The role of organic sulphur in the copper–molybdenum–S interrelationship in ruminant nutrition

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1. The ability of organic and inorganic sulphur to influence the copper and molybdenum metabolism of sheep was compared in a series of three 2 × 2 factorial design experiments. In each experiment, four groups of five to seven hypocupraemic ewes were repleted with a basal diet supplemented with 6 mg Cu/kg and containing S and Mo at one of two concentrations, 1 or 4 g S and 0·5 or 4·5 mg Mo/kg respectively. Sodium sulphate (Expt 1), methionine (Expt 2) or cysteine (Expt 3) were used as the S sources. Cu and Mo concentrations in plasma were estimated in each experiment and in Expt 3 the concentrations of Cu in liver and Mo in urine were also estimated.

2. The effects of the three S sources on Cu and Mo metabolism were similar. Repletion of the plasma Cu pool was unaffected by Mo alone, reduced by S alone and totally inhibited by Mo + S. Plasma Mo was greatly increased by Mo supplements, slightly decreased by S supplements and unaffected by Mo and S supplements given together.

3. In Expt 3 the treatments were found to affect urinary Mo and plasma Mo in a similar manner; S prevented dietary Mo from increasing Mo excretion. The only group to show a significant repletion of the liver Cu pool was that given Mo alone.

4. Supplementation of the diet with organic S significantly reduced the within-treatment variation in plasma Cu and Mo, liver Cu and urinary Mo.

5. It is suggested that variations in dietary S and Mo within the normal range for herbage affect the Cu and Mo metabolism of the grazing animal, and that total S rather than inorganic S is the more useful measurement in the context of the Cu–Mo–S interrelationship.

The discovery by Dick (1953*a*) that dietary molybdenum inhibited the storage of copper in the liver of sheep revealed a classic example of the complex interrelationships which can exist between nutrients. Dick (1953*b*) subsequently found a further factor, present in lucerne hay but not in oat hay, which potentiated the Cu–Mo antagonism. The factor was present in lucerne ash and its effects were qualitatively simulated by sodium sulphate. Dick therefore concluded that inorganic SO_4 was the additional factor involved in the Cu–Mo antagonism. In recent field and experimental studies of the Cu–Mo–sulphur interrelationship in ruminant nutrition, emphasis was also given to the inorganic S component in the ruminant diet (Bingley & Anderson, 1972; Thornton, Kershaw & Davies, 1972; Todd, 1972). Further evidence that SO_4 is still considered to be the crucial third factor in the interaction is evident from the recent review by Underwood (1972).

Organic S provides most of the S in herbage (70%) (Hartmans & Bosman, 1970; Furrer, 1966) and shares many of the properties of inorganic SO_4 in ruminant nutrition. Both S sources are rapidly degraded in the rumen to yield sulphide (Bosman, 1965, 1966; Hume & Bird, 1970; Suttle, 1974*b*) and they have similar reducing effects on Cu availability when added to a low-Mo diet (Suttle, 1974*b*). The possibility that organic S might also share the property of potentiating the Cu–Mo antagonism has,

therefore, been studied. A preliminary report of some of these results has been published (Suttle, 1973).

The effects of S on Mo metabolism have been largely neglected since results of the pioneer studies of Dick (1956) indicated that SO_4 decreased the retention of Mo by sheep. The effects of organic and inorganic S on Mo metabolism have also been studied in these experiments.

EXPERIMENTAL

Design of experiments

The effects on the S–Mo interaction of varying the dietary S source were studied in three Cu-repletion experiments using the procedure described by Suttle (1974*a*), and using a 2×2 factorial design. In each experiment, four groups of initially hypocupraemic ewes were repleted for 35 d with a basal semi-purified diet (Suttle & Field, 1968), supplemented with 6 mg Cu/kg. They received diets containing either no supplementary Mo or 4 mg Mo as ammonium molybdate/kg, and either no supplementary S, or 3 g S/kg. A different S source was used in each experiment; $NaSO_4$, methionine (Met) and cysteine (Cys) were used in Expts 1, 2 and 3 respectively. For convenience the groups have been designated O, Mo, S and Mo + S according to the supplement given: S is replaced by the appropriate S source when this is specified. The unsupplemented diet (O) contained 0·5 mg Mo and 1 g total S/kg, and all diets were given at a rate of 0·8 kg/d in two equal meals. There were seven, five and six replicates in Expts 1, 2 and 3 respectively.

