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The demonstration of protein-bound ^{99}Mo-di- and trithiomolybdate in sheep plasma after the infusion of ^{99}Mo-labelled molybdate into the rumen

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1. Protein-bound, trichloroacetic acid- (TCA) insoluble ^{99}Mo appeared in plasma a few hours after the introduction of ^{99}Mo-labelled molybdate (30 mg Mo) into the rumen of sheep maintained on a basic diet supplemented with elemental sulphur (3 g S/d).

2. Most of the ^{99}Mo could be displaced from its protein carrier in vitro and the labelled compounds displaced were identified by Sephadex G-25 chromatography as di- and trithiomolybdate. Tetrathiomolybdate was not detected.

3. In control experiments protein-bound, TCA-insoluble ^{99}Mo predominated in plasma after the direct administration of [^{99}Mo]tetrathiomolybdate, either into the rumen or intravenously. The ^{99}Mo could be displaced in vitro and [^{99}Mo]tetrathiomolybdate identified, although [^{99}Mo]trithiomolybdate was also present. The study provides direct evidence of thiomolybdate synthesis and absorption in ruminants in vivo.

A role for thiomolybdates in Mo-induced hypocupraemia and molybdenosis in ruminants was suggested as part of the comprehensive hypothesis outlined by Suttle (1974) and by Dick *et al.* (1975). These authors envisaged that thiomolybdates could arise in the rumen from the interaction of dietary Mo and sulphide, depress the availability of dietary copper, and, if absorbed, interfere with Cu metabolism in the tissues. There is some indirect evidence for this, tetrathiomolybdate was highly toxic to weanling rats maintained on a semi-synthetic low-fibre diet containing 3 μg Cu/kg (Mills *et al.* 1981), although adult rats (Fell *et al.* 1979) and sheep (Suttle, 1980) are less sensitive.

While there is no direct evidence of thiomolybdate synthesis and absorption in ruminants in vivo, Mills *et al.* (1978) reported that the characteristic absorption spectrum of tetrathiomolybdate was detectable in vitro after the incubation of ammonium *p*-molybdate (10 mg Mo/l) and S sources (20–50 mg S/l) with sieved rumen contents for 18 h under carbon dioxide at 39°. The spectrum was not detected in rumen fluid obtained from cattle maintained on a diet containing (/kg dry matter) 5 mg Mo, 11 mg Cu and 3·8 g S. However, the authors estimated that spectrophotometry would be too insensitive to detect the compound in vivo at dietary Mo concentrations of less than 100 mg/kg. Bray *et al.* (1982) detected a mixture of thiomolybdates with a predominance of trithiomolybdate in the liquid phase of digesta from an artificial rumen system (Rusitec); the digesta sulphide levels reported varied between 14·6 and 19·2 μg S/ml at Mo input levels of 4, 8 and 12 μg Mo/ml. However, Clarke & Laurie (1979) predicted from in vitro studies that rumen concentrations of sulphide and Mo would favour the formation of di- and trithiomolybdate, whereas the extensive synthesis of tetrathiomolybdate would occur only after a relatively long period of time at high dietary S:Mo values.

There is evidence that some chemical modification occurs during the passage of Mo through the rumen (Mason *et al.* 1978; Kelleher *et al.* 1982). Thus, while the infusion of [^{99}Mo]molybdate into the duodendum causes the appearance of trichloroacetic acid (TCA)-soluble ^{99}Mo in plasma, rumen administration leads to the appearance in plasma of ^{99}Mo which is TCA-insoluble and protein-bound, particularly at higher dietary sulphur

levels. TCA-insoluble Mo (and Cu) also appears in plasma after the infusion of di-, tri- and tetrathiomolybdates ($MoO_2S_2^{2-}$, $MoOS_3^{2-}$ and MoS_4^{2-} (Mason *et al.* 1980; Mason *et al.* 1982; Kelleher *et al.* 1982).

The present study was undertaken to identify the protein-bound Mo compounds present in plasma after the rumen infusion of ^{99}Mo-labelled molybdate, and to attempt to provide direct evidence of thiomolybdate formation and absorption in vivo.

