Effects of dietary molybdenum and sulphur on the distribution of copper in plasma and kidneys of sheep

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1. A 30-week study has been made in growing ewe lambs of the effects of dietary supplementation with molybdenum, or Mo plus inorganic sulphate on the distribution of copper in their blood plasma and kidneys.

2. The addition of 25 mg Mo and 5 g SO$_4$*/kg diet increased Cu concentrations in plasma and kidney and decreased those in liver. Plasma caeruloplasmin activities (ferroxidase; EC 1.16.3.1) were unaffected.

3. Subcellular fractionation of the kidney cortex and gel filtration of the plasma and kidney cortex cytosol showed that the increased Cu content of these tissues was associated with abnormalities in the distribution of Cu. It appeared that both Cu and Mo were associated with the same proteins.

4. Dietary supplementation with Mo alone (25 mg/kg) had no effect on plasma or kidney Cu concentrations, suggesting that S metabolism is involved in the formation of the abnormal Cu-binding proteins in plasma and kidney.

5. The results are discussed in relation to the induction of Mo-induced Cu-deficiency states and the possible involvement of thiomolybdates.

Although molybdenum and sulphur in combination have long been recognized as antagonists of copper metabolism, especially in ruminant animals, the mechanisms whereby they induce a Cu-deficiency state are still not fully understood (Suttle, 1974). Effects on the availability of Cu (Suttle, 1975), its urinary excretion (Smith, Field & Suttle, 1968; Marcilese, Ammerman, Valsecchi, Dunavant & Davis, 1970) and on caeruloplasmin synthesis have been reported (Gaballah, Abood, Kapsalis & Sturdivant, 1965; Marcilese, Ammerman, Valsecchi, Dunavant & Davis, 1969). Liver Cu concentrations are usually reduced in ruminants fed on diets supplemented with Mo and S (Dick, 1956; Smith et al. 1968; Marcilese et al. 1969), indicating depletion of Cu reserves, but this is not always associated with a decrease in plasma concentrations of Cu, as is normally found in Cu-deficient animals. Indeed, increased plasma Cu concentrations have been reported in sheep offered such diets (Dick, 1956), with some of this Cu present in an unusual, ‘residual’ form, being neither caeruloplasmin nor direct-reacting Cu (Suttle & Field, 1968; Smith & Wright, 1975a, b) which together are thought to account for virtually all plasma Cu in normal animals.

Kidney Cu concentrations are often elevated in these animals and urinary excretion of Cu may also be increased (Smith et al. 1968; Marcilese et al. 1970). It is possible, therefore, that the ‘residual’ Cu fraction in plasma constitutes a non-available form of Cu which does not accumulate in the liver but is removed by the kidney and ultimately excreted in the urine (Suttle, 1974). However, little attention has been paid to the nature of the Cu-containing fractions in the plasma, except for the studies of Smith & Wright (1975b), and none to that in the kidneys of Mo- and S-supplemented animals. This is the subject of the present investigation, of which a preliminary report has already been published (Bremner, 1976). It was found that the changes in plasma and kidney Cu concentrations and distribution only occurred after dietary supplementation with both Mo and S. Furthermore, there was a consistent tendency for both metals to occur in the same chromatographic fractions, suggesting some association of the metals with the same proteins.
EXPERIMENTAL

Animals and treatment

Twelve Finn-Dorset x Suffolk ewe lambs, aged about 12 weeks and weighing about 22 kg, were allocated at random to three treatment groups, each of four animals. The lambs were individually penned indoors and were offered ad lib. the diet of Suttle & Field (1968) which was further supplemented with (g/kg) 9.7 CaHPO₄·2H₂O and 3.1 CaCO₃. The concentrations of Cu and Mo (mg/kg) and of S (g/kg) in this basal diet were 9.8, 0.5 and 0.8 respectively. Lambs in treatment group A were given the basal ration whereas those in groups B and C received in addition supplements of 25 mg Mo/kg (as ammonium molybdate) or 25 mg Mo/kg plus 5 g SO₄²⁻/kg (as Na₂SO₄) respectively. The lambs were weighed weekly and blood samples collected at regular intervals. They were slaughtered after 30 weeks. Livers and kidneys were removed immediately after slaughter and stored at −20°C.

