The fate of ^{99}Mo-labelled sodium tetrathiomolybdate after duodenal administration in sheep: the effect on caeruloplasmin (*EC* 1.16.3.1) diamine oxidase activity and plasma copper

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(*Received* 13 *August* 1979 – *Accepted* 17 *December* 1979)

1. The effect of acute duodenal infusion of ^{99}Mo-labelled sodium tetrathiomolybdate on caeruloplasmin (ferroxidase; *EC* 1.16.3.1) was examined in sheep. The diamine oxidase activity of this enzyme with respect to two substrates, *p*-phenylenediamine and *o*-dianisidine (both at their apparent K_m concentrations) was inhibited.

2. The ^{99}Mo appeared rapidly in plasma and was at first present predominantly in a trichloroacetic acid insoluble form; inhibition of oxidase activity was related to the levels of TCA-insoluble Mo. The behaviour of the copper prosthetic groups of caeruloplasmin was altered since some plasma Cu precipitated with the protein fraction after TCA treatment. The appearance of TCA insoluble Cu was related to the level of TCA-insoluble ^{99}Mo and corresponded to the inhibition of diamine oxidase activity.

Molybdenum compounds, usually administered experimentally as molybdate (MoO_4^{2-}) or paramolybdate ($Mo_7O_{24}^{2-}$) salts, interfere with the absorption and metabolism of copper in animals and man (Agarwal, 1975). The interference is not due to a simple chemical reaction of dietary or tissue Cu with molybdate but by complex three-way interactions between Cu, Mo and sulphur. The form of the interactions varies quantitatively between ruminants and non-ruminants (see reviews by Suttle, 1974; Pitt, 1976; Mason, 1978). In ruminants increased dietary S usually exacerbates the effects of Mo, although Mo retention is decreased (Dick, 1953; Mason, Lamand *et al.* 1978) and there is evidence (Bremner & Young, 1978) that addition of sulphate to an Mo-supplemented diet improves both growth rate and haematological status.

In normal animals approximately 95% of plasma Cu is present as the prosthetic groups of caeruloplasmin (ferroxidase *EC* 1.16.3.1; Cp), and is readily released from the Cp apoprotein by treatment with trichloroacetic acid (TCA; 50 g/l). The oral administration of Mo to sheep increases plasma Cu levels, at least initially, but decreases TCA solubility since some of the plasma Cu then precipitates with the plasma proteins (Smith & Wright, 1975). The appearance of this new fraction, which also contains Mo is, in sheep, dependent on dietary S (Bremner, 1975). Mason, Cardin *et al.* (1978) showed that this effect of Mo in guinea-pigs was also increased by dietary S, but only when S was administered as sulphide. It is thus probable that the higher sensitivity of the ruminant to Mo compared to the non-ruminant is due to rumen reduction of S compounds to sulphide. The nature of the TCA insoluble Mo–Cu–protein complex is not established but Bremner & Young (1978) suggested that the change in behaviour following long-term administration of Mo was due to the appearance of molybdo-proteins with a high affinity for Cu.

Dick *et al.* (1975) proposed that the sensitivity of ruminants to Mo compounds was due to rumen formation of thiomolybdates ($MoO_nS_{(4-n)}^{2-}$, where $n = 1$–4) by the interaction of Molybdate and sulphide. Mills *et al.* (1978) produced good evidence supporting this scheme by showing that tetrathiomolybdate (TTM) prepared from ammonium paramolybdate decreased Cu absorption and provoked the appearance of TCA-insoluble Cu in plasma. However, direct evidence for rumen synthesis of tetrathiomolybdate is limited.

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0007-1145/80/3389-1712 $01.00

Mason, Lamand *et al.* (1978) showed that the passage of ^{99}Mo molybdate through the ovine rumen modified its behaviour particularly when the diet was high in S. The modification increased the TCA insolubility of the isotope in plasma and the intestinal absorption of the modified form was reduced by increasing the dietary Cu.

Sodium TTM inhibits the diamine oxidase activity of ovine and human Cp in vitro (Kelleher & Mason, 1979); for *o*-dianisidine and human purified Cp in vitro the mechanism appears competitive (J. Mason, unpublished results). The extent of inhibition observed would obviously depend on the concentrations of substrate and inhibitor relative to their K_m and K_i values. This could explain the failure to detect decreased Cp activities (Mason, Cardin *et al.* 1978; Mills *et al.* 1978) except where long-term Mo administration reduced tissue Cu levels, despite apparent profound changes in the form of plasma Cu (Mason, Cardin *et al.* 1978).