During each repletion experiment, the changes in total (T) Cu and caeruloplasmin (CP) Cu, were measured by collecting weekly blood samples. Direct-reacting (DR) Cu (Suttle & Field, 1968) was measured after 21 d only. Effects on repletion of the liver Cu pool were measured in Expt 3 by taking an initial liver biopsy sample at the beginning of the experiment and another liver sample when the animals were slaughtered at the end of the experiment. Effects on Mo metabolism were assessed by estimating plasma Mo concentrations in each experiment and urinary Mo excretion in Expt 3.

Analytical methods

Plasma Cu. T Cu was determined by atomic absorption spectrophotometry using the standardization technique described previously (Suttle, 1974*a*). CP Cu (μg Cu/ml) was estimated by the method of Smith & Wright (1974) and the equivalent values expressed in IU/ml were obtained using the conversion factor of Suttle (1974*a*). DR Cu was estimated by the method of Suttle & Field (1968) and the amount of residual Cu was calculated by difference (T − (CP + DR)).

Cu in diets and tissues. Dietary and liver Cu concentrations were estimated by the methods described by Suttle & Field (1968) after digestion of the sample in a nitric acid–perchloric acid mixture (Thomson & Blanchflower, 1971). In Expt 3, the initial liver sample was taken by the aspiration biopsy technique of Dick (1944): samples at slaughter were taken from the caudate, ventral and dorsal lobes, and the results were pooled to give a mean concentration for the whole liver.

Mo in diets, plasma and urine. Mo contents of diets were estimated by the method of Bingley (1959) and Mo concentrations for plasma and urine by a modification (Suttle, Thornton & Alloway, 1975) of the method of Stanton & Hardwick (1967).

S content was determined using a modification of the method described by Lachicha Garrido (1964); the initial digestion procedure was that of Mottershead (1971).

Urinary creatinine. Urinary creatinine content was determined by the method of Bonsnes & Taussky (1945) for spot samples obtained by inserting Foley catheters into the bladder on day 35 of Expt 3. Total daily creatinine output was predicted from the relationship with live weight given by Field, Sykes & Gunn (1974) and total urinary Mo excretion was obtained from the Mo:creatinine ratio for the urine sample.

Statistical analysis. Animals were allocated to treatments on the basis of their performance in a uniformity trial (Suttle, 1974*a*) and each parameter was analysed for an experiment of randomized block design (Snedecor, 1956). Homogeneity of variance was checked by the application of Bartlett's test and any parameter lacking homogeneity was subjected to a logarithmic transformation before determining levels of significance for the treatment effects. However, treatment means with separate SEs, determined before transformation, are given in the figures and tables in order to indicate the heterogeneity and preserve the physiological impact of the results.

RESULTS

Plasma Cu

T Cu. In each experiment T Cu generally increased linearly with time within treatment groups and presentation of the results has, therefore, been simplified by giving the mean responses in T Cu after 35 d repletion (Fig. 1). Supplementing the diet with organic S significantly reduced the within-treatment variation and the data for Expts 2 and 3 were, therefore, logarithmically transformed. In other respects the effects of the treatments were similar in each experiment. Dietary Mo had no effect on repletion but each S source when given alone reduced the response in T Cu; the effect was most marked for Cys ($P < 0{\cdot}01$; Expt 3) and was least marked for Met (not significant; Expt 2). In the presence of Mo and S, repletion of the plasma Cu pool was almost totally inhibited, giving a Mo–S interaction which was significant with Mo + SO_4 ($P < 0{\cdot}01$) and Mo + Met ($P < 0{\cdot}05$) and least evident with Mo + Cys ($P < 0{\cdot}5$).

DR Cu. The mean DR Cu concentration in plasma after 21 d repletion for each experiment is given for each group in Table 1. The addition of S to the diet generally reduced DR Cu concentrations and the effects were significant in two of the three experiments ($P < 0{\cdot}01$ in Expt 1; $P < 0{\cdot}05$ in Expt 3). Mo tended to increase DR Cu and the effect was significant for the pooled data ($P < 0{\cdot}01$) but there was no Mo–S interaction. DR Cu accounted for only a small proportion of T Cu in any group.