Analytical methods

Tissue samples were digested with a mixture of conc. nitric, perchloric and sulphuric acids (4:1:0.5, by vol). Mo concentrations were measured after acid digestion by the methods of Bingley (1959) or of Bradfield & Strickland (1975). In some cases Mo present in column subfractions was measured directly on the Model 63 Carbon Rod Atomizer (Varian Associates Ltd, Walton-on-Thames, Surrey). Plasma Cu concentrations were determined by atomic absorption spectrophotometry on the Varian AA5 spectrophotometer (a) after dilution of the sample with 4 vol. distilled water (total Cu) or (b) after precipitation of proteins with 50 g/l trichloroacetic acid (TCA-soluble Cu). Direct-reacting Cu was measured by the method of Suttle & Field (1968). Caeruloplasmin (ferroxidase I; EC 1.16.3.1) was determined by the method of Smith & Wright (1974) using the standardization procedure of Rice (1962). Statistical analysis of results was by analysis of variance.

Fractionation of plasma and kidneys

The kidney cortex was separated from the medulla and homogenized with ice-cooling in 2.5 vol. 0.01 M-Tris-acetate, pH 7.4, using an X-1020 homogenizer (Scottish Instrument Centre, Edinburgh). The homogenate was centrifuged at 70000 g for 1.5 h and the supernatant fraction collected.

Gel filtration of all the kidney supernatants and of some plasma samples was carried out at room temperature on columns of Sephadex G.75 (900 x 26 mm), Sephadex G.100 (600 x 26 mm) or Sephadex G.200 (900 x 26 mm) (Pharmacia Ltd, Uppsala, Sweden) with 0.01 M-Tris-acetate (pH 7.4) as eluant. The flow rates were about 35, 20 and 5 ml/h respectively; 5.0-7.0 ml fractions were collected.

The Sephadex G.200 column was calibrated as described by Andrews (1965), with the following proteins of known molecular weight, bovine γ-globulin, ovine caeruloplasmin, human transferrin, bovine serum albumin and myoglobin.

Low-molecular-weight forms of Mo-binding fractions were isolated by ultra-filtration through Pellicon membranes, using an Immersible Molecular Separator Kit (Millipore (UK) Ltd, London). Polyacrylamide gel electrophoresis was done by the method of Davis (1964), using 70 g photopolymerized gel/l and electrolyte buffer containing 4.9 mm-Tris and 38.5 mm-glycine (pH 8.3). Gels were stained with Amido Black and were scanned using a Gilford 240 Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio, USA).

Subcellular fractionation of the frozen kidney cortex from each animal in Groups B and C was carried out by differential centrifugation. The kidney cortex was homogenized, with
Table 1. Effect of molybdenum and sulphate supplementation on plasma copper concentrations, caeruloplasmin activities and haematological status of sheep given a basal diet (treatment group A) or diets with added Mo or (Mo + SO₄²⁻) (treatment groups B and C)*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Mo supplement (mg/kg)</td>
<td>0</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Concentration of SO₄²⁻ supplement (g/kg)</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Total plasma Cu concentration (µg/ml)</td>
<td>0.89</td>
<td>1.10</td>
<td>1.14</td>
</tr>
<tr>
<td>Plasma TCA-soluble Cu concentration (µg/ml)</td>
<td>0.94</td>
<td>1.06</td>
<td>0.69</td>
</tr>
<tr>
<td>Caeruloplasmin activity (units/l)†</td>
<td>15.6</td>
<td>18.8</td>
<td>14.2</td>
</tr>
<tr>
<td>Blood haemoglobin concentration (g/l)‡</td>
<td>103</td>
<td>89</td>
<td>113</td>
</tr>
<tr>
<td>Packed cell volume‡</td>
<td>0.36</td>
<td>0.32</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* For details of diet see p. 326.
† One unit of activity is defined as the amount catalysing the transformation of 1 µmol substrate/min.
‡ Measured after 17 weeks only.

RESULTS

Body-weight gain. There were no significant differences in the growth rates of the lambs in the control group (A) and the Mo- and SO₄²⁻-supplemented group (C), their final weights being 60.5 ± 3.7 and 59.9 ± 2.8 kg respectively. Lambs in group B, receiving only the Mo supplement, grew more slowly than the other animals and their final weights were only 44.2 ± 3.1 kg.