MATERIALS AND METHODS

Experimental animals

Two male castrated sheep, a Cheviot cross, weighing 44 kg (sheep A) and a Suffolk cross, weighing 48 kg (sheep B), were maintained on a basic diet of concentrate (400 g/d) and hay (*ad lib.*). Elemental S (3 g/d) was added to the concentrate and this was fed at 10.00 hours each day. The ^{99}Mo-labelled compounds were given by stomach tube into the rumen at 14.00 hours.

[^{99}Mo]molybdate

The [^{99}Mo]molybdate used for infusion was obtained as ammonium molybdate (specific activity 2 mCi/mg) from Amersham International Ltd, England. Upon receipt, the consignment was made alkaline with 200 μl NaOH(1 M) to prevent polymerization. This stock solution was used to label solutions of sodium molybdate (BDH, England). [^{99}Mo]tetrathiomolybdate was prepared and purified as in Mason *et al.* (1982).

Blood samples and plasma ^{99}Mo

Plasma samples were obtained and the concentrations of TCA-soluble and TCA-insoluble ^{99}Mo determined as reported by Mason *et al.* (1978). Plasma samples for gel filtration and chromatography were stored at $-20°$.

Sephadex gel filtration and chromatography

Thiomolybdates can be separated and identified by elution through Sephadex since the retardation, arising presumably as a result of matrix interactions, is characteristic: tetra- > tri- > dithiomolybdate (Mason *et al.* 1982). Molybdate is not retarded.

To displace and identify the ^{99}Mo-labelled protein-bound compounds present in plasma, unlabelled tetra- or trithiomolybdate (0·45–0·8 mg Mo), prepared and purified using Sephadex G-25 chromatography as in Mason *et al.* (1982), was added to 1–4 ml heparinized plasma. The treated plasma was then immediately passed through a column of Sephadex G-25 (325 or 350 × 22 mm). Elution, at 4°, was with 0·05 M-phosphate buffer at pH 7·5 with a flow rate of approximately 50 ml/h and fractions of 8 or 9 ml collected. The distribution of radioactivity (as ^{99}Tc) was determined after the attainment of isotopic equilibrium (48 h) (Mason *et al.* 1978) using a Packard 5230 gamma liquid scintillation counter and the fractions containing protein detected by optical density measurements at 280 nm. The unlabelled thiomolybdate added to samples could easily be distinguished; its identity and distribution was, however, checked by determining the absorption spectra of the coloured fractions.

Removal of non-protein-bound ^{99}Mo

Most samples examined were subjected to a preliminary rapid gel filtration using short column (100 × 22 mm) of Sephadex G-25 before treatment with unlabelled thiomolybdates. Elution, of 1–4 ml heparinized plasma, was with 0·05 M-phosphate buffer, pH 7·5, at 4°. Fractions of approximately 5 ml were collected. The protein-containing eluate, and thus the protein-bound ^{99}Mo, approximately fraction no. 4, was retained for the displacement and chromatographic studies; subsequent fractions containing unbound ^{99}Mo were rejected.