The experiments described involve the administration of ^{99}Mo-labelled sodium TTM to sheep via a duodenal cannula to avoid any modification of the molecule in the rumen. The objectives were: (*a*) to determine whether TTM is absorbed and to ascertain whether its subsequent plasma distribution and excretion pattern resembled that of the rumen-modified molybdate (Mason, Lamand *et al.* 1978); (*b*) to determine whether and at what concentrations TTM inhibited the diamine oxidase activity of caeruloplasmin in vivo using substrates at apparent K_m concentrations; (*c*) to examine the effect of TTM on plasma Cu.

MATERIALS AND METHODS

Animals

Four male sheep of the Texel breed were fitted with duodenal cannulas located immediately post pylorus. Animals nos. 1 and 2 received a basic diet containing 4·8 mg Cu, 0·35 mg Mo and 1·1 g S/kg. (Mason, Lamand *et al.* 1978); animals nos. 3 and 4 received the same diet supplemented with elemental S at a level of 3 g/kg diet. All sheep were fed *ad lib.* throughout and were given the diet for at least 4 weeks prior to experimental use. The sheep were housed in metabolism cages and fitted with harnesses for the collection of urine and faeces (Mason, Lamand *et al.* 1978). Their weights were 40, 75, 50·5 and 80 kg for animals nos. 1, 2, 3 and 4 respectively.

Preparation of ^{99}Mo-labelled sodium TTM

A labelled TTM preparation was made by passing hydrogen sulphide through a solution (50 ml, 0·24 M) of sodium molybdate, Na_2MoO_4 (Prolabo) containing 0·5 mCi $Na_2{}^{99}MoO_4$ (Commissariat à l, Energie Atomique, France) for 12 h. The solution was then purged with nitrogen for 20 min in an attempt to eliminate residual sulphide. The TTM was then administered immediately. Although the 465 nm peak characteristic of tetrathiomolybdate predominates in the absorption spectrum the presence of a slight shoulder in the 392 nm region indicates the presence of some residual trithiomolybdate in this type of preparation.

Administration of the ^{99}Mo-labelled TTM

[^{99}Mo]TTM (10 ml, 734 mg) was introduced directly into the duodenum of each sheep via the duodenal cannulas using a Foley balloon catheter pre-inflated in the cannula to prevent reflux back into the cannula.

Samples

Urine and faeces were collected and ^{99}Mo counted as described by Mason, Lamand *et al.* (1978). Plasma samples were obtained and the quantity of TCA-soluble and TCA-insoluble ^{99}Mo measured and calculated as described by Mason, Lamand *et al.* (1978). TCA-soluble Cu was estimated by atomic absorption spectrophotometry.

Enzyme Assays

p-Phenylenediamine (PPD) oxidation. This was estimated as follows. EDTA (1·3 mM, 0·1 ml) followed by the sheep plasma (0·2 ml) were added to 1 ml PPD (Carlo Erba Divisione Chimica Industriale, Milan) in acetate buffer (0·8 M, pH 6·3) at 37° to give a final concentration of 2 mM-PPD. After 15 min incubation at 37° the reaction was stopped with sodium azide (9·75 g/l; 2 ml). The extinction values at 525 nm were read against a reagent blank incubated at the same time to allow for auto-oxidation of PPD. PPD samples from different sources have widely varying rates of auto-oxidation; with the PPD sample used auto-oxidation was very low. Assays were carried out in duplicate.

o-Dianisidine oxidation. This was assayed as follows. Sheep plasma (0·2 ml) and 2 mM-EDTA (0·1 ml) were pre-incubated for 5 min with 0·8 M-acetate buffer at pH 5·5 (1·5 ml). The test tubes were prepared for each assay, for 5 and 15 min incubation respectively. To start the reaction *o*-dianisidine dihydrochloride (Sigma) (0·2 ml, 15 mM, final concentration in the incubation mixture 1·5 mM) were added to each test tube. The reactions were stopped after 5 and 15 min incubation at 37° by the addition of 9 M-sulphuric acid (2 ml). The slight turbidity which develops was removed by centrifugation at 40000 *g* for 10 min, in some groups of sheep the turbidity appears slowly and the samples are best left overnight before centrifugation. The colour is stable. The extinction at 540 nm was then measured and the activity taken as the difference between the 5 and 15 min samples. All assays were carried out in duplicate.

