Identification of thiomolybdates in digesta and plasma from sheep after administration of ^{99}Mo-labelled compounds into the rumen

By J. PRICE, A. MARIE WILL, G. PASCHALERIS* AND J. K. CHESTERS

Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

(*Received 20 October 1986 – Accepted 9 February 1987*)

1. At 16 h after the rapid injection of ^{99}Mo-labelled compounds into the rumen of sheep maintained on dried grass (6·2 mg molybdenum/kg dry matter (DM), 4·3 g sulphur/kg DM), labelled thiomolybdates associated with the digesta solids or bound to plasma macromolecular species were displaced from their carriers in vitro and identified by Sephadex G25 chromatography.

2. After molybdate injection, the thiomolybdates displaced from rumen, duodenal and ileal solids were predominantly the trithio- and tetrathio- species. Dithiomolybdate was present to a relatively minor extent. Trace amounts of di- and trithiomolybdates were detected in the liquid phase of digesta from the duodenum.

3. Whether injected into the rumen as molybdate or tetrathiomolybdate, bound ^{99}Mo appearing in plasma was present mainly as di- and trithio- species. The tetrathio- species appeared in trace amounts in plasma only after tetrathiomolybdate injection, despite its existence almost exclusively in this form in rumen digesta.

4. The present study provides direct evidence for thiomolybdate synthesis within the rumen and indicates that while the effects of thiomolybdates in inhibiting copper absorption are likely to be due to tri- and tetrathiomolybdates, post-absorptive effects on Cu metabolism are probably due to di- or trithiomolybdate.

The adverse effect of increased dietary molybdenum on the utilization of copper by ruminants has been attributed to the formation of thiomolybdates in the rumen (Suttle, 1974; Dick *et al.* 1975). The antagonism, potentiated by dietary sulphur, was envisaged as a progressive reaction of rumen sulphide with molybdate (MoO_4^{2-}) leading to the formation of thiomolybdates (general formula: MoO_nS_{4-n}, where n is 0–3); these would then react with Cu in the gut reducing its availability and, if absorbed, interfere with Cu metabolism in the tissues.

Evidence for rumen synthesis of thiomolybdates has been accumulating. Mills *et al.* (1978) reported that the characteristic absorption spectrum of tetrathiomolybdate was detectable in the liquid phase of strained rumen contents incubated in vitro with Mo and S compounds providing 10 mg Mo/l and 20–50 mg S/l. Tetrathiomolybdate has also been detected spectrophotometrically (El-Gallad *et al.* 1983) in the liquid phase of bovine rumen fluid after addition of molybdate to the diet to provide 100 mg Mo/kg dry matter (DM). Using a different approach, Bray *et al.* (1982) reported a substantial conversion of MoO_4^{2-} to tri- and tetrathiomolybdates in an artificial rumen system constantly infused with a solution containing 4–12 mg Mo/l. The thiomolybdates remained predominantly in the liquid phase of the digesta. However, in these investigations the concentrations of Mo in the diet or incubated rumen suspensions greatly exceeded those normally found when Mo inhibits utilization of Cu by grazing ruminants. Furthermore, from studies on the relative availability of Cu present in digesta fractions from sheep maintained on a ration containing 11·6 mg Mo/kg DM, Price & Chesters (1985) concluded that the factors inhibiting Cu utilization were associated mainly with the digesta solids.

The extent to which molybdate is chemically modified at rumen Mo and S concentrations typical of those encountered in field cases of Mo-induced Cu deficiency remains uncertain. From studies in vitro, Clarke & Laurie (1979) predicted that rumen conditions would favour the formation of di- and trithiomolybdates rather than tetrathiomolybdate.

* Present address: Ministry of Agriculture Veterinary Laboratory, Erythrou Stavros 38, Kavala, Greece.

Although the appearance of di- and trithiomolybdates in plasma from sheep (Mason *et al.* 1982) and cattle (Hynes *et al.* 1985) given $^{99}MoO_4^{2-}$ as a single dose into the rumen would tend to support this view, the absence of tetrathiomolybdate from plasma could also be explained by its failure to be absorbed.

The present study was undertaken to identify the Mo species present in the solid and liquid phases of digesta of sheep maintained on a ration containing less than 10 mg Mo/kg DM and to relate the findings to the forms of Mo appearing in the plasma. Studies were also carried out to ascertain whether or not tetrathiomolybdate is absorbed following its introduction into the rumen. Since it has been postulated that Mo inhibits Cu absorption through formation of insoluble 'Cu–thiomolybdates', the effect of Cu on Mo species in rumen digesta was also investigated in vitro.

MATERIALS AND METHODS

Experimental animals

Four male castrate sheep weighing 65–75 kg were cannulated in the rumen (sheep no. 1), the rumen and duodenum approximately 100 mm beyond the pylorus (sheep nos. 2 and 3) or rumen and ileum approximately 200 mm before the ileo-caecal junction (sheep no. 4). They were maintained on dried grass (1·2 kg/d) sprayed with a solution of ammonium molybdate to increase the Mo content by 5 mg Mo/kg DM. The dried grass used throughout these investigations contained (/kgDM) 6 mg Cu, 1·2 mg Mo and 4·3 g S and was given at 09·00 and 17·00 hours. The ^{99}Mo-labelled compounds (molybdate, 0·2–2 mCi ^{99}Mo or tetrathiomolybdate, 0·2 mCi ^{99}Mo) were injected rapidly in a 50 ml volume directly into the rumen at 17·00 hours.

