Effect of dietary molybdenum and sulphur on the copper status of hypercuprotic sheep after withdrawal of dietary copper

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A trial was conducted to determine the efficacy of dietary molybdenum (Mo) and sulphur (S) in reducing liver copper (Cu) levels in sheep at risk of Cu toxicity. Sheep were fed 75 mg Cu/day during a preliminary period to establish high Cu levels in their liver. For a period of 47 days the sheep were fed varying levels of Mo (0 – 140 mg/day) and S (0 – 4 g/day) with no added dietary Cu. Within 8 days of the commencement of Mo feeding, sheep in the highest Mo groups (140 mg/day) developed Mo toxicity. Six sheep in these groups were slaughtered on day 13, the remainder were fed 70 mg Mo and 50 mg Cu per day. Sheep fed 70 mg Mo/day (without Cu) showed a 40% reduction in liver Cu compared with groups receiving no Mo supplement. When extra Cu was fed with 70 mg Mo, no reduction in liver Cu was observed. Elevated kidney and plasma Cu levels were found in all groups receiving supplementary Mo. A dietary supplement of 70 mg Mo for a restricted period is recommended to reduce liver Cu in sheep (SA Mutton Merinos) provided dietary Cu levels are simultaneously reduced.

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Die doeltreffendheid waarmee molibdeen (Mo) en swawel (S) die koper (Cu) in die lewers van skape met hoë vlakke Cu in hul lewers kan verlaag, is ondersoek. Gedurende 'n voorbereidingsperiode het die skape 75 mg Cu/dag ontvang sodat Cu in hul lewers kon opgaar. Molibdeen (0 – 140 mg/dag) en S (0 – 4 g/dag) is vir 'n periode van 47 dae aan die skape gevoer. Binne 8 dae na aanvang van die proefperiode het die skape in die hoogste Mo-groepe (140 mg) tekens van Mo-vergiftiging getoon. Ses skape is op dag 13 geslag, terwyl die oorblywende skape in die groepe 70 mg Mo en 50 mg Cu per dag ontvang het. Die skape op 70 mg Mo/dag (sonder Cu) het 'n verlaging van 40% in lewer-Cu teenoor die 0-Mo-groepe getoon. Waar ekstra Cu saam met 70 mg Mo gevoer is, is geen verlaging in lewer-Cu waargeneem nie. Verhoogde nier- en plasma-Cu-peile is in al die behandelings wat Mo ontvang het, gemeet. 'n Mo-peil van laer as 70 mg/skaap/dag word vir 'n beperkte periode aanbeveel om Cu uit die lewers van skape (SA Vleismerino's) te verwyder indien hul Cu-innames ook verlaag word.

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Chronic copper (Cu) toxicity in sheep is of an insidious nature, with a passive accumulation of Cu in the liver without observable clinical signs until the final stages when a haemolytic crisis occurs (Todd, 1969; Underwood (1977). Usually only a small proportion of a flock will undergo this crisis (Todd, Gracey & Thompson, 1962; Todd, 1969). However, the hepatic Cu concentration may remain at a high level (Ross, 1970; Olson, Fontenot & Failla, 1984), and sheep may succumb to Cu toxicosis long after the withdrawal of any additional Cu from their diets (Bracewell, 1958; Gopinath & Howell, 1975).

Many different recommendations are to be found in the literature concerning methods of preventing the accumulation of Cu in the liver. These recommendations include different dietary levels of molybdenum (Mo) and sulphur (S) for varying periods or the feeding of high levels of zinc (Zn) (van Ryssen, 1982). The availability of Cu at different levels of Mo and S can be predicted from formulae proposed by Suttle & McLauchlan (1976) as accepted by the ARC (1980).

In practice, Cu is often withdrawn from the sheep's diets when cases of Cu toxicity are noticed. In addition Cu taken in at relatively low levels may accumulate in the livers over a number of years, as observed in the Karoo region of South Africa (Bath, 1979). To prevent further deaths in such cases, it seems advisable to reduce the level of Cu in the livers. The antagonistic action of Mo plus S against Cu in ruminants is believed to occur primarily in the digestive tract, and then in the body (Dick, Dewey & Gawthorne, 1975; Harker, 1976).