RESULTS

The appearance of both TCA-insoluble and TCA-soluble Mo in plasma after the duodenal administration of ^{99}Mo-labelled TTM (734 mg) for the four sheep used is recorded in Figs. 1 and 2. This can be compared with the levels of plasma Cp diamine oxidase activity (Figs. 3–6) with respect to dianisidine and PPD oxidation (both substrates present at the apparent K_m concentrations of 1·5 and 2 mM respectively). Plasma TCA-soluble Cu levels over the same period are also shown in Figs. 3–6.

Plasma ^{99}Mo

Radioactivity appeared very quickly in blood and for the first hour after administration when quantities were maximal the ^{99}Mo was predominantly in the TCA-insoluble fraction. Absorption of TTM from the small intestine was evidently rapid and relatively efficient; 47·7, 51·6, 50·7 and 75·7% of the dose was recovered in the faeces for sheep nos. 1, 2, 3 and 4 respectively after 120 h. Most of this must have represented unabsorbed rather than re-excreted Mo since 95–99% of the faecal ^{99}Mo appeared in the first 40 h after administration. Despite this, the total amounts found in blood in the TCA-insoluble fraction at any time never represented more than 1–2% of the dose administered. Concentrations of TCA-insoluble ^{99}Mo were calculated to be 0·96, 0·76, 0·8 and 0·52 $\times$ 10^{-5} M for sheep nos. 1, 2, 3 and 4 respectively 60 min after administration when concentrations were approximately maximal.

By contrast the TCA-soluble plasma Mo exhibited a completely different pattern, the label appeared more slowly to reach a maximum some 6–8 h after administration, in addition the rate of decrease was markedly affected by the level of dietary S. It was higher in animals nos. 3 and 4 (Fig. 2) which received the basic diet supplemented with 3 g elemental S/kg diet.

The pattern observed in a control experiment (sheep no. 3 receiving an S-supplemented diet) in which an equivalent quantity of Mo was administered duodenally as sodium molybdate (578 mg) differed and was similar to the absorption pattern already reported

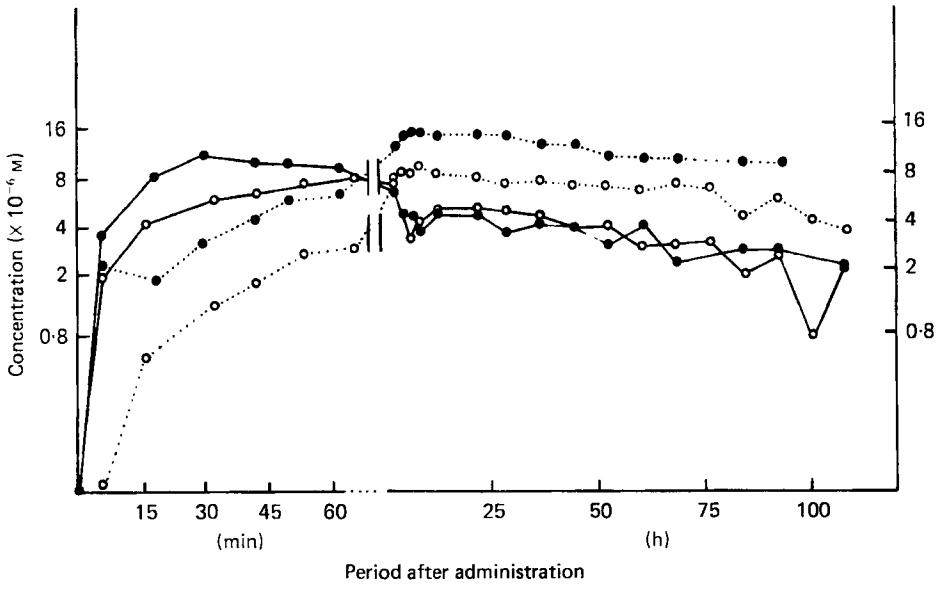

Fig. 1. Level of labelled plasma molybdenum after duodenal administration of ^{99}Mo sodium tetrathiomolybdate (734 mg) for sheep given the basal diet. Sheep no. 1 TCA insoluble Mo (● —— ●), TCA soluble Mo (● ... ●). Sheep no. 2 TCA insoluble Mo (○ —— ○), TCA soluble Mo (○ ... ○).