^{99}Mo compounds

The ^{99}Mo was obtained from ^{99m}Tc generators (Amersham International plc, Amersham, Bucks) which contain the Mo isotope adsorbed on a small column of alumina. After eluting the daughter isotope ^{99m}Tc from the column in 20 ml saline (9·0 g sodium chloride/l), $^{99}MoO_4^{2-}$ was displaced from the alumina in 15–20 ml 0·1 M-sodium hydroxide. Portions of this stock solution (specific activity approximately 20 mCi/mg Mo, 30 mg Mo/l) were diluted to 50 ml with distilled water and the pH adjusted to 7 with hydrochloric acid before administration to sheep.

^{99}Mo-labelled tetrathiomolybdate was prepared by passing hydrogen sulphide gas through a solution of sodium molybdate (10 ml, 20 mg Mo/l) containing 0·2 mCi $^{99}MoO_4Na_2$ in 0·2 M-sodium phosphate buffer, pH 6, for 1 h. Excess H_2S was then removed by passing nitrogen through the solution for 5 min. Labelled tetrathiomolybdate was purified immediately before administration to sheep by passage through a column of Sephadex G25 (22 × 150 mm) using Tris–HCl buffer (10 mM, pH 7·6) as eluant. The absence of di- and trithiomolybdates from fractions containing tetrathiomolybdate was verified spectrophotometrically using the values of Clarke & Laurie (1979).

Blood samples

Preliminary studies indicated that maximal ^{99}Mo activities were attained in plasma 16–20 h after the rapid injection of labelled Mo compounds into the rumen. In subsequent experiments samples of jugular blood were therefore obtained in heparinized tubes 16 h post-infusion and the proportion of plasma ^{99}Mo insoluble in trichloroacetic acid (50 g/l; TCA) determined by difference after counting whole and deproteinized plasma. Free ^{99}Mo species and those bound to macromolecular material in plasma (Kelleher *et al.* 1983) were separated by gel filtration on a Sephadex G25 column (22 × 350 mm) using a modification

of the technique described by Mason *et al.* 1982). Plasma (3 ml) was eluted with Tris–HCl buffer (10 mM, pH 7·6) and fractions of approximately 4 ml collected. The macromolecular species in plasma, detected by measurement of absorbance at 280 nm, eluted mainly in fractions nos. 5 and 6, fraction no. 6 being retained for identification of bound ^{99}Mo species.

Digesta samples

Samples of rumen, duodenal and ileal digesta (200 ml) were obtained 16 h after dosing and the rumen samples strained through a single layer of coarse muslin gauze to obtain a fluid fraction representative of the digesta flowing out of the reticulo-rumen. Portions of digesta (30 ml) were fractionated by centrifugation at 2200 ***g*** for 10 min, the supernatant fraction removed and centrifuged at 30000 ***g*** for 1 h and the distribution of ^{99}Mo activity between the two centrifuged pellets and the liquid phase determined. Because virtually all the label associated with the solid phase of digesta was found in the 2200 ***g*** pellet (except in ileal samples), this fraction was isolated from strained rumen fluid (10 ml), duodenal and ileal digesta (5 ml) and used in subsequent studies. The pellets were washed three times with Tris–HCl buffer (10mM, pH 7·6) before displacement and identification of bound ^{99}Mo species. Losses of radioactivity which occurred in washing the 2200 ***g*** pellets were low with rumen (< 7%) and duodenal (< 2%) samples but substantial (18%) with ileal samples.

Identification of Mo species

The oxythio- and thio- anions of Mo may be separated by chromatography on Sephadex G25 columns (Zumft, 1978) and identified from their characteristic elution profile (Mason *et al.* 1982). Using the technique applied to plasma by Mason *et al.* (1982), ^{99}Mo species were displaced from plasma macromolecular material and digesta solids by exchange with unlabelled tetrathiomolybdate (ammonium salt) prepared by the method of Tridot & Bernard (1962) and purified in Tris–HCl buffer (10 mM, pH 7·6) by Sephadex G25 column chromatography. Tetrathiomolybdate (1 ml, 0·5 mg Mo/ml) was added to 4 ml of the eluate containing bound ^{99}Mo from plasma or to washed digesta solids suspended in 4 ml Tris–HCl buffer. The treated plasma fraction was chromatographed immediately whereas 0·5 h was allowed for exchange with digesta solids before centrifugation (30000 ***g***, 1 h) and chromatography of the supernatant fraction on Sephadex G25 (columns 22 × 470–500 mm). The liquid phase (3 ml) from digesta was chromatographed untreated or 0·5 h after addition of unlabelled tetrathiomolybdate. Elution was with Tris–HCl buffer (10 mM, pH 7·6) at a flow rate of 20 ml/h and fractions of approximately 3·5 ml were collected. Although the pH of the liquid phase from digesta varied with the sampling site in the digestive tract, all samples were eluted with buffer at pH 7·6 to minimize hydrolysis and provide separation of the thiomolybdates, if present.

