Cu may be bound to albumin (Hynes *et al.* 1984), and the possibility exists that this albumin Cu may be recaptured by liver for excretion via bile. Based on liver fractionation studies following ^{67}Cu and ^{99}Mo administration (S. R. Gooneratne, B. Laarveld and D. A. Christensen, unpublished results), the release of liver Cu appears to be responsible for the proposed initial increase in albumin Cu (Hynes *et al.* 1984) and the decrease in ^{67}Cu in the liver. Bile ^{67}Cu and ^{99}Mo in peaks B and B^1 reported here probably represent albumin-bound Cu and Mo excretion through the trans-hepatocellular model as described by Kressner *et al.* (1984). The time-interval required for such a transfer to take place appears to coincide with the time-intervals at which bile peak(s) B (or B_1 and B_2) appeared. The reason for the biphasic appearance of peaks B_1 and B_2 in some sheep after ^{67}Cu administration and the appearance of a single peak (B_1) after TTM administration is not clear, but the lambs which showed this anomaly consistently produced this pattern in subsequent experiments also. However, there were other animals which consistently showed one B peak after ^{67}Cu administration and two B peaks (B_1 and B_2) after TTM administration (Gooneratne *et al.* 1989*a*).

As discussed previously, several bile Cu proteins appear to originate from the plasma compartment. However, biochemical (Mullock *et al.* 1978; Kloppel *et al.* 1986) and

morphological (De Duve, 1963) evidence suggests that expulsion of hepatocytic lysosomal products is the most important route of disposal of undigestible residues including Cu (Gooneratne *et al.* 1980; Jones *et al.* 1984). This is probably represented by bile Cu peaks C and C^1. We did not establish which Cu proteins constitute these peaks. Degradation of MT occurs mostly in the lysosomes (Bremner, 1981) although cytoplasmic degradation has also been described recently (Chen & Failla, 1988). The half-lives of the two major isometallothioneins, MT-1 and MT-2, in the livers of Cu-injected rats are 15 and 18 h respectively (Mehra & Bremner, 1985). This disappearance of MT may be due to its transfer to lysosomes and some of this Cu may be temporarily stored or degraded within the lysosomes and subsequently excreted into bile. But in sheep most of the Cu taken up by the liver accumulates in primary lysosomes which later become secondary and tertiary lysosomes (Gooneratne *et al.* 1980). Little is known about the role of MT in the biliary excretion of Cu. Sato & Bremner (1984) showed that only 1–2% of bile Cu is excreted bound to MT, but a high proportion of aggregated and lysosomal degradation products of MT are present in bile. Kressner *et al.* (1984) showed that the biliary Cu processed via lysosomes reaches bile canaliculi after a lag period. The time-interval taken for lysosomal discharge of Cu into bile may depend on the animal's metabolic requirement (Bremner, 1981) and on the presence of in vivo chelators which can remove Cu from the MT (Mehra & Bremner, 1985). Based on these findings it appears that peaks C and C^1 constitute Cu released from MT and other forms of Cu discharged from the lysosomal system.

TTM administration produced two other peaks of ^{67}Cu and ^{99}Mo at 40 and 54 h. It is not certain whether these represent discharges of different forms of Cu from the lysosomal system at specific times or whether they represent Cu processed via other organelles such as the nucleus. Approximately 20% of the Cu in the nuclear fraction represents a temporary storage form (Lewis & Laemmli, 1982). MT is present in the nucleus (Banerjee *et al.* 1982) and TTM administration decreases Cu in this fraction (Kelleher & Ivan, 1985).

The concentration of Cu in the liver of sheep does not influence the biliary Cu concentration or the total output of Cu in bile (Gooneratne *et al.* 1988), unlike the situation in cattle (Phillippo & Graca, 1983). Thus the higher susceptibility of sheep to Cu toxicity may be related to their poor ability to excrete excess Cu into bile. The significance of other pathways such as endogenous secretion in gastrointestinal juices and saliva is not known. But evidence from the present study shows that these routes of Cu excretion may be of importance, as the fraction of endogenous ^{67}Cu excreted into faeces after iv administration of ^{67}Cu was of similar magnitude to that excreted in bile. Similar observations were made in cattle where approximately equal proportions of Cu appear to be excreted into bile (Phillippo & Graca, 1983) and into the gastrointestinal tract (Charmley *et al.* 1982).

The increase in faecal ^{64}Cu excretion following TM administration has been reported (Mason *et al.* 1988). However, the present study describes for the first time increased excretion of endogenous faecal Cu, free of bile, due to TTM administration. We assumed this to be gastrointestinal secretions, although the nature of the faecal Cu was not identified. The marked faecal ^{67}Cu excretion after TTM in lambs is a significant finding. It is an added advantage when TTM is used as a treatment for Cu toxicity in sheep (Gooneratne *et al.* 1981*b*). It also means that under practical farming conditions when a diet high in Mo and S is given, TM formed in the rumen would not only prevent intestinal absorption of Cu, but also remove tissue Cu secreted (via plasma) back into the intestinal lumen for excretion via faeces. Stable Cu excretion in faeces during the radiotracer study period was also increased and this was again most marked after intraduodenal TTM administration. But the small number of lambs used in the present study and the large variation in faecal stable Cu excretion does not permit reasonable interpretation of such results.

Increases in urinary Cu excretion following TTM administration were observed in all lambs. This was partly due to increases in urine volume. Similar increases in urine Cu excretion have also been reported in sheep fed on excess Mo and S (Marcilese *et al.* 1970), and in Wilson's disease patients given TTM (Walshe, 1986) but is contradicted by Mason *et al.* (1988). The latter authors' observations were based on iv administration of trithiomolybdate, whereas TTM is a more potent decoppering agent than other TM (Suttle & Field, 1983).

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