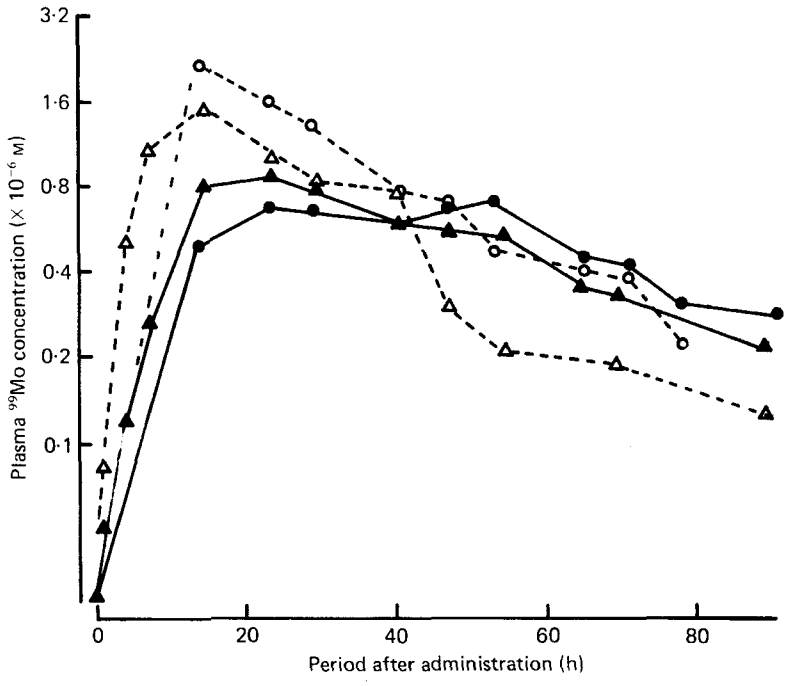

Fig. 1. The concentrations of labelled molybdenum in plasma after the infusion of 30 mg Mo, as [^{99}Mo]molybdate, into the rumen. Sheep A, (●——●) TCA-insoluble Mo; (○- -○) TCA-soluble Mo. Sheep B, (▲——▲), TCA-insoluble Mo; (△- -△), TCA-soluble Mo.

The TCA-insoluble/protein-bound and TCA-soluble/unbound fractions corresponded closely (J. Letters, unpublished results).

RESULTS

Molybdate infusion

The infusion of ^{99}Mo-labelled molybdate (approximately 2 mCi ^{99}Mo and 30 mg Mo) into the rumen was followed by the rapid appearance of ^{99}Mo in plasma. The pattern, shown in Fig. 1, was similar for the two animals used. Initially the radioactivity was mainly TCA-soluble but TCA-insoluble ^{99}Mo also appeared, but at a lower rate. Both fractions attained maximum levels approximately 15–20 h post-infusion; thereafter the TCA-soluble ^{99}Mo fraction decreased more rapidly.

Animal A

Sephadex G-25 chromatography of plasma samples treated with unlabelled tetrathiomolybdate in vitro, showed three distinct peaks of radioactivity, that is the fractions eluted from 90–135, 145–200 and 220–300 ml in the examples of elution profiles shown in Fig. 2(*a*) of samples obtained 29 and 52 h after infusion. Control experiments showed that molybdate, dithiomolybdate and trithiomolybdate elute in these positions. Some residual radioactivity remained associated with the plasma proteins at approximately 60 ml (Fig. 2(*a*)). Plasma samples run without the addition of tetrathiomolybdate showed only two peaks; the two later peaks eluting in the position of di- and trithiomolybdate were absent and the corresponding radioactivity remained associated with the fractions containing proteins (50–60 ml). No significant levels of radioactivity were detected in the fractions containing