The columns were calibrated with MoO_4^{2-}, paramolybdate ($Mo_7O_{24}^{6-}$) and the thiomolybdates, including monothiomolybdate. Di- ($MoO_2S_2^{2-}$), tri- ($MoOS_3^{2-}$) and tetrathiomolybdate (MoS_4^{2-}) were prepared by passing H_2S through solutions of sodium molybdate in Tris–HCl buffer (10 mM, pH 7·6, 20 mg Mo/l). Monothiomolybdate, because of its instability and the ease with which reaction of molybdate with HS^- proceeds to dithiomolybdate, was prepared as an equilibrium mixture of mono- and dithiomolybdates by introducing a small quantity of H_2S into the air space above the molybdate–buffer solution (100 mg Mo/l) in a 10 ml tube and inverting the stoppered tube to mix the reactants. The void volume (V_o) for each column was determined as the elution volume of bovine serum albumin and the volume of the stationary phase (V_s) as the volume difference between V_o and the elution volume of the potassium ion added as potassium chloride (0·2 ml, 2 M) to Mo standards (5 ml) before chromatography. Because columns of differing packed bed

Table 1. *Distribution of radioactivity in digesta from sheep 16 h after rapid injection of $^{99}MoO_4^{2-}$ into the rumen*
(Mean values and standard deviations for single determination/sheep; no. of sheep given in parentheses)

	Distribution of ^{99}Mo (% of total activity in digesta)				
	Rumen (*n* 4)		Duodenum (*n* 2)		
Digesta fraction	Mean	SD	Mean	SD	Ileum (*n* 1)
2200 *g* pellet	68	7	87	4	52
30000 *g* pellet	6	1	12	3	14
30000 *g* supernatant fraction	26	2	1	1	34

volume were used, the elution behaviour of each Mo species was defined by its distribution coefficient, K_d, calculated as $(V_e - V_o)/V_s$, where V_e is the elution volume of Mo species in the calibration and experimental samples.

Unlabelled thiomolybdates used in column calibration were identified by their electronic spectra before and after elution (Aymonino *et al.* 1969; Clarke & Laurie, 1979) while the elution positions of molybdate and paramolybdate were determined by graphite-furnace atomic-absorption spectrophotometry (Model no. 9000; Pye Unicam) and that of K^+ by flame-emission spectrophotometry (Model no. A460; Perkin Elmer).

RESULTS

^{99}Mo species associated with digesta solids after $^{99}MoO_4^{2-}$ injection into the rumen

In rumen, duodenal and ileal digesta a major proportion of the label was associated with the solids, mainly the 2200 *g* pellet, 16 h after injection of molybdate into the rumen (Table 1). After displacement from the washed 2200 *g* solids a number of Mo species was separated by Sephadex G25 chromatography. A typical elution profile for Mo associated with the rumen 2200 *g* solids (sheep no. 1) is shown in Fig. 1. The radioactivity eluted in five peaks, three of which had distribution coefficients greater the unity, indicating retardation by the column, and were identified as di-, tri- and tetrathiomolybdates. The identities of the Mo species, K_d 0·08 and 0·77, eluting before K^+ remain unknown, although the broad shoulder (K_d 0·86) of the latter peak appeared in the position of monothiomolybdate. A substantial proportion of ^{99}Mo was not displaced from the previously described pellet by the single treatment with tetrathiomolybdate. Attempts to solubilize further ^{99}Mo by repeating the displacement procedure after ultrasonic disintegration of the treated pellet resulted in an increase in displacement of the label from 51 to 65%.

Because the elution profile (not shown) of Mo species displaced from the rumen 30000 *g* pellet (sheep no. 1) was similar qualitatively and quantitatively to that of the 2200 *g* pellet from the same sheep, and made only a minor contribution to the total radioactivity associated with the rumen solids in all sheep, no further studies were carried out on this fraction.

When $^{99}MoO_4^{2-}$ was added directly to unlabelled 2200 *g* solids obtained from sheep no. 1 before ^{99}Mo injection and the solution after displacement with tetrathiomolybdate was chromatographed, labelled di-, tri- and tetrathiomolybdates were absent from the elution profile (Fig. 1). While this finding demonstrates that the thiomolybdates ($K_d > 1$) identified after ^{99}Mo injection had originated in the rumen and were not artefacts arising from

