SULPHUR METABOLISM AND EXCRETION STUDIES
IN RUMINANTS

III. The Effect of Sulphur Intake on the Availability of Copper in Sheep

P. R. BIRD*

Summary

In Merino wethers, increasing the intake of cystine-S or inorganic SO₄⁻S under steady state feeding conditions significantly increased ruminal sulphide concentrations and decreased the flow of soluble copper to the omasum. The curvilinear regression of soluble Cu outflow (y, mg/day) on ruminal S= levels (x, μg/ml) was y = 5.57 -2.36x + 0.491x² -0.0333x³ (r = 0.95; P<0.001), but the flow of soluble Cu was not significantly correlated with the flow of protein from the rumen (r = 0.48). A highly significant depression in potentially available Cu occurred when the S-intake was increased from 0.6 g/day to 1.95 to 3.4 g/day but levels of ruminal S= greater than about 2μg S/ml did not further reduce this flow below approximately 1.75 mg soluble Cu/day.

The significance of increased dietary total sulphur intake per se upon the Cu status of sheep is discussed.

I. INTRODUCTION

The influence of inorganic SO₄²⁻ and MoO₄²⁻ on the absorption and retention of Cu in sheep was first demonstrated by Dick (1953 a, b). Subsequent work (Dick 1954 a, b, 1956 a, b; Wynne and McClymont 1955, 1956; Mylrea 1958; Cunningham, Hogan and Lawson 1959a, b; Evans and Davis 1963; Hogan, Money and Bluyney 1968) indicated that increasing the dietary SO₄²⁻ and MoO₄²⁻ intake reduced the rate of liver Cu storage. An interaction between inorganic SO₄²⁻ and MoO₄²⁻ has been clearly demonstrated in the sheep with respect to the mobilisation of Cu (Dick 1954 a), the reduction of liver and blood Cu levels and the production of dystrophic wool (Wynne and McClymont 1956). A similar effect upon liver Cu storage in cattle was shown by Mylrea (1958). Some of the data of Dick (1953 b, 1954 a) and of Wynne and McClymont (1956) indicate that the addition of inorganic SO₄²⁻ reduced liver Cu storage.

Inorganic SO₄²⁻ may impede the membrane transfer of MoO₄²⁻ (Dick 1954 b, 1956 a), and thus or otherwise impede the transport and storage of Cu (Mills 1960; Spais, Lazaridis and Agiannidis 1968). Mills (1960) observed that MoO₄²⁻

* Institute of Agriculture, University of Western Australia, Nedlands, Western Australia.
supplementation increased ruminal sulphide levels and suggested that MoO₄²⁻ may increase the rate of SO₄²⁻ reduction. Inorganic SO₄²⁻ is readily reduced to sulphide in the rumen (Spais, Lazaridis and Agiannidis 1968), little SO₄²⁻ is absorbed as such from the rumen (Bray 1969), and little SO₄²⁻ flows out of the forestomach (Bird and Hume 1970; Bird and Moir 1970). Most inorganic SO₄²⁻ ingested passes through the ruminal S= pool prior to absorption. When iron sulphide is fed, the rate of Cu accumulation in the liver is significantly reduced (Dick 1954 a).

The long term effect of SO₄²⁻, administered either intravenously or orally to sheep, is to increase Mo retention (Dick 1956 a). In the rat, an increased concentration of Mo in the body may decrease the activity of the Cu-containing enzyme sulphide oxidase, thereby inactivating tissue Cu in the insoluble CuS form (Mills 1960; Spais, Lazaridis and Agiannidis 1968).

An examination has now been made of the influence of the level and form of dietary sulphur upon the amount of Cu potentially available for absorption by the sheep.

II. MATERIALS AND METHODS

(a) Experimental Conditions

The collection procedures and diets were described in detail by Hume and Bird (1970). A 4 x 4 Latin square design was employed, using mature Merino wethers fitted with omasal and ruminal cannulae. The four treatments differed only in the amount and form of sulphur (Table 1) added to the basal diet which contained 2.6 per cent N, half as gelatin and the remainder as urea. The mineral supplement supplied 10 p.p.m. Cu (as CuCl₂) and 1.9 p.p.m. Mo (as Na₂MoO₄). The actual daily intakes are shown in Table 1. The animals were fed 65 g D.M. at two-hour intervals to simulate steady state conditions, and Cr⁵¹ EDTA was used to estimate the rate of fluid flow.

(b) Analytical Procedures

Sulphide content of strained rumen fluid and omasal digesta was determined immediately after collection by the method of Bird and Fountain (1969).

The omasal samples collected each day were combined within each treatment and centrifuged at 35,000 x G for 35 min to precipitate cellular debris and insoluble copper sulphides. The soluble Cu content of the cell-free supernatant was determined by atomic absorption spectroscopy (AAS).