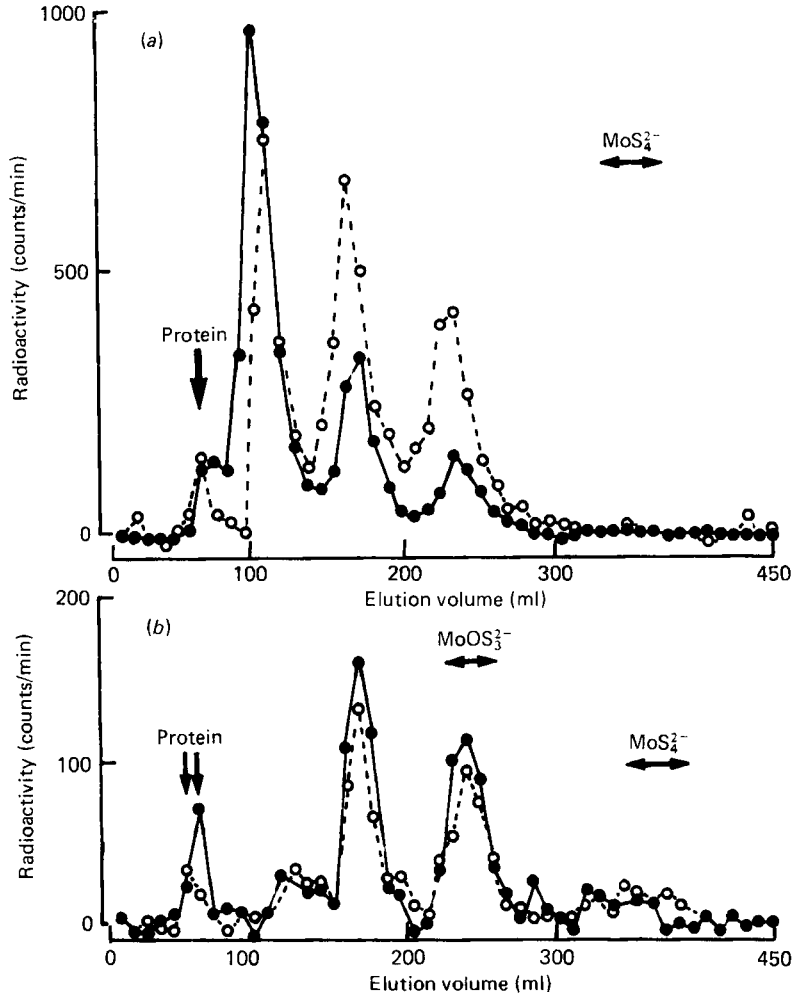

Fig. 2. Sephadex G-25 chromatography of plasma samples from sheep A administered [^{99}Mo]molybdate (30 mg Mo) via the rumen. Column bed volume 123 ml. (*a*) A plasma sample (1 ml) treated with 500 μl 9 mM-sodium tetrathiomolybdate (0·45 mg Mo). The samples were obtained 29 h (●——●) and 52 h (○- -○) post-infusion. (*b*) A plasma sample (2 ml) obtained 40 h post-infusion subjected to a preliminary Sephadex G-25 gel filtration to remove non-protein-bound ^{99}Mo before treatment with trithiomolybdate (○- -○) or tetrathiomolybdate (●——●) (0·45 mg Mo).

the unlabelled tetrathiomolybdate eluted at approximately 350 ml (indicated in Fig. 2) with any sample from any stage of the experiment.

Figure 2(*b*) shows that a preliminary gel filtration with the short Sephadex G-25 column before thiomolybdate treatment virtually eliminated the peak (90–140 ml) corresponding to [^{99}Mo]molybdate. A near identical elution profile (Fig. 2(*b*)) was obtained when trithiomolybdate was used as an alternative to tetrathiomolybdate to displace the ^{99}Mo from the protein fraction.

Animal B

The results obtained from a second experiment with sheep B were very similar. Over the duration of the experiment, samples, from 14–64·5 h, gel-filtered to remove unbound ^{99}Mo