CP Cu. The results for CP Cu were essentially the same as those for T Cu. CP Cu accounted for virtually all the response in T Cu in Expt 2 and 3 but in Expt 1 this was not so. Mean increases in CP Cu concentrations were 370, 394, 239, 2 (SE 75) μg/l (31·9, 34·0, 20·6, 0·2 (SE 6·5) IU/l) for groups O, Mo, SO_4, Mo + SO_4 respectively: the

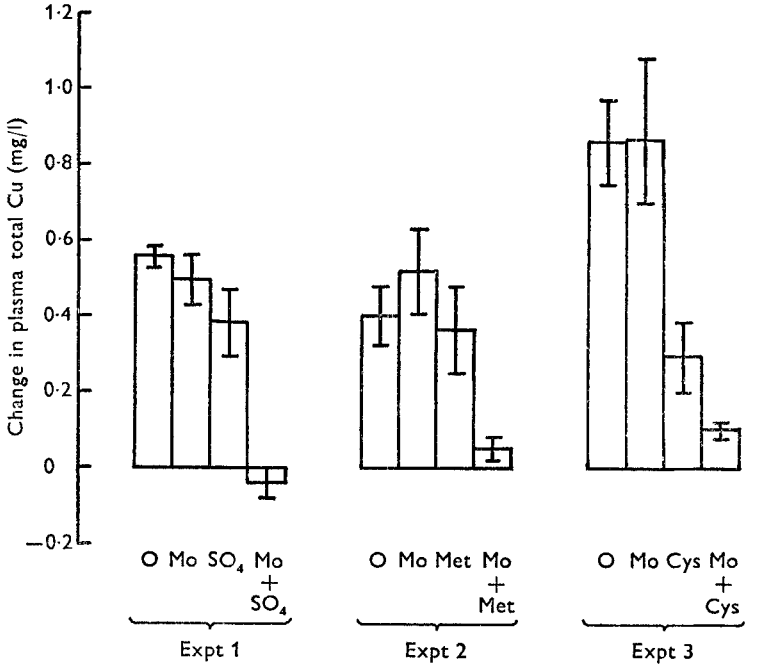

Fig. 1. Effects of dietary supplements of molybdenum (4 mg Mo as ammonium molybdate/kg) and sulphur (3 g/kg) as sulphate (Expt 1), methionine (Met) (Expt 2) or cysteine (Cys) (Expt 3), on the responses in plasma total Cu (mg/l) of initially hypocupraemic ewes given a Cu-supplemented diet (O) for 35 d. Mean values for groups of seven, five and six sheep respectively, for Expts 1, 2 and 3; vertical bars represent standard errors of the means. For details of experimental procedures, see p. 412.

Table 1. *Effects of dietary molybdenum and organic or inorganic sulphur supplements on direct-reacting copper† concentration (μg/l) in plasma of groups of initially hypocupraemic ewes given a Cu-supplemented diet for 21 d*

(Mean values for groups of seven, five and six sheep respectively, for Expts 1, 2 and 3)

Expt‡	Form of S supplement	Supplement				SE of means	Statistical significance of effects of:		
		O	Mo	S	Mo+S		Mo	S	Mo × S
1	Sulphate	24	25	18	22	2	NS	**	NS
2	Methionine	24	31	22	29	6	NS	NS	NS
3	Cysteine	35	45	5	28	7	NS	*	NS

O, No supplement; Mo, 4 mg Mo as ammonium molybdate/kg diet; S, 3 g S/kg diet; NS, not significant.

* $P < 0.05$, ** $P < 0.01$.

† For details, see Suttle & Field (1968).

‡ For details, see p. 412.

corresponding 'non-CP' Cu responses were 167, 79, 147, −37 (SE 63) μg/l. The treatment effects on CP Cu in Expt 1 were nevertheless similar to those in Expt 2 and 3.