Samples of the drinking water were analysed for Cu content and duplicate 1 g samples of the diets were digested with the reagents described by Eden and Green (1940) and diluted to 50 ml for copper and molybdenum analyses by

<table>
<thead>
<tr>
<th>Treatment</th>
<th>S-source</th>
<th>S-intake(g)</th>
<th>Cu-intake(mg)</th>
<th>Mo-intake(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Basal</td>
<td>0.61</td>
<td>8.70 (±0.07)</td>
<td>2.0</td>
</tr>
<tr>
<td>B</td>
<td>A++++inorganic SO₄</td>
<td>1.94</td>
<td>8.80 (±0.10)</td>
<td>2.0</td>
</tr>
<tr>
<td>C</td>
<td>A++cystine</td>
<td>1.95</td>
<td>8.74 (±0.10)</td>
<td>2.0</td>
</tr>
<tr>
<td>D</td>
<td>A+ SO₄+cystine</td>
<td>3.42</td>
<td>8.78 (±0.11)</td>
<td>2.0</td>
</tr>
</tbody>
</table>
AAS. The sensitivity for 1 per cent absorption was 0.08 p.p.m. Cu and 0.7 p.p.m. Mo.

The recovery of Cu added to strained rumen fluid (SRF) and cell-free supernatant (CFS) was examined. Rumen fluid collected from sheep 24 h after giving feed containing 1 g total S was gassed with N₂ for 2 h to remove free H₂S and 5 mg Cu (as CuSO₄) was then added to a 500 ml portion of the SRF. In a second experiment, rumen digesta collected 6 h after feeding a ration containing 3 g total S were heated at 80°C for 20 min to inactivate the microflora, the pH was adjusted to 4.5 with HC₁ and N₂ gas was bubbled through the SRF before centrifuging one portion at 35,000 x G. Five mg Cu (as CuCl₂) was then added separately to 500 ml of SRF and 500 ml of CFS after determining the S= concentration in each solution. Sulphide, from the reduction of 200 μg SO₄—S, was passed into open tubes containing 20 ml of SRF or CFS prior to centrifugation. Cu analyses are recorded in Table 2 and Cu determinations were corrected for the recovery found.

III. RESULTS

The average daily intake of Cu, Mo and S is shown in Table 1. There were no significant differences in daily water intake, feed intake or the intake of either Cu or Mo.

Inorganic sulphate treatments (B and D) increased ruminal (P<0.0 1) and omasal S= levels (P<0.01), but cystine treatments (C and D) increased both ruminal (P<0.001) and omasal S= concentration (P<0.001) even further (Table 3). The rate of reduction of cystine is apparently greater than that of sulphate.

Likewise, SO₄= (B and D) and cystine (C and D) reduced the concentration of soluble Cu in omasal digesta (P<0.01 and P<0.001 respectively) and the flow of soluble Cu to the abomasum (P<0.05 and P<0.001 respectively) (Table 3). The regression of soluble Cu outflow (γ, mg/day) on ruminal S= concentration (x μg S/ml) is shown in Figure 1. While the linear regression γ = 4.18 — 0.51 x (r = 0.79) is highly significant (P<0.001), the curvilinear

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SRF</td>
<td>SRF</td>
</tr>
<tr>
<td>B + C</td>
<td>0.120</td>
<td>0.106</td>
</tr>
<tr>
<td>B + H₂S + C</td>
<td>—</td>
<td>0.071</td>
</tr>
<tr>
<td>B + Cu</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B + Cu + C</td>
<td>2.871</td>
<td>1.440</td>
</tr>
<tr>
<td>B + Cu + H₂S + C</td>
<td>2.462</td>
<td>0.753</td>
</tr>
</tbody>
</table>

B — basal solutions  
C — centrifuged solutions  
Cu — 10 p.p.m. as CuSO₄ (in experiment 1) or CuCl₂ (in experiment 2)  
H₂S — 10 p.p.m. S= added to the solutions
TABLE 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Osmal Cu concentration (µg/ml)</th>
<th>Osmal S= concentration (µg/ml)</th>
<th>SRF S= concentration (µg/ml)</th>
<th>Cu flow (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.660±0.056</td>
<td>0.58±0.06</td>
<td>0.54±0.06</td>
<td>4.7±0.196</td>
</tr>
<tr>
<td>B</td>
<td>0.37±0.02</td>
<td>3.02±1.03</td>
<td>1.91±0.05</td>
<td>2.83±0.048</td>
</tr>
<tr>
<td>C</td>
<td>0.275±0.012</td>
<td>5.33±1.36</td>
<td>4.26±1.07</td>
<td>1.92±0.089</td>
</tr>
<tr>
<td>D</td>
<td>0.280±0.031</td>
<td>5.95±0.89</td>
<td>3.47±0.56</td>
<td>2.03±0.144</td>
</tr>
</tbody>
</table>

Each result is the mean of four observations ± S.E.m. Similar superscripts within columns indicate significant differences between treatments. viz. a,b,c — P<0.001
d,e,f — P<0.01
g,h — P<0.05

regression y = 5.57 - 2.36x - 0.49x² - 0.0333x³ (r = 0.95) gives a better fit to the data, and the additional precision gained is significant (P<0.05).