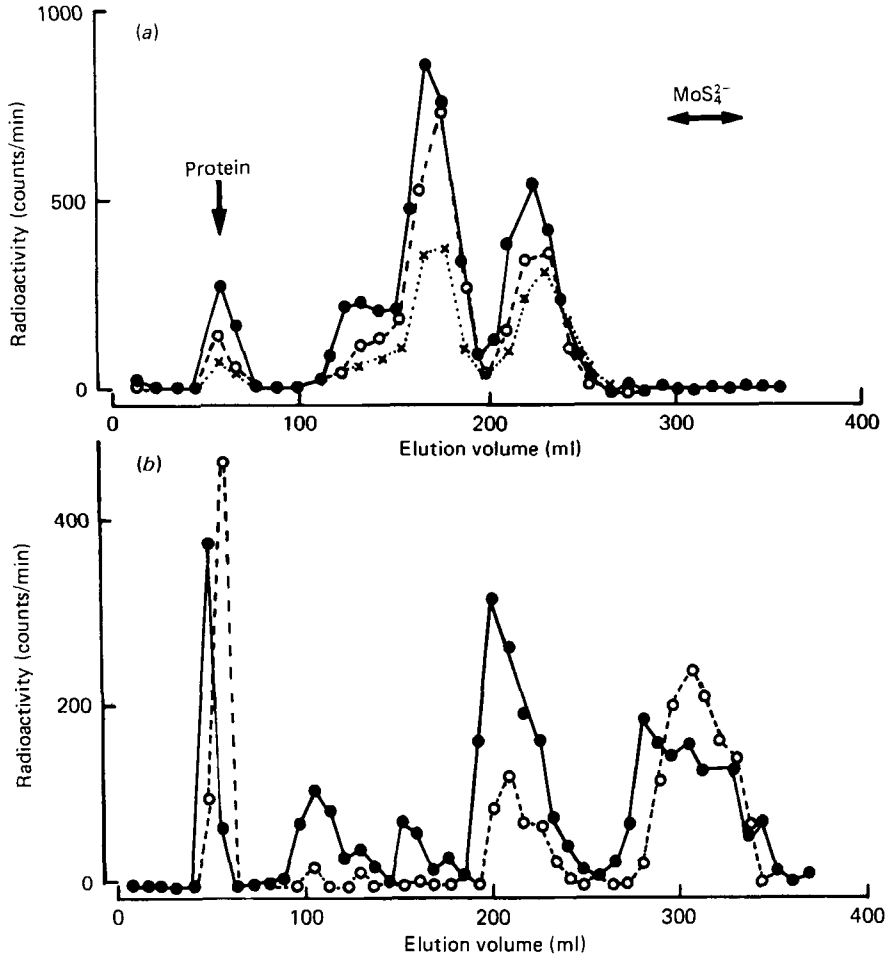

Fig. 3. Sephadex G-25 chromatography (column bed volume 133 ml) of plasma samples (4 ml). (*a*) Samples from sheep B obtained 23·5 h (●——●), 40·5 h (○- -○), and 64·5 h (x··x) after the administration of [^{99}Mo]molybdate (30 mg Mo) via the rumen. All samples were subjected to a preliminary G-25 gel filtration to remove unbound ^{99}Mo before treatment with tetrathiomolybdate (0·8 mg Mo). (*b*) Prefiltered, treated plasma samples obtained 2 h after rumen administration of [^{99}Mo]tetrathiomolybdate (16·5 mg Mo) (●——●) or 5 min after intravenous administration (1·1 mg Mo) (○- -○).

and treated with tetrathiomolybdate before passage through the G-25 Sephadex column (bed volume 133 ml), showed the peaks corresponding to di- and trithiomolybdate. The elution profiles of samples obtained at 23·5, 40·5 and 64·5 h are shown in Fig. 3(*a*). No [^{99}Mo]tetrathiomolybdate is apparent.

Tetrathiomolybdate infusion

Since any [^{99}Mo]tetrathiomolybdate formed might have been subject to specific retention in the rumen or have undergone aquation or oxidation during the displacement procedure in vitro, three control experiments were carried out:

Expt. (*a*) Rumen infusion of ^{99}Mo-labelled tetrathiomolybdate (16·5 mg Mo).

Expt. (*b*) Direct intravenous injection of [^{99}Mo]tetrathiomolybdate (1·1 mg Mo).

Expt. (*c*) Direct addition of [^{99}Mo]tetrathiomolybdate to a plasma sample before G-25 chromatography.

As demonstrated previously (Kelleher *et al.* 1982) rumen administration of tetrathiomolybdate gave predominantly TCA-insoluble ^{99}Mo in plasma, a level of $0{\cdot}5 \pm 0{\cdot}02 \times 10^{-6}$ M between 0·5 and 4 h post-infusion. This is comparable to the levels after molybdate infusion. Some results from Expt. (*a*) are shown in Fig. 3(*b*). ^{99}Mo peaks co-eluted (exactly) with the tetrathiomolybdate used for displacement (between 280 and 350 ml). A [^{99}Mo]trithiomolybdate peak was also apparent; after the rumen infusion this was a major peak; after intravenous administration (Expt. (*b*)) it was less important (Fig. 3(*b*)). The same pattern was observed in all the samples examined, that is 0·5, 1 and 2 h from control Expt. (*a*) and 5 and 15 min samples from Expt. (*b*). It is noteworthy that considerably more radioactivity remained with the protein fraction eluting after 50–60 ml (Fig. 3(*b*)). In the in vitro control, Expt. (*c*) (not shown), only peaks corresponding to residual protein-bound ^{99}Mo and [^{99}Mo]tetrathiomolybdate were apparent.