The formulae published by the ARC (1980) suggest relatively low levels of Mo and S supplementation but these refer to control during high Cu intake and therefore may be ineffective in reducing established high levels of hepatic Cu. Gooneratne, Howell & Gawthorne (1981) administered thiomolybdate intravenously to sheep and successfully reduced liver Cu concentrations. Unfortunately thiomolybdate preparations are not readily available. The addition of high levels of Mo and S to sheep diets for limited periods of time would appear to be a practical alternative to the injection of thiomolybdates.

A trial was therefore conducted in which the livers of sheep were loaded with Cu during a preliminary period. Additional Cu was then withdrawn from the diet and different levels of Mo and S were included in the diet to determine the effect on Cu status in the body. The occurrence of possible health problems such as Mo toxicity or Cu deficiency in the sheep was also investigated.

Material and Methods

Animals, Treatments and Procedure

During a preliminary period of 50 days, 70 SA Mutton Merino ewe lambs (10 months old) received an average of 75 mg Cu per day via the feed to establish an accumulation of Cu in their livers. Plasma aspartate transaminase (AST; EC 2.6.1.1) levels in the sheep were measured to monitor the risk of a haemolytic crisis (Hogan, Money & Blayney, 1968; MacPherson & Hemingway, 1969). The added Cu was withdrawn from the diet on day 50. The lambs were then allocated at random to seven treatments of 10 sheep per treatment. Four levels of Mo, as ammonium molybdate, were fed to the lambs, viz. 0, 35, 70 and 140 mg per sheep per day. The S level was either fixed at 2 g of added S (as Na_2SO_4) or given in a fixed ratio to Mo, viz. 70 mg Mo : 2 g S (Table 1). Each lamb received 225 g concentrate (containing the mineral supplements) per day plus veld hay (mainly *Themeda triandra*) *ad libitum*. Feed intakes per group and individual bodymass changes were recorded.

Table 1 Treatments and average daily molybdenum (Mo), sulphur (S) and copper (Cu) intakes of the lambs during the high-Mo, low-Cu stage of the trial

			Treatment		Average intake/ lamb/day		
Groups	Number of sheep	Duration (days)	Mo (mg)	S (g)	Mo (mg)	S (g)	Cu (mg)
A	10	12	140	4	99[a]	3,6	4,3
A Cu	7	34	70	2	74	3,9	48,5
B	10	12	140	2	113[a]	2,9	4,3
B Cu	7	34	70	1	75	3,0	53,9
C	10	46	70	2	69	3,7	5,4
D	10	46	35	1	41	3,3	5,4
E	10	46	35	2	42	3,9	5,0
F	10	46	0	0	0,4	2,0	5,7
G	10	46	0	2	0,3	3,9	5,2

[a]Intakes depressed owing to lowered feed consumption.

Eight days after the onset of the experimental period lambs in both the 140 mg Mo groups showed serious symptoms of Mo toxicity. On the 13th day the six most seriously affected lambs from these two treatment groups were killed and autopsies performed. The two treatments supplying 140 mg Mo/day were changed to supply *ca* 70 mg Mo plus 50 mg Cu per day to the lambs (Table 1). All the lambs were slaughtered after 47 days on the experimental rations. Liver and kidneys were collected and liver wet masses determined. Liver and kidney cortices were sampled and dried for further analyses.

Analytical methods

The following analyses were performed according to the methods described by van Ryssen & Stielau (1980): dietary Mo, Cu, Zn, Mn, Fe, Ca, P and crude protein; Mo and total Cu in organs and plasma; packed cell volume (PCV); and haemoglobin (Hb) concentration in whole blood. Dietary sulphur was measured by the magnesium nitrate method for plants (AOAC, 1980). Erythrocyte (RBC) counts on whole blood were performed using an electronic particle counter. Plasma AST and alkaline phosphatase (AP; EC 3.1.3.1) levels were estimated using Boehringer Mannheim standard kits (Boehringer Mannheim GmbH Diagnostica, West Germany) and caeruloplasmin (ferroxidase; Cp; EC 1.16.3.1) activity in plasma as described by Smith & Wright (1974).

Statistical analyses

The exclusion of the two high Mo treatments complicated the proper interpretation of results relating to Mo and S ratios. These were therefore only compared in analyses of variance. Although the A Cu and B Cu treatments are not directly comparable to the other treatments, the results are presented, mainly in relation to Treatment C.