Plasma Cu distribution. The total Cu concentrations in plasma were greatest in group C and least in group A (Table 1). These differences were evident within a few weeks and persisted throughout the experiment. As no consistent change in concentrations occurred during the 3- to 30-week period, the values quoted in Table 1 are based on the means of seven estimations for each animal over this period. Concentrations of TCA-soluble Cu in the plasma of groups A and B were similar and were significantly greater than those in group C (P < 0.05). There was good agreement between the concentrations of total and TCA-soluble Cu in both groups A and B, but in group C only about 50% of the total Cu was TCA-soluble. Although no significant differences were found in caeruloplasmin activities in the three treatment groups, the activities did tend to follow the concentrations of TCA-soluble Cu. Measurements of direct-reacting Cu were not made routinely throughout the experiment, but the concentrations at weeks 3 and 7 were similar with over-all means and standard errors in groups A, B and C of 0.19 ± 0.03, 0.16 ± 0.02 and 0.70 ± 0.23 µg/ml respectively.

Plasma Mo concentrations over the period 4–17 weeks were 0.90 ± 0.08 µg/ml in group C and 16.3 ± 0.9 µg/ml in group B. Concentrations in group A were too low (<0.2 µg/ml) for reliable estimation by the analytical method used (Bingley, 1959).

The haematological status of the lambs was assessed after 17 weeks. There was a slight,
Table 2. Effect of molybdenum and sulphate supplementation on the concentrations (mg/kg fresh tissue) of copper and Mo of liver and kidney cortex from sheep given a basal diet (treatment group A) and diets with added Mo or (Mo+SO₄²⁻) (treatment groups B and C)*

(Mean values for four sheep/treatment)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Approximate SE of differences between means</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Concentration of Mo supplement (mg/kg) 0</td>
<td>25</td>
</tr>
<tr>
<td>Concentration of SO₄²⁻ supplement (g/kg) 0</td>
<td>0</td>
</tr>
<tr>
<td>Liver Cu concentration 229</td>
<td>180</td>
</tr>
<tr>
<td>Liver Mo concentration 10</td>
<td>76</td>
</tr>
<tr>
<td>Kidney Cu concentration 50</td>
<td>60</td>
</tr>
<tr>
<td>Kidney Mo concentration 06</td>
<td>86</td>
</tr>
<tr>
<td>Kidney TCA-soluble Cu concentration 37</td>
<td>30</td>
</tr>
<tr>
<td>Kidney TCA-insoluble Cu concentration 13</td>
<td>30</td>
</tr>
</tbody>
</table>

* For details of diet see p. 326.
† Only three animals/treatment.

Fig. 1. Relationship between concentrations of copper and molybdenum in the kidney cortex of sheep receiving the basal diet (○) (group A), the basal diet plus Mo (□) (group B) or the basal diet plus Mo and sulphate (●) (group C); for details of diet, see p. 326.
Table 3. Effect of molybdenum and sulphate supplementation on the subcellular distribution of copper and Mo in the kidney cortex of sheep given a diet with added Mo (group B) or (Mo\(+SO_4^{2-}\)) (group C)*

(Mean values, with standard error, for four sheep/treatment)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>B</th>
<th>C</th>
<th>SE of differences between means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Cu (mg/kg fresh cortex)</td>
<td>6·0</td>
<td>9·6</td>
<td>1·8</td>
</tr>
<tr>
<td>Proportion of Cu in subcellular fractions:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear</td>
<td>0·16</td>
<td>0·22</td>
<td>0·04</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>0·23</td>
<td>0·33</td>
<td>0·03</td>
</tr>
<tr>
<td>Microsomal</td>
<td>0·09</td>
<td>0·08</td>
<td>0·02</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>0·52</td>
<td>0·37</td>
<td>0·06</td>
</tr>
<tr>
<td>Concentration of Mo (mg/kg fresh cortex)</td>
<td>8·6</td>
<td>4·6</td>
<td>1·8</td>
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<td>Proportion of Mo in subcellular fractions:</td>
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</tr>
<tr>
<td>Nuclear</td>
<td>0·13</td>
<td>0·20</td>
<td>0·04</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>0·16</td>
<td>0·39</td>
<td>0·02</td>
</tr>
<tr>
<td>Microsomal</td>
<td>0·11</td>
<td>0·10</td>
<td>0·03</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>0·60</td>
<td>0·31</td>
<td>0·03</td>
</tr>
</tbody>
</table>

* For details of fractionation and diet see p. 326.

but significant, decrease in haemoglobin concentrations and packed cell volumes of the animals in group B \((P < 0·01)\) (Table 1).