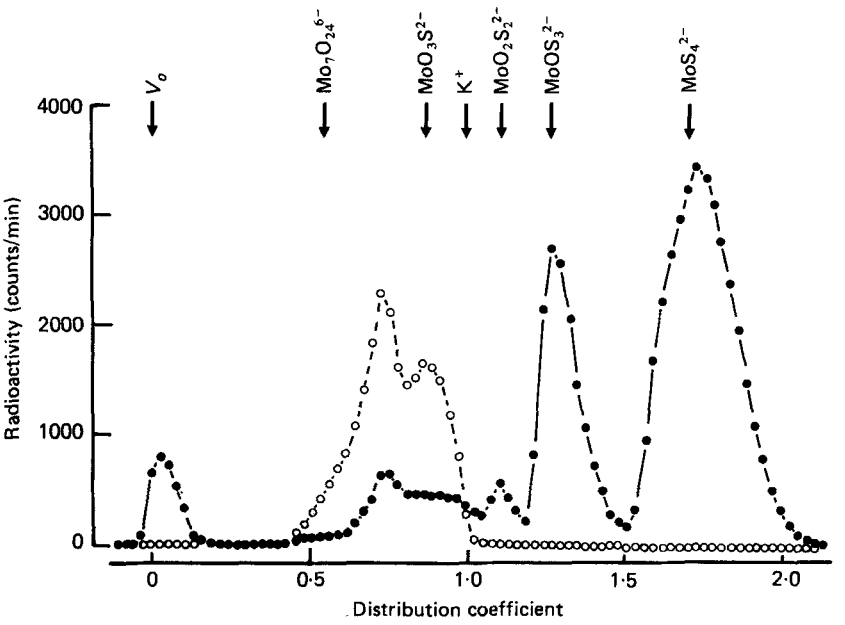

Fig. 1. Sephadex G25 chromatography (column bed volume 180 ml) of ^{99}Mo species displaced from washed 2200 *g* pellets isolated from rumen fluid (10 ml, sheep no. 1). ^{99}Mo species were displaced from the solids by treatment with unlabelled tetrathiomolybdate 0·5 h after addition of $^{99}MoO_4^{2-}$ (5 μCi) directly to the pellet (○---○) or 16 h after injection of $^{99}MoO_4^{2-}$ (1·8 mCi) into the rumen (●—●). V_o, void volume.

reaction in vitro between $^{99}MoO_4^{2-}$ and MoS_4^{2-} during the displacement procedure, labelled molybdate added to the pellet did not elute in the position expected for this anion. In calibration runs both MoO_4^{2-} and its polymeric form $Mo_7O_{24}^{6-}$ eluted in the same position (K_d 0·54) whereas ^{99}Mo added to the pellet as molybdate eluted in two poorly separated peaks (K_d 0·77 and 0·86) after displacement.

The elution profiles for radioactivity displaced from rumen-, duodenal- or ileal-washed 2200 *g* solids were similar, but the proportions of individual ^{99}Mo species varied characteristically (Table 2). A proportion of the activity from all digesta samples eluted as a peak (K_d 0·76–0·78) with a broad shoulder similar to that observed when $^{99}MoO_4$ was added to rumen solids in vitro. The identity of the Mo species in this fraction remains uncertain, but activity eluted in the shoulder has been tentatively attributed to monothiomolybdate. All activity eluting after K^+ has been attributed to thiomolybdates and appeared in three well-defined peaks. While dithiomolybdate was present consistently as a minor component in all digesta, the proportions of tri- and tetrathiomolybdates varied considerably between sheep and sampling site in the digestive tract. In sheep nos. 2 and 3, cannulated in the rumen and duodenum, the proportion of activity displaced as trithiomolybdate was markedly lower with duodenal than rumen solids while the proportion of tetrathiomolybdate remained unchanged. The decrease in trithiomolybdate was also accompanied by a substantial increase of radioactivity in the combined peak and shoulder eluting just before K^+. The possibility that these differences resulted from the fall in pH of digesta on passage from rumen to duodenum was investigated in vitro by applying the usual displacement and chromatographic procedures to 2200 *g* centrifuged pellets prepared from untreated (pH 6·9) and acidified (pH 2·9) rumen fluid from sheep no. 2. Adjustment of pH precipitated and

Table 2. *^{99}Mo species separated by chromatography on Sephadex G25 after displacement from the washed 2200 g pellets from rumen, duodenal and ileal digesta by unlabelled tetrathiomolybdate*

Mobility of ^{99}Mo fraction (K_d)	Identity of ^{99}Mo species	^{99}Mo activity in column fractions (% of activity displaced)						
		Rumen				Duodenum		Ileum
		1*	2	3	4	2	3	4
< 0·10	Unknown	5·7	1·3	4·9	3·1	11·2	5·3	0·0
0·75–0·95	Unknown	12·3	25·4	11·7	17·3	47·4	37·6	14·1
1·10	Dithiomolybdate	3·8	4·7	3·9	4·5	3·5	2·4	5·0
1·28	Trithiomolybdate	23·1	51·0	62·0	28·8	21·4	31·6	37·7
1·71	Tetrathiomolybdate	55·1	17·6	17·5	46·2	16·5	23·1	43·2
		^{99}Mo activity displaced (% of activity in 2200 g solids)						
		51	56	52	41	55	63	51

K_d, distribution coefficient. $K_d = (V_e - V_o)/V_s$, where V_e is the elution volume of Mo species, V_o is the void volume of the column and V_s is the volume of the stationary phase calculated as the elution volume of the potassium ion minus V_o.

* Sheep number.