Analyses of variance were performed on the data with the use of the computer program Genstat (Genstat V Mark 4.03 (c) 1980 Lawes Agricultural Trust, Rothamsted Experimental Station). Logarithmic transformations were employed to reduce differences in variance between treatments in the case of liver and kidney analyses.

Results

Preliminary period

No sign of Cu toxicity was observed during this period. Although the AST levels in plasma increased, no dramatic increases indicating a pending haemolytic crisis were observed. Plasma Cu levels showed a slight but consistent increase from the pre-experimental collection to the end of the high Cu feeding period (Table 4).

Experimental period

Clinical signs

In Group A, one sheep was lame in the hind limbs by day 9 after introduction of the diet and by day 11 was unable to stand. On day 12 a further two sheep were lame and by day 13 four sheep out of the group of 10 were visibly lame.

One sheep in Group B was observed with fore-limb lameness by day 9 and by day 10 four of the group were partially lame, all involving the fore-limbs. The severity of lameness appeared to fluctuate, but by day 13 two of the group were unable to stand.

In Group C, two animals showed fore-limb stiffness and lameness by day 16 which persisted until day 18 and then recovered. Several of the sheep in Groups A and B had noticeable diarrhoea by day 10 of the trial and by day 12 three of the animals in Group A were scouring severely. A few sheep in Group C developed diarrhoea for a few days.

Bodymass, feed and mineral intakes

Average bodymass for each group at the start of the trial was 36 kg and 38 kg at the end of the trial. No differences were observed in bodymass changes consequent to treatment effects.

During the experimental period the feed intakes of Groups A and B were depressed, resulting in mineral intakes well below those intended (Table 1). When these two diets were changed to A Cu and B Cu, the feed intakes returned to a level similar to that in the other groups. The average concentrations of other minerals (determined on a DM basis) in the diets were : 0,63% Ca, 0,22% P, 63 mg Zn/kg, 145 mg Fe/kg and 161 mg Mn/kg in a ration containing 10,1% crude protein.

Post-morten changes

Six of the most severly affected sheep from Groups A and B were slaughtered on day 13 and examined. Lesions were found in all except one of the radius/ulna-metacarpal joints and the tibial-metatarsal joints. The articular cartilage of these joints was thin and showed mild inflammation. There were visible erosions into the articular cartilage in all cases, the erosions varying from shallow penetrations to complete erosion and involvement of calcified bone. Uneroded areas of articular cartilage showed patchy, mild inflammation. No abnormal secretions were apparent in the joint cavities and synovial fluid

was sparse. In the worst clinically affected sheep the ulnar articular surface of the elbow joint (humerus-ulna) showed erosions and petechiae. The articular surface of other joints (stifle, hip, shoulder, carpals, tarsals) showed no erosions but the articular cartilage was also thin and mildly reddened.

Organs and tissues

To simplify interpretation the results are grouped into all the treatments excluding those with additional Cu (Table 2) and the three treatments where 70 mg Mo per sheep were fed (Table 3).

From Table 2 it is evident that an intake of 70 mg Mo per day (Treatment C) decreased ($P < 0,01$) hepatic Cu concentration relative to the 0-Mo groups, from approximately 1000 mg/kg DM to 611 mg Cu/kg DM; a reduction of *ca* 40%. An intake of 40 mg Mo and the same level of S (Treatment E) as that in treatment C resulted in a reduction ($P < 0,05$) of 33% in hepatic Cu compared with the 0-Mo treatment. Increased dietary Mo increased the Mo levels in the livers and kidneys and the Cu in the kidneys substantially ($P < 0,01$). In the treatments where Mo was added, the higher S levels at the same Mo intakes (Treatments E vs D) resulted in significant increases in kidney Cu ($P < 0,01$) and Mo ($P < 0,05$) levels. When no Mo was supplemented, S did not have any effect on the mineral levels in the livers or kidney cortices.