Tissue concentrations of Cu and Mo. In both liver and kidneys the concentrations of Mo were greatest in group B and least in group A (Table 2). The animals in group C had the lowest liver Cu concentrations but the greatest kidney Cu concentrations. There were, however, no differences between groups A and B in the concentrations of Cu in either the livers or kidneys of the lambs. There was a significant relationship between the concentrations of Cu and Mo in the kidneys of the lambs in group C (Fig. 1), which could be expressed by the equation: \(y = 1·54x + 2·38\) (SE of regression coefficient 0·25, \(P < 0·01\)), where \(x\) and \(y\) are the concentrations (mg/kg) of Mo and Cu respectively in the fresh kidney cortex. There was no correlation between Cu and Mo concentrations in the kidneys from group B.

Kidney Cu concentrations in one animal in group A (A 1) were 34 mg/kg which was nearly seven times that found in the other animals in this group and in the range encountered in Cu-intoxicated animals (Bremner, Young & Mills, 1976). This value was excluded in making the comparisons between groups.

As the occurrence of anomalous Cu-binding fractions in the plasma of group C animals could be surmised from their insolubility in 5 % TCA solution, the effect of this acid on the solubility of the Cu in the kidney homogenates was studied (Table 2). Concentrations of TCA-insoluble Cu were greatest in group C and there was a non-significant trend for the concentrations of TCA-soluble Cu to be greatest in group A. Only 0·30 of the Cu in samples from group C was therefore acid-soluble, compared with 0·50 and 0·70 from those in groups B and A respectively. It is noteworthy that in the kidneys from sheep A 1, 0·85 of the Cu was acid-soluble, suggesting that the lower solubility of Cu in group C was not a consequence of their greater Cu content.

Distribution of Cu and Mo in kidney. Subcellular fractionation of isotonic homogenates of the kidney cortex of animals in groups B and C showed that addition of SO_4^{2-} to the Mo-supplemented diet reduced the proportion of Cu in the cytosol from 0·52 to 0·37 and increased that present in the mitochondrial fraction \((P < 0·05)\) (Table 3). Similarly, the
Table 4. Effect of molybdenum and sulphate supplementation on the concentrations of copper in the sub-fractions isolated by gel filtration of the kidney cortex of sheep given a basal diet (treatment group A) or diets with added Mo or (Mo + SO\textsubscript{4}\textsuperscript{2−}) (treatment groups B and C)*

<table>
<thead>
<tr>
<th>Concentration of Mo supplement (mg/kg)</th>
<th>Concentration of SO\textsubscript{4}\textsuperscript{2−} supplement (g/kg)</th>
<th>No. of samples</th>
<th>Proportion of Cu in supernatant</th>
<th>Concentration of Cu (mg/kg kidney cortex) in Fraction 1</th>
<th>Concentration of Cu (mg/kg kidney cortex) in Fraction 2</th>
<th>Concentration of Cu (mg/kg kidney cortex) in Fraction 3</th>
<th>Proportion of Cu in fraction 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment group</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<td>3</td>
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<tr>
<td>B</td>
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<td>25</td>
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</tr>
<tr>
<td>C</td>
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<td>-</td>
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</tr>
</tbody>
</table>

* For details of diet and fractionation by gel filtration see p. 326.

Fig. 2. Separation on Sephadex G.75 of the supernatant fraction from a homogenate of kidney cortex from one sheep in treatment group A, receiving only the basal diet. Kidney copper concentration was 6.1 mg/kg. For details of experimental procedures, see p. 326; 7 ml fractions were collected. The positions of fractions 1-3 are shown.

Fig. 3. Separation on Sephadex G.75 of the supernatant fraction from a homogenate of kidney cortex from one sheep in treatment group C, receiving the basal diet with 25 mg molybdenum and 5 g sulphate/kg. Kidney copper concentration was 14.2 mg/kg. For details of experimental procedures, see p. 326; 7 ml fractions were collected. The positions of fractions 1-3 are shown.
Fig. 4. Separation on Sephadex G.100 of the supernatant fraction from a homogenate of kidney cortex (12.7 mg copper/kg) from one sheep in treatment group C, receiving the basal diet supplemented with 25 mg molybdenum and 5 g sulphate/kg. For details of experimental procedures, see p. 326; 5 ml fractions were collected. The concentrations of total Cu (○), TCA-soluble Cu (○), TCA-insoluble Cu (△) and total Mo (■) and the positions of fractions 1–3 are shown.

proportion of Mo present in the cytosol of the sheep in group C was only half of that in group B (P < 0.001), whereas the proportion in the mitochondrial fraction was more than doubled to nearly 0.40. The proportions of Cu and Mo present in the nuclear fractions were usually less than those in the mitochondrial and cytosolic fractions and tended to increase on the addition of SO₄²⁻ to the diet, although this trend was not significant.