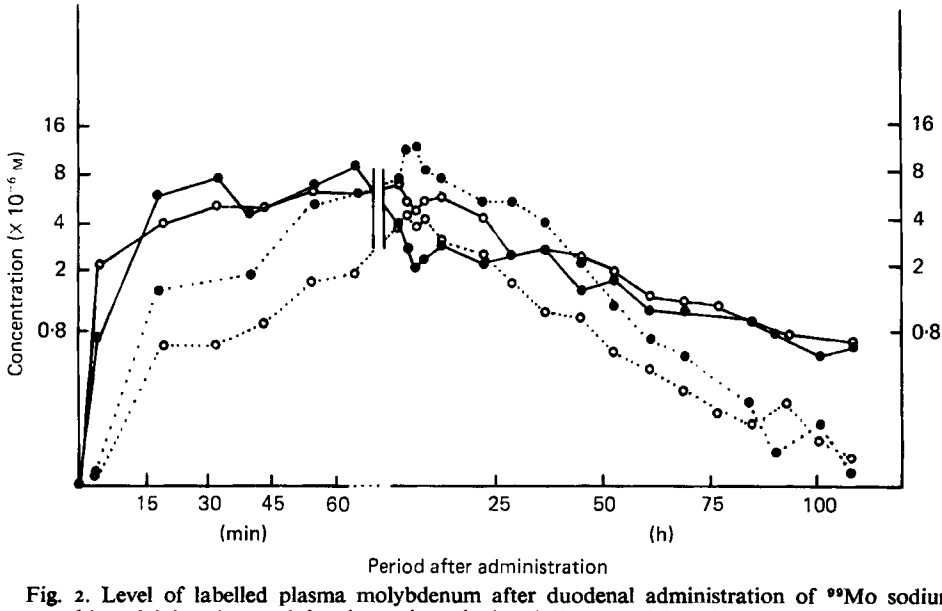

Fig. 2. Level of labelled plasma molybdenum after duodenal administration of ^{99}Mo sodium tetrathiomolybdate (734 mg) for sheep given the basal diet + 3g S/kg. Sheep no. 3 TCA insoluble Mo (● —— ●), TCA soluble Mo (● ... ●). Sheep no. 4 TCA insoluble Mo (○ —— ○), TCA soluble Mo. (○ ... ○).

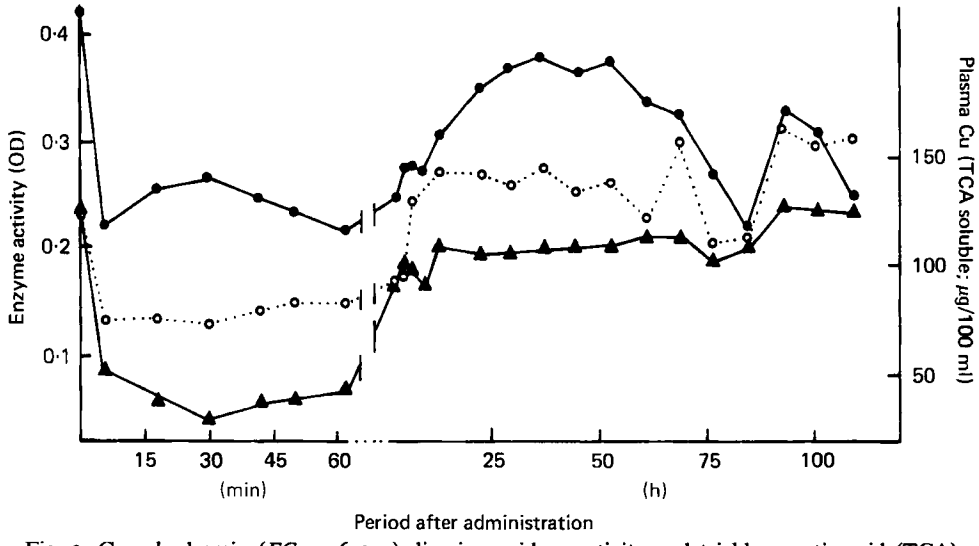

Fig. 3. Caeruloplasmin (*EC* 1.16.3.1) diamine oxidase activity and trichloroacetic acid (TCA) soluble plasma copper after duodenal tetrathiomolybdate administration for sheep no. 1 given the basal diet. 1.5 mM-*o*-dianisidine (● —— ●), 2 mM-*p*-phenylenediamine substrate (○ ... ○), TCA soluble Cu (▲ —— ▲).