Table 2 Effect of various levels of dietary molybdenum (Mo) and sulphur on the copper (Cu) and Mo concentrations of livers and kidney cortices of lambs on low Cu rations (values expressed on DM basis)

	Liver*			Kidney cortex*	
	Cu				
Treatment	(mg/kg)	(mg)	Mo (mg/kg)	Cu (mg/kg)	Mo (mg/kg)
C	611[a]	67,5[a]	59[a]	348[a]	275[a]
D	759	83,3	19[d]	199[d]	158[b]
E	675[c]	79,9[c]	33[d]	295[a]	222[c]
F	996[b]	105,5[b]	3[b]	22[b]	2,7[d]
G	1008[b]	110,3[b]	2[b]	23[b]	2,2[d]
SED**	130	14,4	7	41	32

*Values within columns with different superscripts denote significance: b – c at $P < 0,05$; a – b, a – d, b – d, c – d at $P < 0,01$ levels of significance

Statistical analyses based on logarithmic tranformations.

**SED = Standard error of difference of means.

Table 3 Average copper (Cu) and molybdenum (Mo) concentrations of liver and kidney cortices of sheep receiving approximately 70 mg Mo/day, with or without additional Cu (values expressed on DM basis)

	Livers*			Kidney cortex*	
	Cu				
Treatments 70 mg Mo	(mg/kg)	(mg)	Mo (mg/kg)	Cu (mg/kg)	Mo (mg/kg)
C	611[a]	67,5[a]	59[a]	348[a]	275[a]
A Cu	869[c]	120,3[b]	45	274	188[b]
B Cu	971[b]	117,9[b]	33[b]	215[b]	154[b]

*Values within columns with different superscripts denote significance: a – c at $P < 0,05$ and a – b at $P < 0,01$ levels of significance. Statistical analyses based on logarithmic transformations.

Furthermore, none of the treatments had a significant effect on the concentration of Zn in the livers and kidneys.

At the Mo intakes of 70 mg/day (Table 3) the liver Cu levels were significantly lower when no Cu (Treatment C) was fed compared to the addition of 50 mg Cu/sheep/day in Treatments A Cu ($P < 0,01$) and B Cu ($P < 0,05$). It seems reasonable to accept that these differences would have been more pronounced if the sheep in groups A Cu and B Cu did not receive 140 mg Mo/day for a period of 12 days. The addition of Cu to the diet at 1 g S intake (treatment B Cu) resulted in a lower ($P < 0,01$) level of accumulation of Mo in the livers and kidneys and in Cu in the kidneys of the sheep compared to when no Cu was added (Treatment C).

Blood analyses

Plasma Cu and Mo. On day 13 after the introduction of Mo, the Cu levels in plasma (Table 4) of all the Mo treatments were higher ($P < 0,01$) than in the no-Mo groups. These Cu levels subsequently maintained the same treatment effects ($P < 0,01$) but were lower on days 41 and 47 than on day 13. The plasma Cu concentrations in both the 70 mg Mo plus Cu treatments were lower than the 70 Mo without the additional Cu.

Table 4 Average total plasma copper (Cu) concentrations of lambs receiving a high level of dietary Cu during the preliminary period and different levels of molybdenum (Mo) and sulphur (S) without added Cu for a further 47 days

	Total plasma copper (mg/l)*				
	Preliminary period		Experimental period		
Treatments	Day 0	Day 50	Day 13	Day 41	Day 47
A	0,90	1,07	2,12[a]	1,44**	1,42**
B	0,93	1,12	2,26[a]	1,16***	1,26***
C	0,97	1,11	2,24[a]	1,84[ac]	1,91[a]
D	0,95	1,07	1,99[a]	1,53[ad]	1,59[ae]
E	1,03	1,01	2,20[a]	1,67[a]	1,96[a]
F	0,85	1,10	1,02[b]	0,82[b]	0,93[b]
G	0,95	1,07	1,08[b]	0,83[b]	0,88[b]
SED+	0,083	0,093	0,146	0,118	0,116

*Values within columns with different superscripts denote significance: a – b and a – e at $P < 0,01$; c – d at $P < 0,05$ levels of significance.

**Treatment changed to 70 Mo, 2 S and 40 Cu; not included in statistical analyses.

***Treatment changed to 70 Mo, 1 S and 40 Cu; not included in statistical analyses.

+SED = Standard error of difference of means.

Plasma Mo levels (Table 5) on day 13 correlated with the level of Mo intake. On day 47 Mo levels were lower than corresponding readings on day 13. At the same S intake, the addition of Cu (A Cu) resulted in a reduction ($P < 0,01$) in plasma Cu (treatment C).