Fractionation of the Tris-acetate homogenates of the kidney cortex revealed a similar decrease in the proportion of Cu present in the cytosol from the Mo- and SO₄²⁻-supplemented sheep (Table 4). Separation of the cytosol on Sephadex G.75 yielded three Cu-containing fractions (1–3) similar to those described previously for sheep liver (Bremner & Marshall, 1974). Examples are shown in Figs 2 and 3 for kidneys from groups A and C respectively. The mean concentrations of Cu in fractions 1–3 are given in Table 4. There was no significant difference between treatment groups in the concentrations of Cu in fraction 3, despite the elevated Cu content of the group C kidneys. A greater proportion of the total Cu was therefore present in fraction 3 in the kidneys of groups A and B compared with those of group C.

The concentrations of Cu in fraction 2 were not affected by treatment, but concentrations in fraction 1, which contained high-molecular-weight proteins, were greatest in group C (P < 0.05) (Table 4). While about 0.9 of the Cu in fraction 1 from the kidneys of group A was generally soluble in acid solution, only about 0.1 of the Cu in fraction 1 was acid-
soluble in samples from groups B and C. The Mo present in the cytosol of the kidneys from groups B and C was detected only in fraction 1 (not shown). This was demonstrated more clearly when the kidney cytosol of group C was fractionated on Sephadex G.100, which gave better resolution of the high-molecular-weight proteins. Three main Cu-containing fractions were still obtained, with a large proportion of the Cu occurring in fraction 1, which was eluted at the void volume of the column and therefore had a molecular weight of over 150000 (Fig. 4). Although two separate peaks of TCA-soluble Cu were detected within this fraction, they only accounted for about 0.1 of the total Cu, whereas all the Cu in fractions 2 and 3 was acid-soluble. Mo was present only in fraction 1, and its elution profile tended to follow that of the acid-insoluble rather than acid-soluble Cu.

**Distribution of Cu and Mo in plasma.** Fractionation of the plasma from the Mo- and SO_4^{2-}-supplemented lambs on Sephadex G.200 revealed the presence of five Cu-containing fractions (Fig. 5). One of these was eluted at the void volume of the column (fraction 1) and one (fraction 3) contained all the caeruloplasmin activity (not shown). Fraction 5 had the same elution volume as bovine serum albumin and the approximate molecular weights of fractions 2 and 4 were calculated from their elution volumes to be 177000 and 90000 respectively. On the polyacrylamide gel electrophoresis of fraction 5 (tube 43), only one protein band was detected (Fig. 6), and this had the same mobility as plasma albumin. The same protein band occurred also in fraction 4, but other proteins were also present in large amounts (Fig. 6). The mobilities of these suggested that they consisted of a mixture of lipoproteins and α- and β-globulins. It was not possible to determine with which of these proteins the Cu was associated. The relative proportions of Cu in fractions 1-5 were variable,
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Fig. 6. Separation by polyacrylamide gel electrophoresis of fraction 4 (a) and fraction 5 (b) from the plasma of a sheep in treatment group C receiving the basal diet with 25 mg molybdenum and 5 g sulphate/kg. For details of experimental procedures see p. 326. Fractions 4 and 5 were isolated by gel filtration on Sephadex G.200 (see Fig. 5). Acrylamide concentrations were 7% and the electrolyte buffer contained 4.9 mM-Tris and 38.5 mM-glycine (pH 8.3). Gels were stained with Amido Black and scanned as described on p. 326.

but fractions 1 and 2 together never accounted for more than 0.2 of the total plasma Cu. Less than half of the total Cu was usually present as caeruloplasmin. In the separation shown in Fig. 5 of a plasma sample containing 1.64 µg Cu/ml, the proportions of Cu in fractions 1–5 were 0.06:0.08:0.36:0.36:0.14 respectively. Practically all the Cu present in fractions 1, 2, 3 and 5 was acid-soluble, whereas only about half of the Cu in fraction 4 was soluble. This fraction therefore contained most of the TCA-insoluble Cu in the plasma.