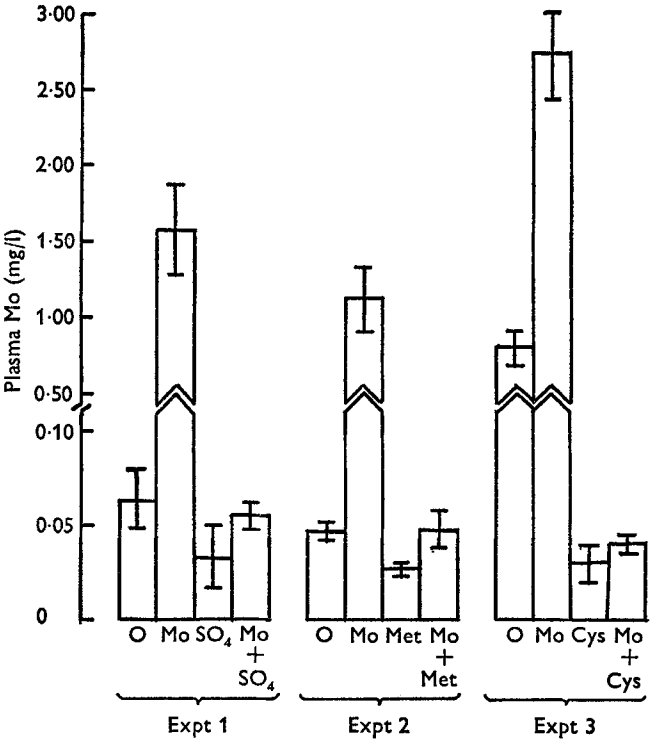

Fig. 2. Effects of dietary supplements of molybdenum (4 mg Mo as ammonium molybdate/kg) and sulphur (3 g/kg), as sulphate (Expt 1), methionine (Met) (Expt 2) or cysteine (Cys) (Expt 3), on plasma Mo (mg/l) in initially hypocupraemic ewes after 21 d repletion. Mean values for groups of seven, five and six sheep respectively, for Expts 1, 2 and 3: vertical bars represent standard errors of the means. For details of experimental procedures, see p. 412.

Plasma Mo

The effects of dietary Mo and S on the concentrations of Mo in plasma after 21 d were essentially similar regardless of the S source used (Fig. 2). The addition of Mo alone to the diet significantly increased the within-treatment variation and logarithmic transformations of the data were again done before complete analysis of variance. Plasma Mo concentrations were increased by 1–2 mg/l in the Mo groups ($P < 0{\cdot}001$) but the effect was totally eliminated by simultaneously adding organic or inorganic S to the diet. Addition of S alone tended to reduce plasma Mo concentrations: Cys was most effective ($P < 0{\cdot}001$) and SO_4 was least effective ($P < 0{\cdot}05$), but the Mo–S interactions were significant ($P < 0{\cdot}05$) with the exception of Mo × Cys. Values for groups O and Mo in Expt 3 were much higher than the corresponding values in Expts 1 and 2.

Urinary Mo

Urinary Mo excretion was estimated from the Mo:creatinine ratio in the urine on the last day of Expt 3 and the effects of the dietary treatments generally followed those described for plasma Mo. The mean total outputs of Mo in urine (mg/d) were

(mean ± SE) 0·562, 0·240; 0·109, 0·034; 1·314, 0·233; 0·464, 0·046 for groups O, Cys, Mo, Mo + Cys respectively. Dietary S reduced the variation within treatment groups ($P < 0·01$). Analysis of the logarithmically transformed data indicated that S reduced ($P < 0·001$) and Mo increased ($P < 0·001$) the urinary excretion of Mo and that the effects were independent.

Cu in liver and bile

The mean initial liver Cu concentrations and the increase in liver Cu after 35 d repletion in Expt 3 are given in Table 2. Cys significantly decreased the within-treatment variation and reduced the response in liver Cu ($P < 0·05$), whereas Mo increased the response provided that Cys was not added: there was, therefore, a significant Mo × S interaction ($P < 0·01$). Only the group given Mo showed a significant increase in liver Cu during repletion. The dietary treatments had no effect on concentrations of Cu excreted in bile (Table 2).