Haematological measurements. Throughout the experiment PCV(%), Hb levels (g/100 ml) and RBC counts ($\times 10^6$/ml) remained relatively constant at average levels of 30,8, 11,6 and 10,1 respectively, without any significant differences between treatments or any indication of anaemia in the sheep.

Plasma caeruloplasmin activity. The Cp activity in Treatment A tended to be lower than the activity on other treatments on days 13 and 41. No significant differences in Cp activity were observed between treatments during the collections on day 47 of the experimental period (Table 6).

Table 5 Average molybdenum (Mo) concentrations in plasma at two stages of the Mo plus sulphur feeding period

Plasma molybdenum concentration (mg/l) on:			
Day 13		Day 47	
Treatment	Mo	Treatment	Mo
A	2,87[a]	A Cu	0,93
B	2,91[b]	B Cu	1,64[a]
C	2,29	C	1,86[b]
D	1,53[e]	D	1,30[c]
E	1,54[f]	E	1,24[d]
F	0,002[c]	F	0,26[e]
G	0,028[d]	G	0,11[f]
SED[+]	0,204	SED[+]	0,117

*Values within columns with superscripts a – b, c – d and e – f denotes no statistically significant differences between those treatments. All other differences were significantly different ($P < 0,01$).
[+]SED = Standard error of the difference.

Table 6 Caeruloplasmin activity of plasma during different stages of the molybdenum/sulphur supplementation

	Caeruloplasmin activity (OD/min/l on:		
Treatment	Day 13	Day 41	Day 47
A	71,5[a]	74,4[a]**	71,1**
B	86,3	94,6[b]**	69,7**
C	85,6	88,3	68,5
D	95,5[c]	88,4	79,2
E	85,7	85,6	74,8
F	87,4	84,9	71,6
G	81,3	82,9	65,8
SED[+]	7,34	9,11	6,38

*Values within columns with different superscripts denote significance: a – b at $P < 0,05$ and a – c at $P < 0,01$ levels of significance.
**Treatment A Cu and B Cu respectively.
[+]SED = Standard error of difference.

Plasma enzyme levels. The AST levels in the plasma of some sheep showed elevated levels towards the end of the preliminary period and during the Mo treatment period but these levels did not follow a specific pattern according to treatment. AP concentrations in plasma were estimated during the Mo treatment period (Table 7). On day 13, AP levels of the two 140 Mo treatments were significantly lower than those of the other treatments. At later collections the differences were less pronounced though the levels in the treatments receiving no Mo tended to be consistently higher than the other treatments.

Discussion

Molybdenosis

A frequent clinical sign of Mo toxicity in ruminants is the development of Cu deficiency in the animal in the form of, *inter alia* anaemia, achromotrichia, steely wool, low plasma, and hepatic Cu levels (Hogan, Money, White & Walker, 1971; Underwood, 1977; Ward, 1978). In the present trial, none of the haematological parameters measured, nor the caeruloplasmin levels, indicated a copper-deficient condition in any sheep. The high liver and plasma Cu levels also ruled out a Cu deficiency. However, because of the accumulation of inert

Table 7 Plasma alkaline phosphatase levels during various stages of molybdenum/sulphur supplementation

	Alkaline phosphatase* (U/l) on:		
Treatment	Day 13	Day 27	Day 40
A	199[a]	322[c]**	343**
B	273[c]	360**	386**
C	305[e]	391	400
D	405[b]	433	433
E	309	325	337[b]
F	412[b]	444[b]	437
G	424[bf]	417	458[c]
SED[+]	59,0	58,1	57,4

*Values within columns with different superscripts denotes significance: c – b, e – f at $P < 0,05$ and a – b at $P < 0,01$ levels of significance.
**Treatment A Cu and B Cu respectively.
[+]SED = standard error of difference of means.

Cu-Mo compounds in plasma, plasma Cu concentration is not a reliable parameter of Cu deficiency at high Mo intakes (van Ryssen & Stielau, 1981).