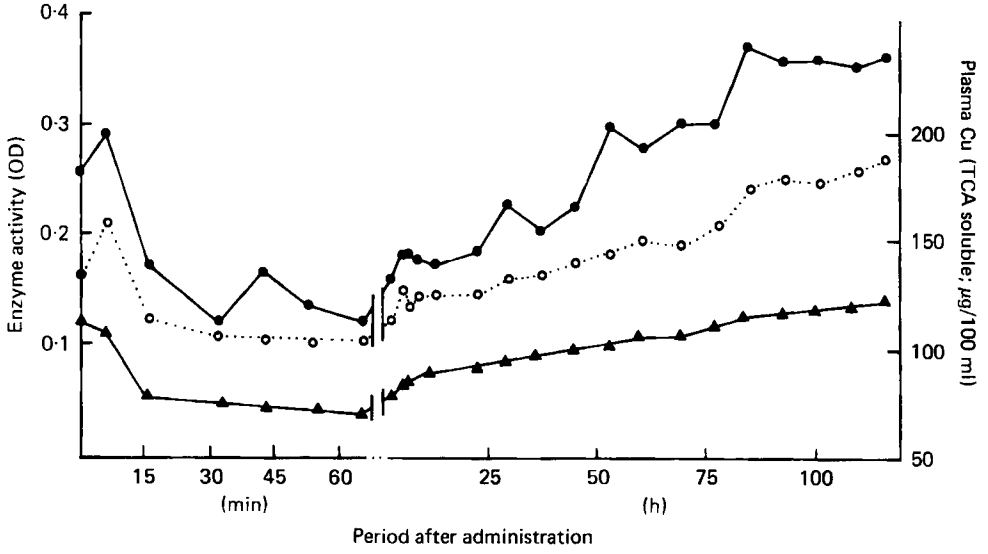

Fig. 4. Caeruloplasmin (*EC* 1.16.3.1) diamine oxidase activity and trichloroacetic acid (TCA) soluble plasma copper after duodenal tetrathiomolybdate administration for sheep no. 2 fed the basal diet. 1.5 mM-*o*-dianisidine (● —— ●), 2 mM-*p*-phenylenediamine substrate (○ ... ○), TCA soluble Cu (▲ —— ▲).

for trace amounts of ^{99}Mo-labelled molybdate administered duodenally by Mason, Lamand *et al.* (1978). That is, rapid absorption reaching a plasma maximum 2 h after administration and a clear predominance of TCA-soluble ^{99}Mo throughout the experimental period. The TCA-soluble : TCA-insoluble value in the control experiment was fairly constant at 10:1. The total amount found in plasma was also much greater i.e. 7% of the dose 2 h after administration.