About half of the Mo in the plasma was in protein-bound form and was eluted in fractions 1–5 (Fig. 5). About two-thirds of this was present in fraction 4 (0.31 µg Mo/ml plasma in the sample described in Fig. 5) with the rest distributed among the other fractions. The remainder of the plasma Mo was eluted near the total volume of the column, along with low-molecular-weight peptides and amino acids (not shown). In a separate study involving the ultrafiltration of five plasma samples, containing 1.00±0.25 µg Mo/ml, 0.54±0.06 of the Mo was found to be in diffusible form, a finding which is consistent with the gel filtration results.

Fractionation on Sephadex G.200 of plasma from sheep fed on the control diet yielded only three Cu-containing fractions (1–3). The elution profile was similar to that shown in Fig. 5, except that no detectable Cu was found in fractions 4 and 5. The relative proportions of Cu in fractions 1–3 were almost identical to that shown in Fig. 5. Caeruloplasmin was detected only in fraction 3 and all the Cu was soluble in 5% TCA.

DISCUSSION

These results confirmed that Mo and SO₄²⁻ supplementation of sheep diets can cause an increase in plasma Cu concentrations (Dick, 1956; Suttle & Field, 1968; Smith et al. 1968). Caeruloplasmin activity was unaffected, the additional Cu in the plasma occurring as direct-reacting Cu and in a form where the Cu was insoluble in TCA solution, as reported by Smith & Wright (1975a, b). Less than one-half of the plasma Cu in the Mo- and SO₄²⁻-supplemented sheep was found to be in the caeruloplasmin-containing fraction after separation of the plasma on Sephadex G.200. Practically all the additional Cu present in the plasma of these animals, including that which was insoluble in TCA, was present in two fractions of lower molecular weight, eluted from the column just before or with the albumin.
The presence of only one protein band on gel electrophoresis of fraction 5, with the same mobility as plasma albumin, strongly suggests that the Cu in this fraction was bound to albumin. This is consistent with the increased plasma concentrations of direct-reacting Cu in these animals and the claim that this consists mainly of albumin-bound Cu (Gubler, Lahey, Cartwright & Wintrobe, 1953).

Albumin was also present in fraction 4, but in view of the differences in the elution profiles of the Cu of fraction 4 and plasma albumin, it seems more likely that the Cu was associated with some of the other proteins present in this fraction as shown in Fig. 6. These had an approximate molecular weight of 90000. They were not identified but it is significant that most of the protein-bound Mo was also present in fraction 4. It is possible therefore, that both metals were associated with the same protein or proteins, but further studies will be required to establish this with certainty.

It is commonly accepted that plasma Cu is bound mainly to caeruloplasmin and to a lesser extent to albumin (Underwood, 1971). However, in the Mo- and SO$_4^{2-}$-treated sheep this was clearly not so. Furthermore, even in the control sheep in this experiment, other Cu-binding proteins were detected in fractions 1 and 2. These were not identified, but they accounted for about 0.2 of the total plasma Cu and contained only TCA-soluble Cu.

Although the TCA-insoluble Cu in the plasma tended to be associated with Mo-containing proteins, only about half of the Mo in the plasma from group C animals was protein-bound. The remainder was present in an ultrafiltrable form, possibly as inorganic MoO$_4^{2-}$ as reported by Scaife (1956), and had no effect on the TCA-solubility of Cu when added to control plasma (unpublished observations). It is noteworthy that no TCA-insoluble Cu was present in the plasma of sheep receiving Mo alone, despite their high plasma Mo concentrations.

Dietary supplementation with Mo and SO$_4^{2-}$ can influence Cu metabolism in many ways. In this experiment, Cu concentrations in liver were decreased and those in kidney increased, which is in accord with previous observations (Smith et al. 1968; Marcilese et al. 1969, 1970). It has also been found by others that plasma $^{44}$Cu clearance rates may be decreased, synthesis of caeruloplasmin impaired and urinary excretion of Cu increased in animals on similar diets (Smith et al. 1968; Marcilese et al. 1969, 1970). This sequence of events could arise if a particular Cu fraction in plasma was unavailable for normal metabolic processes and was taken up by the kidney before its excretion in the urine. This view is supported by the existence in the kidney of a relationship between Cu and Mo similar to that noted in the plasma. The increase in kidney Cu concentration was related to the Mo concentration, the atomic ratio of the increment in Cu to that of Mo being about 2. Furthermore, the additional renal Cu was largely insoluble in TCA solution and, more importantly, the Cu and Mo tended to accumulate together in the same sub-fractions after subcellular fractionation and gel filtration of the cytosol. The distribution of Cu was therefore quite different from that normally observed when kidney Cu concentrations are increased, as most of the Cu is then associated with metallothionein (fraction 3 in Figs 2 and 3) (Bremner, Hoekstra, Davies & Williams, 1977). Finally, the effects of Mo on the concentration and distribution of Cu in the kidneys, as in the plasma, occurred only in the animals receiving both Mo and SO$_4^{2-}$ supplements.