Mo *per se* is acutely toxic to ruminants. The typical symptoms include diarrhoea, low food intake, joint and connective tissue lesions (Pitt, 1976; Underwood, 1977; Ward, 1978). At a level of 140 mg Mo/sheep/day, these clinical signs were observed in the present trial. Sheep are reported to be less sensitive than cattle to Mo toxicity (Ward, 1978). Bingley (1974) recorded no clinical signs of Cu deficiency and did not mention any clinical signs of Mo toxicity in sheep fed 120 mg Mo and 7,4 g sulphate per day for a period of 29 months. In the present trial, however, a certain degree of diarrhoea and lameness was observed at 70 mg Mo/sheep/day, although this disappeared after a few days. Suttle & Field (1968) reported a similar recovery after a few days in sheep given 50 mg Mo plus 10 g sulphate per kg feed.

Hogan, *et al.* (1971) reported that blood-AP activity tends to increase in animals suffering from molybdenosis, though van der Schee, Garretsen & van der Berg (1980) observed a decrease in serum-AP level of sheep receiving high levels of Mo. The plasma-AP levels of the sheep suffering from Mo toxicity in the present trial were significantly lower than the levels in the other treatment. At the lower Mo intakes, no abnormal clinical signs were recorded, in agreement with observations by Gooneratne, *et al.* (1981) and van Ryssen, Botha & Stielau (1982).

Effect of Mo and Cu status

The occurrence of systemic effects, such as elevated plasma and renal Cu levels, have often been observed in sheep receiving high levels of dietary Mo (Dick, 1956; Suttle, 1974; van Ryssen & Stielau, 1980, 1981). In the present trial, these increases in plasma and kidney Cu levels were observed when Mo was added to the diets, even though dietary Cu levels were low. In the two treatments where additional Cu was added (Treatments A Cu and B Cu) these systemic effects were less pronounced than in the corresponding 70 mg Mo treatment without Cu. This would suggest that the source of Cu measured in these systemic effects was not of a dietary origin. This observation supports the suggestion by Dick, *et al.* (1975) and Gooneratne, *et al.* (1981) that dietary Mo in the presence of S will bind with Cu in the digestive tract and residual unbound thiomolybdates may be absorbed and react with Cu

in the body. This is also evident from the changes in liver Cu concentrations and total liver Cu content in the present trial. When 70 mg Mo plus Cu was fed as supplement, the liver Cu values remained similar to those where no Mo was added (treatments F and G). When 70 mg Mo was fed without Cu, a 40% drop in liver Cu concentration was observed. It may be concluded that the effect of dietary Mo on the Cu present in the liver of Cu-loaded sheep will depend on level of Cu in the diet. This will influence the accuracy of any recommendations and predictions regarding the reduction of Cu levels in sheep livers. In fact, a recognized treatment for molybdenosis is the addition of Cu to the diet, which will not only overcome a Cu deficiency but reduce the absorption of Mo from the digestive tract (Ward, 1978).

Van Ryssen & Stielau (1981) suggested that the systematic accumulation of Mo-bound Cu in the body at high Mo intakes may also take place in the liver. The reductions in liver Cu levels observed in the present trial may therefore underestimate the effect of Mo on available hepatic Cu, especially at the higher Mo levels.

Because evidence of Mo toxicity was observed in sheep fed 70 mg Mo daily it seems advisable at this stage to recommend levels below this for the removal of Cu from the livers of sheep (SA Mutton Merino) where the risk of Cu toxicity exists. The effectivenss of this treatment will depend upon the level of Cu in the diet.

Effect of sulphur on Cu and Mo status

The two levels of dietary S at 0-Mo supplementation (Treatments F and G) did not differ in their effect on Cu levels in the livers, kidneys or plasma. This observation supports the evidence that the effect of S *per se* on Cu is not systemic but takes place in the digestive tract (Suttle, 1974; Huisingh & Matrone, 1976).

Where comparisons could be made between different levels of S supplementation at the same level of added Mo (Treatments A vs B, A Cu vs B Cu, and E vs D) increased concentrations were measured in kidney cortex Cu and Mo, liver Mo and plasma Cu of those groups receiving the higher levels of S. This increase in Mo accumulation with higher S intakes corresponds with a similar observation by Grace & Suttle (1979) which they ascribed to the formation of poorly excretable thiomolybdate-type complexes at high Mo and S intakes. This is contrary to the depressing effect of S on Mo levels in tissues reported by Dick (1956), Grace & Suttle (1979) and van Ryssen & Stielau (1980) at lower Mo and S intakes.

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