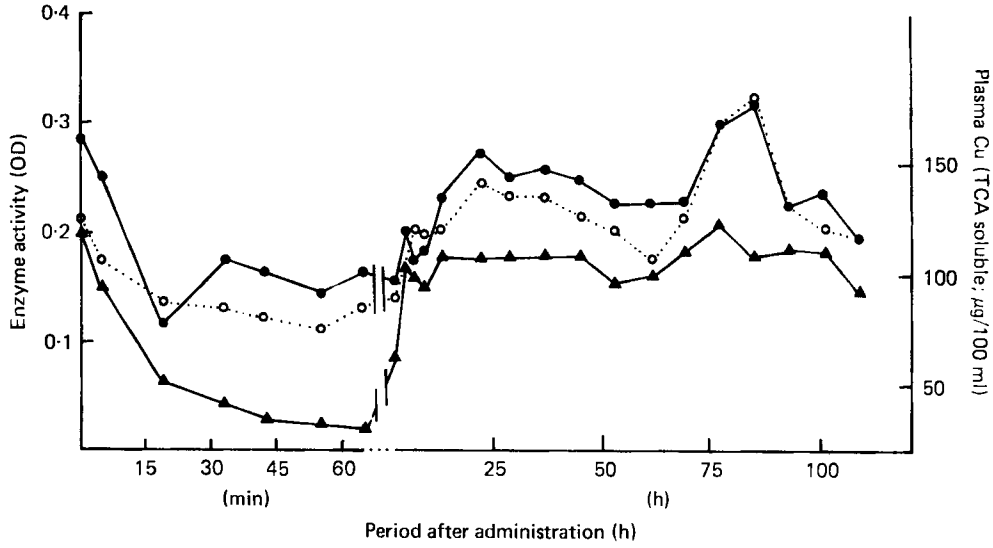

Fig. 5. Caeruloplasmin (*EC* 1.16.3.1) diamine oxidase activity and trichloroacetic acid (TCA) soluble plasma copper after duodenal tetrathiomolybdate administration for sheep no. 3 fed the basal diet + 3 g S/kg. 1.5 mM-o-dianisidine (● —— ●,) 2 mM-*p*-phenylenediamine substrate (○ ... ○), TCA soluble Cu (▲ —— ▲).

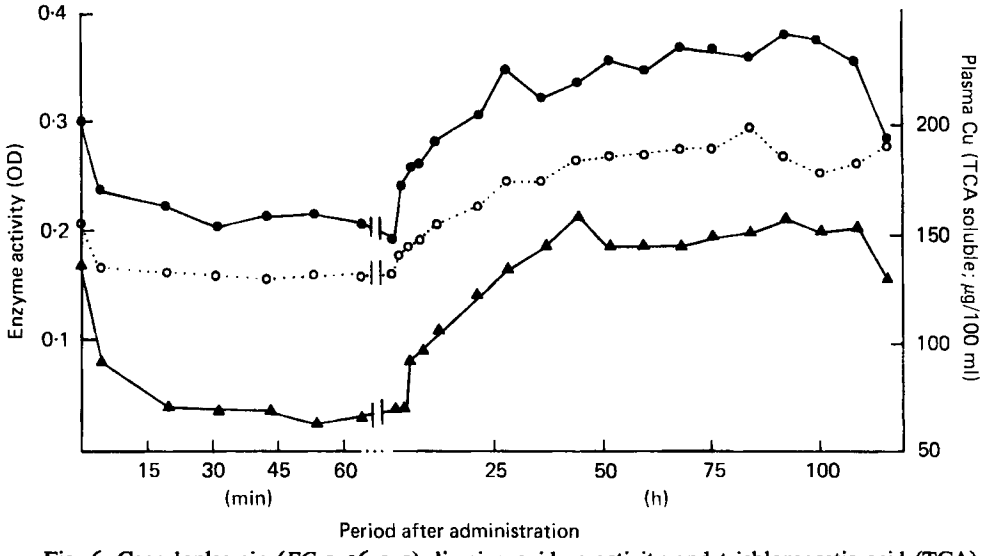

Fig. 6. Caeruloplasmin (*EC* 1.16.3.1) diamine oxidase activity and trichloroacetic acid (TCA) soluble plasma copper after duodenal tetrathiomolybdate administration for sheep no. 4 fed the basal diet + 3 g S/kg. 1.5 mM-*o*-dianisidine (● —— ●), 2 mM-*p*-phenylenediamine substrate (○ ... ○), TCA soluble Cu (▲ —— ▲).

Cp diamine oxidase activity

The changes in Cp diamine oxidase activity with respect to the two substrates, *o*-dianisidine and PPD are shown in Figs. 3–6. Clearly the oxidation of both substrates was inhibited and the extent of inhibition was related to the level of TCA-insoluble Mo in plasma. That is, inhibition was maximal over the first 2–3 h and then decreased as the TCA-insoluble Mo

declined. There was no corresponding relationship between inhibition and TCA-soluble Mo levels. The decrease in Cp diamine oxidase activity corresponded very closely to a decline in the TCA solubility of plasma Cu, also recorded in Figs. 3–6.

No enzyme inhibition or decrease in plasma Cu TCA solubility was observed in the control experiment where an equivalent quantity of sodium molybdate (578 mg) was administered duodenally.