Table 3. *Effect of lowering the pH of strained rumen fluid on the proportions of ^{99}Mo species displaced from the 2200 g pellet*

		Strained rumen fluid		Duodenal digesta
pH		6·9	2·9*	2·9
^{99}Mo activity in 2200 g solids (% of activity in whole digesta)		47	74	84
^{99}Mo activity displaced (% of activity in 2200 g solids)		55	61	55
Mobility of ^{99}Mo fraction (K_d)	Identity of ^{99}Mo species	^{99}Mo activity in column fractions (% of activity displaced)		
< 0·10	Unknown	1·3	8·8	11·2
0·75–0·95	Unknown	25·4	42·7	47·4
1·10	Dithiomolybdate	4·7	2·9	3·5
1·28	Trithiomolybdate	51·0	19·0	21·4
1·71	Tetrathiomolybdate	17·6	18·6	16·5

K_d, distribution coefficient. $K_d = (V_e - V_o)/V_s$, where V_e is the elution volume of Mo species, V_o is the void volume of the column and V_s is the volume of the stationary phase calculated as the elution volume of the potassium ion minus V_o.

* pH adjusted by addition of hydrochloric acid before isolation of the 2200 g centrifugal fraction; digesta from sheep no. 2.

coagulated the suspended solids and increased the proportion of radioactivity isolated in the 2200 g pellet (Table 3). Although the proportion of activity displaceable from the pellet showed little change, acidification decreased exchangeable trithiomolybdate and increased the proportion of Mo species eluting within the range K_d 0·75–0·95. Acidification in vitro thus mimicked the changes occurring between rumen and duodenum in vivo.

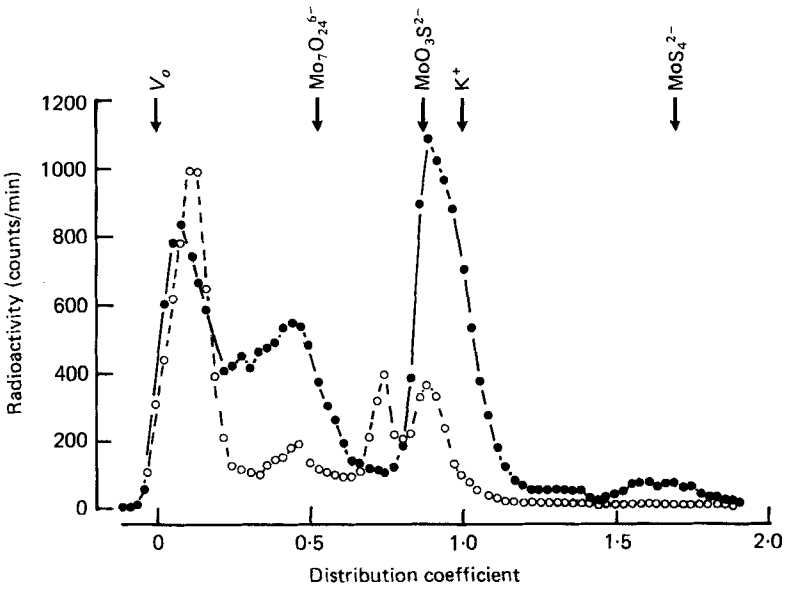

Fig. 2. Sephadex G25 chromatography (column bed volume 190 ml) of ^{99}Mo species in the liquid phase (30000 *g* supernatant fraction) from rumen fluid (sheep no. 1) 16 h after injection of $^{99}MoO_4^{2-}$ (2 mCi) into the rumen. The liquid phase was chromatographed untreated (○---○; 3 ml) and on a later occasion, after addition of unlabelled tetrathiomolybdate (●—●; 5 ml). V_o, void volume.

Mo species in the liquid phase of digesta after $^{99}MoO_4^{2-}$ injection into the rumen

When chromatographed on Sephadex G25, Mo species in rumen 30000 *g* supernatant fraction (3 ml) eluted with distribution coefficients less than unity, indicating the absence of free di-, tri- or tetrathiomolybdate anions; the elution profile shown (Fig. 2) is typical of those obtained for samples from four sheep. ^{99}Mo eluted as four distinct peaks with K_d values of 0·10, 0·48, 0·75 and 0·89, the last of these being tentatively identified as monothiomolybdate. To investigate the possibility that thiomolybdates were present in a bound form, the same sheep was infused with $^{99}MoO_4^{2-}$ on a second occasion and displacement attempted by addition of unlabelled tetrathiomolybdate to 5 ml of the rumen liquid phase. Of the thiomolybdates only tetrathiomolybdate was detected on subsequent chromatography and this comprised approximately 3% of the radioactivity eluted (Fig. 2). Apart from the absence of the peak at K_d 0·77, the elution profile was similar to that of untreated samples.

A typical elution profile for the untreated liquid phase from duodenal digesta (pH 2·9) is shown in Fig. 3 and demonstrates the presence of free trithiomolybdate and a trace of dithiomolybdate, the two species together comprising 35% of the soluble ^{99}Mo but only 0·5% of the total radioactivity in duodenal digesta. The remaining Mo eluted as a poorly defined, broad peak close to the void volume of the column. When the acid pH of the liquid phase from duodenal digesta was neutralized before chromatography, di-, tri- and tetrathiomolybdate were not detected, the elution profile bearing close similarities to that of the untreated liquid phase from rumen digesta. Free thiomolybdates were not detected in the liquid phase from ileal digesta (pH 8·1).