DISCUSSION

A definitive identification of the ^{99}Mo-compounds from chemical or spectral evidence would be difficult to obtain since the concentrations involved are so low. However, since ^{99}Mo-labelled thiomolybdates synthesized in vitro and administered directly also bind to plasma proteins and decline at the same slow rate in vivo (Mason *et al.* 1980; Mason *et al.* 1982; Kelleher *et al.* 1982) and in these experiments elute in identical positions in vitro, it is likely that the identification is correct. The experiments thus appear to be the first demonstration of thiomolybdate formation and absorption in vivo in sheep.

However, the most potentially controversial aspect is perhaps the failure to detect [^{99}Mo]tetrathiomolybdate in any of the plasma samples examined after [^{99}Mo]molybdate infusion, despite the S supplementation of the diet. Several explanations can be offered. First, that some tetrathiomolybdate was formed but was preferentially retained in the digesta after interaction with, for example, Cu or Fe or by adsorption on to the solid phase. Preliminary experiments in vitro with simple solutions show no difference in the rates at which the spectra of tri- and tetrathiomolybdate disappear with step-wise addition of cupric chloride (J. Letters, unpublished results). In vivo the infusion into the rumen of tri- and tetrathiomolybdate (Kelleher *et al.* 1982) at the same Mo levels produces virtually identical plasma patterns and similar percentage absorptions. In our control Expt. (*a*) [^{99}Mo]tetrathiomolybdate was certainly readily detectable after rumen infusion and the levels of plasma TCA-insoluble ^{99}Mo obtained were comparable to the earlier experiments.

A second possibility is that the tetrathiomolybdate formed may have undergone partial oxidation (or aquation), for example, in the intestine or during passage across the intestinal wall. This was suggested by the experiments reported by Mason *et al.* (1982) and Kelleher *et al.* (1982) and by the present control Expt. (*a*). However, in the control experiment significant quantities did remain, whereas no tetrathiomolybdate was ever detected after molybdate infusion. A third possibility is that tetrathiomolybdate is removed more rapidly from circulation. However, in previous experiments (Mason *et al.* 1982), the rates of decrease of the plasma TCA-insoluble fractions, after infusion of individual thiomolybdates, were not very different, in fact the TCA-insoluble fraction after tetrathiomolybdate infusion appears slightly more persistent. Nevertheless, none of these possibilities can be discounted at this stage since there is so little knowledge of thiomolybdate metabolism in the animal.

Interest has perhaps been concentrated on tetrathiomolybdate since the experiments with rats have shown that tetrathiomolybdate is highly toxic (Mills *et al.* 1978), whereas the other compounds are much less effective. However, as Mason (1981) and Clarke & Laurie (1982)

point out, thiomolybdates are sensitive to acid hydrolysis in the order di- > tri- > tetrathiomolybdate. Thus, it is not clear whether a non-ruminant provides a suitable model for the ruminant, since any compound added to the diet is subjected to a low pH environment immediately post-ingestion. In the ruminant, at least in sheep, there appears to be direct absorption from the near neutral rumen (Kelleher *et al.* 1982).

The presence of [^{99}Mo]trithiomolybdate after rumen infusion with [^{99}Mo]tetrathiomolybdate may be significant since Weber *et al.* (1979) conclude that preparations of thiomolybdates at neutral or near-neutral pH are susceptible to hydrolysis and tend to form equilibrium mixtures of thiomolybdate, molybdate and the different ionized forms of sulphide. While these observations have been criticized (Clarke & Laurie, 1982) on the grounds that their preparations may have been impure initially, some oxidation or equilibration must have occurred in the present experiments. The rate constants of aquation and formation of the different thiomolybdates have been determined by Harmer & Sykes (1980).

We suggest that in these experiments the equilibrium mixture in the rumen may not have contained significant quantities of tetrathiomolybdate and support the view of Clarke & Laurie (1982) that di- and trithiomolybdate may be of more pathological importance than the slower-forming tetrathio ion. The present experiments should be regarded as exploratory and further study should be made over a wider range of dietary Cu, Mo and S levels, particularly where Mo is fed continuously. The techniques described allow this kind of investigation to be undertaken for the compounds in plasma and possibly in the rumen.

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