DISCUSSION

^{99}Mo administered via a duodenal cannula as TTM was rapidly absorbed but the experiments described do not establish the form in which ^{99}Mo appeared in plasma. The TCA-soluble and TCA-insoluble ^{99}Mo plasma fractions clearly represented two different forms of Mo, since the decline in TCA-soluble radioactivity was greatly increased by the addition of elemental S (3 g/kg) to the experimental diet. S supplementation has been shown to accelerate the excretion of ^{99}Mo-labelled molybdate administered duodenally (Mason, Lamand *et al.* 1978). The TCA-soluble Mo fraction is thus probably molybdate or at least a form of Mo which competes for the group 6 oxyanion transport system and the most likely explanation for the more rapid decrease is molybdate–sulphate interaction of the type described by Mason & Cardin (1977), at the level of the renal tubule. There is no corresponding interaction between tetrathiomolybdate (MoS_4^{2-}) and sulphate, at least in the rat intestine (J. Mason, unpublished results). By contrast the plasma TCA-insoluble ^{99}Mo was only slightly affected by dietary S supplementation. The slower decrease at the lower S level may have been due to increased molybdate retention and Mo recycling, including presumably a reconversion of molybdate to thiomolybdate in the rumen, rather than a direct effect of sulphate on the TCA-insoluble fraction. Mo recycling has been described by Grace & Suttle (1979). The TCA-soluble Mo probably arises from oxidation or metabolism of TTM since there is a time-lag between the appearance of TCA-insoluble ^{99}Mo and TCA-soluble ^{99}Mo in plasma. Duodenally-administered ^{99}Mo molybdate appears more rapidly and always predominantly in the TCA-soluble fraction.

It is evident from the results that at the level used the introduction of TTM into the duodenum caused a transient inhibition of Cp diamine oxidase activity and an alteration of the post-TCA-treatment behaviour of the prosthetic Cu groups of Cp. Since the decrease in activity is so rapid the effect can hardly be due to reduced synthesis; in any event total plasma Cu levels remained stable over the first few hours. The inhibition is related to the level of TCA-insoluble ^{99}Mo and unrelated to the TCA-soluble ^{99}Mo. Calculation of the concentrations of Mo in the TCA-insoluble fraction show that if the Mo is in the form of TTM then inhibition of this order could have been predicted from results in vitro (Kelleher & Mason, 1979).

The inhibition seen contrasts with failure in previous experiments (Mason, Cardin *et al.* 1978; Mills *et al.* 1978) to observe decreases in Cp diamine oxidase activity despite large increases in TCA-insoluble Cu and falls in the TCA-soluble Cu. A slight decrease (10%) was however noted by El Gallad *et al.* (1977) after intravenous administration. There are a number of possible explanations for previous failures; first the amount of TTM administered was relatively high and was administered rapidly as a single dose. However, despite this, plasma levels were never high, approximately 1% of the dose; secondly the Cp substrates were used at concentrations corresponding to the K_m values determined for plasma rather than the saturating concentrations of the standard assays. For a competitive inhibitor the reduction in substrate concentration would increase the sensitivity to inhibition. Thirdly, it is possible that the different groups working in the field are using different compounds or even different mixtures of compounds as 'TTM' (Weber *et al.* 1979). Mills *et al.* (1978) report that the acute infusion of TTM (0·2–0·5 mg Mo/kg) into circulating plasma neither modified

the activity of Cp nor inhibited the release of Cu from this and higher molecular weight proteins on treatment with TCA. Acute intravenous infusion of this amount would give a theoretical maximal concentration of approximately $7{\cdot}5 \times 10^{-5}$ M, well above the inhibitory concentrations found in the present work. Mills *et al.* (1978) prepared TTM from ammonium paramolybdate whereas the materials used in these experiments were prepared from sodium molybdate since sodium is the predominant rumen cation and the Mo exists in this salt as the simple MoO_4^{2-} ion. A recent paper by Weber *et al.* (1979) demonstrates the complexity and instability of thiomolybdates prepared from potassium molybdate. The latter authors reported rapid bleaching of TTM kept in unsealed containers; this was not a feature of our preparations nor did the enzyme-inhibitory capacity decrease with storage (C. A. Kelleher & J. Mason, unpublished results). Evidently a thorough investigation of thiomolybdate chemistry and comparison of the preparations of the different groups is now desirable. The effect of the low duodenal pH on TTM introduced there or passing through from the rumen should also be investigated since low pH values lead to the formation of insoluble molybdenum sulphides, including MoS_3.