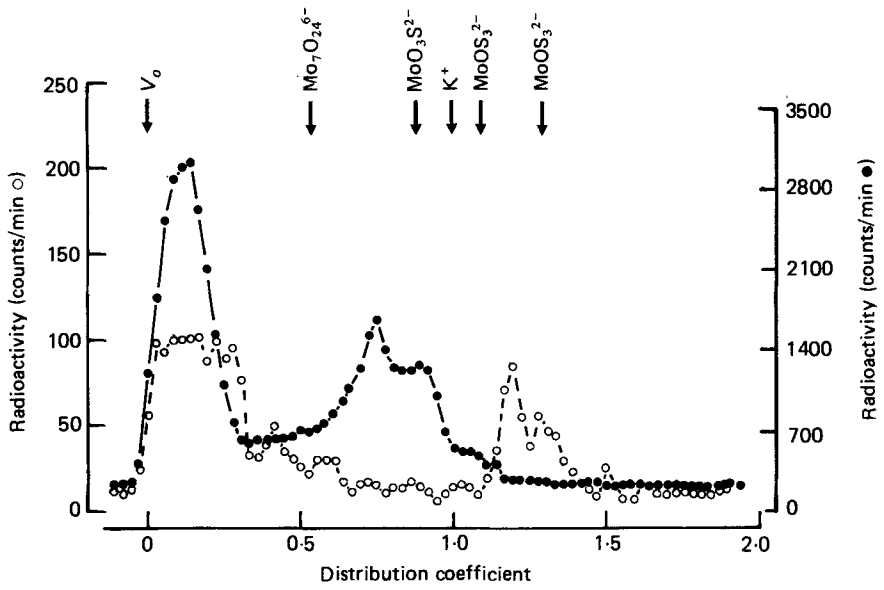

Fig. 3. Sephadex G25 chromatography (column bed volume 190 ml) of ^{99}Mo species in the liquid phase (3 ml, 30000 *g* supernatant fraction, pH 2·9) from duodenal digesta (sheep no. 2) 16 h after injection of $^{99}MoO_4^{2-}$ (0·2–1·9 mCi) into the rumen. The liquid phase was chromatographed untreated (○---○; 0·2 mCi infused) and on a later occasion after adjustment to pH 7 with sodium hydroxide (●—●; 1·9 mCi infused). V_o, void volume.

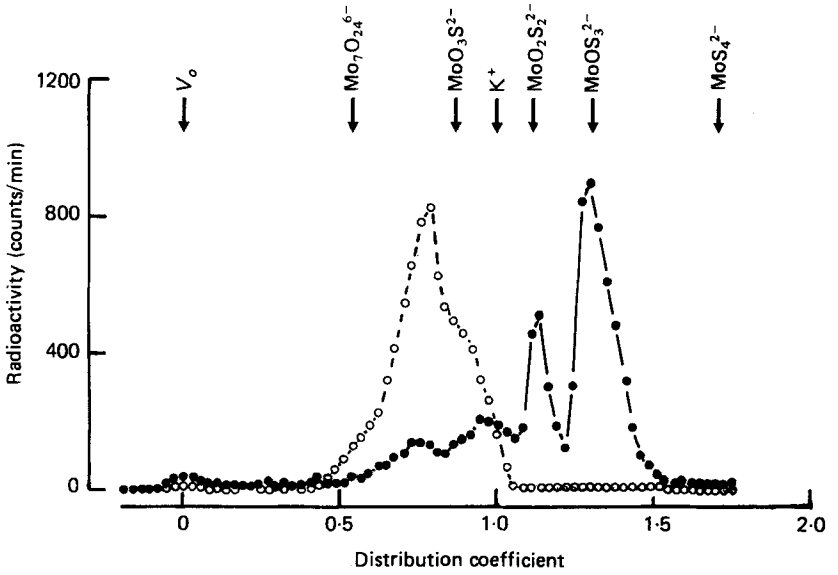

Fig. 4. Sephadex G25 chromatography (column bed volume 190 ml) of ^{99}Mo species displaced from fraction no. 6 proteins isolated by preliminary gel filtration of plasma (3 ml) from sheep no. 1. ^{99}Mo species were displaced by treatment with unlabelled tetrathiomolybdate immediately after direct addition of $^{99}MoO_4^{2-}$ (2·5 μCi) to fraction no. 6 from samples obtained before injection (○---○) or 16 h after injection of $^{99}MoO_4^{2-}$ (1·8 mCi) into the rumen (●—●). V_o, void volume.

Table 4. *^{99}Mo species displaced from the 2200 g pellet from rumen digesta and from plasma after rapid injection of ^{99}Mo-labelled tetrathiomolybdate into the rumen*

(Mean values and standard deviations for three sheep)

Mobility of ^{99}Mo fraction (K_d)	Identity of ^{99}Mo species	^{99}Mo activity (% of total eluted)			
		Rumen 2200 *g* solids		Whole plasma	
		Mean	SD	Mean	SD
< 0·10	Unknown	1·4	0·9	18·6	3·3
0·75–0·95	Unknown	6·1	3·3	48·0	4·4
1·10	Dithiomolybdate	1·7	0·7	18·8	7·1
1·28	Trithiomolybdate	9·0	0·7	11·8	1·0
1·71	Tetrathiomolybdate	81·8	5·5	2·3	1·1
		^{99}Mo activity displaced (% of activity in 2200 *g* solids)			
		55	11		

K_d, distribution coefficient. $K_d = (V_e - V_o)/V_s$, where V_e is the elution volume of Mo species, V_o is the void volume of the column and V_s is the volume of the stationary phase calculated as the elution volume of the potassium ion minus V_o.