DISCUSSION

Organic v. *inorganic S.* In the particular conditions in which these experiments were done, it has been shown conclusively that inorganic and organic S play similar roles in the Cu–Mo–S antagonism. Both S sources potentiated the inhibitory effect of Mo on Cu repletion rate while simultaneously decreasing Mo concentrations in plasma. These similar responses were not unexpected in view of the common metabolic pathways of the two S sources, which are both degraded rapidly to S^{2-} in the rumen. What is surprising is that Dick's (1953*b*) conclusion that inorganic SO_4 was predominantly involved in the Cu–Mo–S antagonism has been so commonly accepted by other workers in quite different field and experimental situations. Dick's (1953*b*) conclusion was based on the quantitative recovery of the 'active ingredient' in lucerne hay in a crude ash residue. However, one would expect much of the organic S to be converted to the inorganic form during the ashing procedure. The contribution of organic S may also have been underestimated by the use of nonspecific methods for determining inorganic S content of foods. It was some years before a specific method for SO_4 was developed (Bingley & Dick, 1967) and difficulties with foodstuffs rich in starch are still reported (Todd, 1972).

The ability of organic S to potentiate the Cu–Mo antagonism may depend on its conversion to S^{2-} in the rumen (cf. Suttle, 1974*c*). Any S in dietary proteins which escapes degradation in the rumen would, therefore, not participate in the interaction. Hume (1974) has found that 40–60% of vegetable protein can escape degradation in the rumen: with free S amino acids, however, this proportion is reduced to 7–13% (Bird & Hume, 1971). The use of S amino acids as the source of organic S in these experiments may, therefore, over-estimate the capacity of organic S in natural foodstuffs to potentiate the Cu–Mo antagonism. However, since organic S is by far the main source of S in most foodstuffs, the capacity of a diet to influence Cu and Mo metabolism of ruminants will still be more closely correlated with total S than with inorganic S concentrations.

Mechanism of the Cu–Mo–S interaction. Several workers have found that high

Table 2. *Expt* 3.† *Effects of dietary molybdenum and cysteine on the accumulation of copper in the liver and Cu excretion in the bile of initially hypocupraemic ewes given a Cu-supplemented diet for* 35 *d*

(Mean values with their standard errors for groups of six sheep)

	Supplement				SE of means	Statistical significance of effects of:		
	O	Mo	Cys	Mo+Cys		Mo	S	Mo×S
Initial liver Cu (μg/g dry matter (DM))	16·5	13·7	9·6	9·9	3·1	NS	NS	NS
Increase in liver Cu (μg/g DM)	4·8±5·7	11·2±6·1	0·1±1·5	−1·9±1·1	—	NS‡	*‡	**‡
Cu excreted in bile (μg/l)	87	61	71	69	9	NS	NS	NS

O, No supplement; Mo, 4 mg Mo as ammonium molybdate/kg diet; Cys, 3 g sulphur as cysteine/kg diet; NS, not significant.
* $P < 0.05$, ** $P < 0.01$.
† For details, see p. 412.
‡ Statistical analysis done using logarithmically transformed data.

Table 3. *Effects of dietary molybdenum and organic or inorganic sulphur supplements on the availability of dietary copper* to groups of initially hypocupraemic ewes given a Cu-supplemented diet for* 21 *d*

Expt†	Form of S supplement	Supplement O	Mo	S	Mo+S
1	Sulphate	0·047	0·042	0·037	0·016
2	Methionine	0·038	0·046	0·032	0·022
3	Cysteine	0·070	0·078	0·039	0·021

O, No supplement; Mo, 4 mg Mo as ammonium molybdate/kg diet; S, 3 g S/kg diet.
* Estimation based on the mean response in plasma total Cu of each group after 21 d repletion relative to that of similar ewes repleted by continuous intravenous infusion of Cu (Suttle, 1974*a*).
† For details, see p. 412.

dietary concentrations of Mo (30–50 mg/kg dry matter (DM)) increase the concentration of S^{2-} in the rumen (Mills, 1960; Hartmans & Bosman, 1970; Bryden & Bray, 1972) but at the lower Mo concentrations used in the present studies, rumen S^{2-} concentrations are unaffected (Suttle, unpublished results). Since a large excess of S^{2-} is generated in the rumen when the diet is rich in S (Bird, 1970; Suttle, 1974*b*), it is unlikely that antagonistic effects of Mo on Cu metabolism are mediated through effects on S^{2-} formation. However, it is possible that S^{2-} is involved through its ability to convert molybdate to thiomolybdate in the rumen (Suttle, 1974*c*).