The precipitation of cellular debris by centrifugation resulted in the loss of 72 to 87 per cent of the 10 p.p.m. Cu added to SRF (Table 2). A further 4 to 7 per cent loss resulted from the addition of 10 p.p.m. S= prior to centrifugation. Only 9 per cent of the 10 p.p.m. Cu added to CFS was apparently precipitated in the absence of added H₂S, but 35 per cent in its presence. The sulphide concentrations in the basal SRF and CFS were 0.3 and 0.2 µg/ml respectively. The extent of Cu precipitation by cellular material is in accord with the report of Mills (1958) who showed that more than 50 per cent of total Cu in rumen liquor and about 70 per cent in abomasal liquor was associated with microbial and plant cell debris.

IV. DISCUSSION

The “soluble” Cu measured in this experiment is taken to represent the Cu potentially available to the sheep, assuming that all precipitable Cu is physiologically inert.

Most of the Cu in the diet was inorganic, given as CuCl₂ in the mineral addition. Organic ligands complex ingested Cu⁺⁺ (Mills 1958, 1960) so that little, if any, free ionic Cu is found in the rumen. The water-soluble complexed Cu is readily absorbed by the rat (Mills 1958). However, the Cu added to the rumen fluid (Table 2) was largely adsorbed by and precipitated from solution with cellular debris, even in the absence of sulphide. In the absence of cellular material (CFS), the recovery of Cu in the water soluble phase was decreased by 26 per cent
after the addition of sulphide. It appears, therefore, that some of the Cu organic complex is also precipitated in the presence of $H_2S$. Nascent sulphide (0.2 p.p.m.) could account for the 0.5 p.p.m. "loss" of Cu from CFS after centrifugation.

The results from Table 3 and Figure 1 indicate that under steady state feeding conditions when ruminal $S^-$ concentrations exceed about 2 $\mu g/ml$ there is no further limitation to the solubility of ingested Cu. It is clear that low ruminal $S^-$ concentrations are very significantly associated with a greater flow of soluble Cu from the rumen and that, up to a certain level at least, there is a diminution in that flow of Cu by increasing the dietary intake of $S$ as cystine or inorganic $SO_4^{2-}$. It follows that, since most of the ingested $S$ passes through the rumen $S^-$ pool, the total sulphur content of pastures is a better parameter than the inorganic $SO_4^{2-}$ content in assessing potentially available Cu, particularly since the inorganic to
organic S ratio in plants may fluctuate according to the SO₄²⁻ and inorganic N supply for protein synthesis. Thus, when N is limiting, the addition of sulphur markedly increases tissue SO₄²⁻. When N is adequate, the proportion of the organic S is greater (Stewart and Porter 1969).

It is possible that the effect of extra sulphide is to produce more ruminal protein. Thus, in vitro work (Table 2) suggests that while H₂S decreases Cu solubility, protein or cell absorption effects per se are of greater importance. In the in vivo experiment (Figure 1), the linear regression of soluble Cu outflow on ruminal sulphide level was not statistically as good a fit as the curvilinear regression. This may indicate that factors other than the direct effect of sulphide are involved. The mean daily microbial protein production in the rumen was 82, 97, 91 and 98 g for treatments A, B, C and D respectively (Hume and Bird 1970). The basal treatment differed significantly (P < 0.05) from the other treatments. However, the linear regression of soluble Cu outflow (y, mg/day) on ruminal protein production (x, g N x 6.25/day) y = 7.49 - 0.05x (r = 0.48) was not significant, neither was the regression of ruminal sulphide level on ruminal protein production (r = 0.27). It is concluded, therefore, that while the presence of particulate material probably accounts for the precipitation of most of the ingested Cu, the additional effect of sulphide per se significantly reduces the amount of Cu available to the animal.

Of the 8.75 mg intake, about 1.75 mg of soluble Cu flows from the rumen per day and is thus potentially available to the sheep at a S²⁻ concentration of 7 µg/ml (Figure 1). The availability of the precipitated Cu fraction is here assumed negligible, but requires investigation, as well as possible precipitation or release in the lower tract. Dick (1954a) found that in crossbred sheep on daily intakes of 0.2 mg Mo and 1.4 g inorganic sulphate, Cu balance could be obtained at Cu intakes as low as 0.5 mg/day.

While increased ruminal S²⁻ concentrations decrease the amount of Cu which may be absorbed, further effects of Mo and SO₄²⁻ upon Cu metabolism within the tissues appear necessary in order to either increase the rate of depletion of body Cu storage or to decrease the availability of stored Cu to produce a Cu deficiency in the sheep.

V. ACKNOWLEDGMENTS
The work was supported by the Australian Meat Research Committee and Sulphur Institute funds. Analytical assistance from Mr. M. Finucane, and advice from Professor R. J. Moir in the preparation of this paper is gratefully acknowledged.

VI. REFERENCES