Mo species in plasma after injection of $^{99}MoO_4^{2-}$ into the rumen

At 16 h after injection, 56 and 31 % of ^{99}Mo in plasma from sheep nos. 1 and 4 respectively were insoluble in TCA. After displacement with unlabelled tetrathiomolybdate, the ^{99}Mo compounds bound to the macromolecular components of plasma (fraction no. 6 isolated by preliminary gel filtration) separated into four peaks when chromatographed on Sephadex G25 (Fig. 4; sheep no. 1). Dithiomolybdate comprised 18 and 17 % and trithiomolybdate 51 and 48 % of the bound radioactivity in plasma from sheep nos. 1 and 4 respectively, with the remainder appearing in two poorly defined peaks with K_d of 0·76 and 0·95. Radioactivity eluting at the void volume of the column was less than 3 % of the total, indicating rapid and virtually complete displacement of ^{99}Mo species by unlabelled tetrathiomolybdate added immediately before chromatography.

When $^{99}MoO_4^{2-}$ was added in vitro to unlabelled fraction no. 6 from plasma and unlabelled tetrathiomolybdate added, radioactivity eluted in an unidentified peak (K_d 0·79) with a broad shoulder (K_d 0·85–0·95) (Fig. 4). On the basis of their K_d, these Mo species were identical to those eluted after addition of $^{99}MoO_4^{2-}$ to the unlabelled 2200 *g* pellet from rumen digesta.

^{99}Mo-labelled tetrathiomolybdate injection into the rumen

Preformed ^{99}Mo-labelled tetrathiomolybdate was injected into the rumen of three sheep and chromatographic separations were carried out on ^{99}Mo species displaced from both rumen 2200 *g* pellets and whole plasma treated with unlabelled tetrathiomolybdate (Table 4). The ^{99}Mo species displaced from the rumen solids eluted predominantly as tetrathiomolybdate but only traces of this species were found in plasma.

Effect of Cu on rumen ^{99}Mo-labelled thiomolybdates

Since Cu^{2+} reacts with the thiomolybdates in vitro forming Cu (I) thiomolybdate complexes which are insoluble in aqueous media (Clarke & Laurie, 1982), the possibility that such compounds may form in rumen digesta was investigated. The washed ^{99}Mo-labelled

2200 *g* pellets (two) from 4 ml strained rumen fluid (sheep no. 2) were suspended in a solution of $CuSO_4.5H_2O$ (5 ml, 0·5 mg Cu/l distilled water, pH 5·9) giving a fivefold molar excess of added Cu over native Mo. After allowing 0·5 h for reaction between the added Cu and ^{99}Mo-labelled thiomolybdates associated with the digesta solids, 84 (SD 2·3)% of the Cu was found to have been removed from solution. Addition of unlabelled tetrathiomolybdate to untreated (two) or Cu-treated (two) pellets displaced 52 (SE 1·7) and 50 (SE 4·6)% of the radioactivity from the solids respectively. The elution profiles of displaced ^{99}Mo species did not alter qualitatively or quantitatively with Cu treatment. However, application of the displacement procedure to the ^{99}Mo-labelled Cu (I) tetrathiomolybdate complex, a crimson-red insoluble complex (CuS_2MoS_2Cu) formed by reaction of aqueous tetrathiomolybdate with an excess of cuprous iodide dissolved in 2 M-ammonium hydroxide, revealed that the $^{99}MoS_4^{2-}$ core in duplicate samples of the complex was completely exchangeable under the normal displacement conditions.

DISCUSSION

The present study provides the first direct evidence for the rumen synthesis of thiomolybdates under dietary conditions similar to those encountered in field cases of Mo-induced Cu deficiency. Calculations based on values from Tables 1 and 2 indicate that approximately 30% of Mo in strained rumen fluid was present, bound to the solid phase, as di-, tri- and tetrathiomolybdates. These species were not detected either in the free or bound state in the liquid phase from rumen digesta. Since unpublished observations in this laboratory have indicated that tetrathiomolybdate, the most stable of the thiomolybdate series, is rapidly hydrolysed when added in low concentration to the isolated liquid phase from rumen fluid or to potassium cyanide (10 g/l) extracts of whole rumen fluid, the present results would suggest that association with the digesta solids confers stability on the thiomolybdates.

In addition to those thiomolybdates positively identified, a further three Mo fractions were evident in the elution profiles of the untreated liquid phase and after displacement from the solid phase. One of these (K_d 0·86) was tentatively identified from its elution position as monothiomolybdate but the extremely low concentration present precluded a definitive identification from chemical or spectral evidence. Because of its instability in vitro the chemical and physical properties of this species have been little studied and its importance in biological systems is unknown.