The inhibition of Cp diamine oxidase activity was transient and must have resulted from a direct but reversible effect on the enzyme. The inhibition diminished as plasma TCA-insoluble ^{99}Mo levels decreased. There was some evidence of a response since the activities and TCA-soluble Cu levels of sheep 2 and 4 actually increased above the starting values towards the final stages (Figs. 4 and 6) and total Cu levels also increased in these animals. The reversibility is to be expected if the mechanism of inhibition is competitive by analogy with the purified human enzyme (C. A. Kelleher & J. Mason, unpublished results). The most likely explanation for the behaviour of the Cp prosthetic Cu groups, that is, apparently increased insolubility in TCA, is that the release from the Cp apo-protein is unaffected but the Cu is then recomplexed by TTM present and precipitates with the proteins. This would seem more probable than the synthesis of new proteins containing both Mo and Cu which may be a feature of long-term TTM administration (Mills *et al.* 1978). It is significant that El Gallad *et al.* (1977) showed that the TCA insoluble Cu in plasma was restored to its normal soluble form on fractionation of the plasma on Sephadex G100. Mo compounds can therefore inhibit Cp diamine oxidase activity. While this may be useful diagnostically it is hardly physiologically relevant since the role of Cp is in iron oxidation and transport of Cu to peripheral tissues (Frieden & Hsieh, 1976). The diagnostic value of the inhibition remains to be established since it was transient and appeared after the infusion of a large amount of TTM into the duodenum. Since TTM or some product of TTM can be absorbed and affect Cp its effect on these other activities should be examined, in addition since relatively little of the dose was actually present in the blood at any point the effect in tissues on other Cu enzymes is worthy of investigation and in particular lysyl oxidase.

J.M. and C.A.K. acknowledge with gratitude the financial support of the National Board for Science and Technology. The authors are indebted to Claudine Lab, J. C. Tressol, G. Montel and M. Lafarge for technical assistance often above the mere line of duty.

REFERENCES

Agarwal, A. G. (1975). *New Sci.* **65**, 260.
Bremner, I. (1975). *Proc. Nutr. Soc.* **35**, 22A.
Bremner, I. & Young, B. W. (1978). *Br. J. Nutr.* **39**, 325.
Dick, A. T. (1953). *Aust. Vet. J.* **29**, 18.
Dick, A. T., Dewey, D. W. & Gawthorne, J. M. (1975). *J. agric. Sci., Camb.* **85**, 567.
El Gallad, T. T., Bremner, I. P. & Mills, G. F. (1977). *Proc. Nutr. Soc.* **36**, 104A.

Frieden, E. & Hsieh, H. S. (1976). *Adv. Enzymol.* **44**, 187.
Grace, N. D. & Suttle, N. F. (1979). *Br. J. Nutr.* **41**, 125.
Kelleher, C. A. & Mason, J. (1979). *Res. vet. Sci.* **26**, 124.
Mason, J. (1978). *Vet. Sci. Comm.* **2**, 85.
Mason, J. & Cardin, C. J. (1977). *Res. vet. Sci.* **22**, 313.
Mason, J., Cardin, C. J. & Dennehy, A. (1978). *Res. vet Sci.* **24**, 104.
Mason, J., Lamand, M., Tressol, J. C. & Lab., C. (1978). *Ann Rech. Vet.* **9**, 577.
Mills, C. F., Bremner, I., El Gallad, T. T., Dalgarno, A. C. & Young, B. W. (1978). In *Trace Element Metabolism in Man and Animals*, p. 150 [M. Kirchgessner, editor]. Weinhenstephan: Arbeitskreis für Tierernahrungsforschung.
Pitt, M. A. (1976). In *Agents and Actions*, vol. 6, p. 758. Basel: Birkhauser Verlag.
Smith, B. S. W. & Wright, H. (1975). *J. Comp. Path.* **85**, 299.
Suttle, N. F. (1974). *Proc. Nutr. Soc.* **33**, 299.
Weber, K. M., Leaver, D. D. & Wedd, A. G. (1979). *Br. J. Nutr.* **41**, 403.

Printed in Great Britain