Cu–Mo–S antagonism and grazing animals. Assuming that the systemic effects of the dietary treatments were small, responses in plasma Cu in the various experiments can be translated into effects on Cu availability at the gut level (Suttle, 1974*a*). The reduction in Cu availability attributable to the combined effects of Mo + S in Expts 1, 2 and 3 ranged from 30 to 60% (Table 3). Furthermore, the marked decreases were obtained by increasing dietary Mo and S concentrations to levels only equivalent to the upper limits of the respective normal ranges for herbage (4·5 mg Mo, 4·0 g S/kg DM; Whitehead, 1966; Miltimore & Mason, 1971). If the Mo and S in pasture interact with Cu, then natural variations in herbage Mo and S should influence the Cu status of the grazing animal. Indeed it has been suggested that 'organic' Mo in herbage is a more effective Cu antagonist than 'inorganic' Mo (Miller, Lesperance, Bohman & Jensen, 1970). Previous assumptions that the Cu–Mo–S interrelationship has little or no effect on the development of clinical Cu deficiency under normal grazing conditions in the UK (Allcroft & Lewis, 1956) and in The Netherlands (Hartmans, 1970; Hartmans & Bosman, 1970) may, therefore, be incorrect.

Effects of S on Mo metabolism. The decreases in plasma and urinary Mo concentrations following S supplementation of the diet are similar to the responses reported by Dick (1956) in sheep given Mo and S supplements simultaneously for relatively long periods. There is also an indication in an unreplicated and uncontrolled experiment with Mo-supplemented cattle that organic S and SO_4-S have similar decreasing effects on plasma Mo (Cook, Lesperance, Bohman & Jensen, 1966). The S effects are probably related to a decrease in Mo absorption, possibly due to the formation of relatively unavailable thiomolybdates in the rumen (Suttle, 1974*c*). In sheep previously loaded with Mo, SO_4 causes a temporary increase in urinary Mo excretion and a marked negative Mo balance (Dick, 1956). These effects have been explained by competition between the chemically similar SO_4^{2-} and MoO_4^{2-} ions for tissue uptake and renal tubular reabsorption (Huisingh, Gomez & Matrone, 1973). In the present experiments, however, such systemic effects of S on Mo metabolism were probably small and the interaction of overriding importance occurred in the gut. Evidence that systemic effects of Mo on Cu metabolism are relatively unimportant is provided by the fact that ewes given diets high in Mo but low in S, tolerated plasma Mo concentrations of 1–2 mg/l, a level some twenty to forty times greater than normal, without showing an inhibitory effect on caeruloplasmin synthesis. By contrast, normal plasma Mo concentrations were found in the groups showing the most marked inhibition of Cu metabolism (Mo + S).

Cu status and the Cu–Mo–S interaction. It has been suggested that the nature of the

Cu–Mo–S interrelationship is affected by differences in the initial Cu status of the animal (Gray & Daniel, 1964). If this were true, the results reported in the current series of experiments would apply only to sheep of low initial Cu status. There is however, no evidence that Cu status effects the nature of the Cu–Mo–S antagonism in sheep. Supplementation with $Mo + SO_4$ is as effective in preventing or treating Cu toxicity in sheep given high Cu intakes (Ross, 1966; Hogan, Money & Blayney, 1968; Kline, Hays & Cromwell, 1971) as it is in inducing Cu deficiency at low Cu intakes (Suttle & Field, 1968, 1969).

It is interesting to note that, at the end of repletion in Expt 3, no significant repletion of the liver Cu pool had occurred in the group given the diet low in Mo and S, although normal plasma T Cu concentrations were found. It appears that priority was given to repletion of the plasma T Cu pool. However, a similar increment in the total liver Cu pool (1·2 mg) would have been represented by an increase of only 3·6 mg/kg in liver Cu concentration (assuming liver DM 300 g): an increase of this order is within the experimental error of the related methods.

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