Although there were similarities in the properties of the soluble Cu- and Mo-containing proteins in kidney and plasma, the proteins had different molecular weights and were clearly not identical. The observed results may therefore reflect a general phenomenon in which Cu and Mo occur together in a range of proteins and subcellular particles. Mills & Mitchell (1971) also found that these metals accumulated in relatively constant stoichiometric ratio in the subcellular organelles of the liver of Mo-supplemented rats. It is possible that the Mo-induced changes in plasma and tissue Cu distribution are a consequence of the forma-
tion of molybdenoproteins with a high affinity for Cu. These probably require S for the Mo or Cu linkage, as Cu distribution was unchanged in the animals receiving no supplementary S.

It may be that the formation of thiomolybdates in the rumen is responsible for some of these effects of Mo on Cu distribution, as suggested by Dick, Dewey & Gawthorne (1975). This suggestion was based partly on the observation that plasma concentrations of TCA-insoluble Cu were increased in animals treated with thiomolybdate, which does not necessarily reflect a change in Cu distribution (El-Gallad, Bremner & Mills, 1977). However, more recent studies (Mills, Bremner, El-Gallad, Dalgarno & Young, 1977) have provided additional evidence supporting the view that Mo-induced changes in Cu metabolism may arise from the formation of thiomolybdates or related compounds in the intestinal tract.

The addition of SO₄²⁻ to the Mo-supplemented diet had a beneficial effect on both the growth and haematological status of the sheep and markedly influenced Mo metabolism. The growth rate of the animals receiving only the Mo supplement (group B) was significantly less than that of the control animals, but was restored to normal by addition of SO₄²⁻ to the diet. This toxic effect of Mo, which occurred without changes in Cu metabolism, may have been associated with the increased tissue and plasma Mo concentrations in these animals. Scaife (1956) also found that daily intakes of 10–50 mg Mo had adverse effects on the growth of sheep receiving a low S diet, with increased excretion of Mo occurring when a diet containing adequate S was fed. These findings are similar to those observed in rats and other simple-stomached animals, in that dietary SO₄²⁻ supplementation alleviated some symptoms of the toxicity of Mo (Van Reen & Williams, 1956). It is more commonly found in ruminant animals that dietary S supplementation exacerbates the toxicity of the metal (Wynne & McClymont, 1956). It may be that the nature of the S–Mo interaction is dependent on the S content of the basal diet, as this was relatively low in the present experiment. A reduction in haematological status has occasionally been reported in sheep receiving Mo + SO₄²⁻ supplements (Suttle & Field, 1968), and Dowdy & Matrone (1968) found that SO₄²⁻ had a synergistic effect on Mo-induced anaemia, but only at low levels of Mo intake.

The large reduction in plasma Mo concentrations in the sheep receiving Mo + SO₄²⁻, compared with those receiving Mo alone, is in accord with the findings of Dick (1956) and his claim that there is a common transport system for MoO₄²⁻ and SO₄²⁻ at the renal level. However, Dick reported increased kidney Mo concentrations in the SO₄²⁻-supplemented animals, whereas in this experiment they were reduced. This reduction occurred mainly in the cytosolic fraction, and there was an increase in the Mo content of the mitochondrial fraction.

It may appear that some of the differences found between treatment groups A and C could be attributed solely to the difference in SO₄²⁻ intakes of the animals and be independent of their Mo intakes, as no animals received only the SO₄²⁻ supplement. However, it has previously been demonstrated in comparable experiments with sheep that the addition of SO₄²⁻ to the diet has no effect on the concentrations of Cu in plasma or kidney (Suttle & Field, 1968; Marcilese et al., 1969, 1970).

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