Differences in the proportions of Mo species from rumen and duodenal solids may be attributed to the fall in pH of the digesta on passage through the abomasum. The persistence of bound tetrathiomolybdates is not unexpected as the susceptibility of the free ionic species to hydrolysis is known to decrease $MoO_2S_2^{2-} > MoOS_3^{2-} > MoS_4^{2-}$ (Harmer & Sykes, 1980). Even though it was bound to the solids, the relative proportion of trithiomolybdate was substantially lower in duodenal digesta, presumably because it had been converted to the Mo species eluting ahead of K^+. The appearance of di- and trithiomolybdates in the liquid phase of duodenal digesta and their disappearance on increasing the pH is at variance with the known sensitivity of these species in acid medium. However, Chesters *et al.* (1985) also observed the formation of dithiomolybdate in KCN extracts of rumen fluid after reducing the pH to 5·5. Although hydrolysis of bound trithiomolybdate in the present study would release HS^-, it seems improbable that this could have occurred to an extent sufficient to maintain detectable levels of di- and trithiomolybdate in the liquid phase. A satisfactory explanation for these observations must await further investigation.

The appearance in plasma of a TCA-insoluble Mo fraction and protein-bound di- and trithiomolybdates but not tetrathiomolybdate after infusion of molybdate into the rumen

is in agreement with the findings of Mason *et al.* (1982) and Hynes *et al.* (1985). However, the appearance of tetrathiomolybdate in plasma after the injection of this species into the rumen, but not after injection of molybdate, led Mason *et al.* (1982) to conclude that tetrathiomolybdate was not formed in significant quantities in the rumen. In contrast, tetrathiomolybdate was barely detectable in plasma after its infusion into the rumen in the present study, despite its presence as the predominant Mo species in the digesta solids. The most likely explanation for these apparently conflicting findings may lie in differences in the quantity of tetrathiomolybdate administered. In those studies where tetrathiomolybdate absorption has been demonstrated directly in ruminants (Mason *et al.* 1980; Kelleher *et al.* 1983), the daily intake of Mo was not only atypically high, but completely in the form of tetrathiomolybdate. At substantially lower Mo intakes, as in the present study and that of Suttle & Field (1983), tetrathiomolybdate was poorly absorbed. Since this species was absent from the liquid phase of duodenal digesta the previously described observations would suggest that as the concentration of tetrathiomolybdate increases in digesta, the binding capacity of the solids becomes saturated resulting in the appearance of tetrathiomolybdate in the liquid phase from which it may be absorbed.

Recent studies (Price & Chesters, 1985) have shown that available Cu in digesta is associated mainly with the solid phase and that it is from this phase that the greatest loss of available Cu occurs on increasing dietary Mo intake. Although the formation of thiomolybdates in the rumen and their persistence in association with the digesta solids throughout the small intestine has been demonstrated, the mechanisms involved in the inhibition of Cu absorption by such compounds remain unknown. The failure to demonstrate an effect of Cu on either the total ^{99}Mo or the relative proportions of individual ^{99}Mo species displaced from rumen solids does not preclude the presence of Cu–thiomolybdate complexes in view of the demonstration that the ^{99}Mo–S core in one such complex was freely exchangeable with the displacing tetrathiomolybdate anion. However, the latter finding suggests it is improbable that Cu–thiomolybdate complexes could account for the fraction of ^{99}Mo (23 % of the total activity in whole rumen fluid) not displaced from the solids, particularly since Chesters *et al.* (1985) reported that a similar percentage of Mo was resistant to extraction with cyanide, a reagent shown to solubilize the Mo–S core from authenticated insoluble Cu–thiomolybdates.

While di-, tri- and tetrathiomolybdates are known to form a range of complexes with Cu in vitro (Nicholson, 1984), the nature and extent of formation of non-absorbable Cu–thiomolybdate complexes in vivo has yet to be determined. Although the findings of the present study suggest that attention should be focused primarily on the tri- and tetrathiomolybdates in the gut, there are indications that the latter may be the more important in inhibiting Cu absorption. Thus Bremner *et al.* (1982) not only failed to inhibit ^{64}Cu absorption in the rat by addition of di- or trithiomolybdate to the diet, but also failed to diminish their systemic effects by increasing dietary Cu intake. In contrast Mills *et al.* (1981) reported that additional dietary Cu reduced the systemic effects of tetrathiomolybdate in the rat by inhibiting its absorption, probably through formation of a non-absorbable Cu–thiomolybdate complex. In sheep given di- or tetrathiomolybdate in a low-S diet, Cu absorption was reduced only by tetrathiomolybdate (Suttle & Field, 1983); this difference was however not apparent when dietary S intake was increased. This latter observation could be explained by a shift in the equilibrium between the two Mo species in digesta towards tetrathiomolybdate as S intake and consequently rumen sulphide concentration (Grace & Suttle, 1979) is increased. We therefore suggest that further study should be made of the relation of dietary S intake and rumen sulphide generation to the formation of individual thiomolybdates in the rumen in view of the apparent differences in their subsequent interaction with Cu before and after absorption.

G. P. was in receipt of financial support from the International Atomic Energy Agency, Vienna.

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Printed in Great Britain