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Comparative studies of copper, molybdenum and sulphur metabolism in the ruminant and the rat

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It is now firmly established that high levels of molybdenum and sulphate in the diet of the sheep result in the depletion of tissue copper stores and the appearance of signs of copper deficiency. The mechanism of action of molybdenum and sulphate in producing these effects is unknown and has been the subject of much speculation (see, e.g., Dick, 1956).

Attempts have been made to study these interactions in non-ruminant animals such as the rat (Gray & Daniel, 1954; Van Reen, 1954; Miller, Price & Engel, 1956;

Mills, Monty, Ichihara & Pearson, 1958), the chick (Arthur, Motzok & Brannion, 1958; Davies, Reid, Kurnick & Couch, 1960) and the rabbit (Arrington & Davis, 1953; Feaster & Davis, 1959). These studies have indicated that the ingestion of food supplemented with molybdenum may cause inhibition of growth, failure of haemoglobin synthesis and the production of skeletal abnormalities. The appearance of these physiological defects is undoubtedly related to an interference with copper metabolism since a concomitant increase in dietary copper level limits, or may entirely prevent, their development. It has, however, been our experience and that of Miller *et al.* (1956) that molybdenum promotes copper accumulation in the liver of the rat, and these findings suggest that molybdenum prevents the physiological utilization of copper after its absorption into body tissues. This enhanced storage of copper in the rat is in marked contrast to the effects of giving supplementary molybdenum to the ruminant under most conditions, for tissue copper reserves usually become depleted in these animals. A further contrast appears when the effects of dietary sulphate on the copper–molybdenum relationships are compared. In the sheep an increase in sulphate intake precipitates a rapid decline in liver copper content when dietary molybdenum is high (Dick, 1956; Wynne & McClymont, 1956) whereas in the rat (Van Reen & Williams, 1956; Miller *et al.* 1956) supplementary sulphate prevents the abnormal copper accumulation in the liver associated with a high molybdenum intake, at the same time improving the rate of growth and permitting normal haemoglobin production and skeletal development. Sulphate supplementation does not fully restore normal growth in the rat and chick (Davies *et al.* 1960) at extremely high levels of dietary molybdenum (above 1000 p.p.m.) but at lower levels the improvement in physiological performance is dramatic. In both the rat and the sheep, sulphate supplementation of the diet leads to an increase in urinary excretion of molybdenum and a fall in the tissue content of molybdenum.

In view of these marked species differences in the action of sulphate, investigations were undertaken to see whether they were associated with different metabolic fates of sulphate in the digestive tract. In the ruminant, sulphate is rapidly reduced to sulphide by the micro-organisms of the reticulo-rumen (Lewis, 1954; Anderson, 1956) whereas in the non-ruminant animal sulphate reduction only proceeds to a much more limited extent in the caecum and colon. Hence we examined the effects of supplementing rat diets with both molybdenum and sulphide. A semi-synthetic purified diet supplemented with copper to a concentration of 3 p.p.m. was used in these studies. The quantity of calcium sulphide given alone was insufficient to affect physiological performance. Animals given both molybdenum (35 p.p.m.) and sulphide (64 p.p.m.) increased in weight rapidly for a short period after which a marked anaemia developed, weight gains ceased and within 7 weeks all animals receiving this treatment had died (Fig. 1). Supplementation of the diet with molybdenum alone caused a less dramatic restriction of weight increase and haematopoiesis, and no deaths occurred on this treatment. Increasing the copper concentration of the basal diet to 25 p.p.m. when sulphide and molybdenum were given simultaneously prevented any interference with haematopoiesis, and weight increased normally. No deaths occurred of animals so treated.
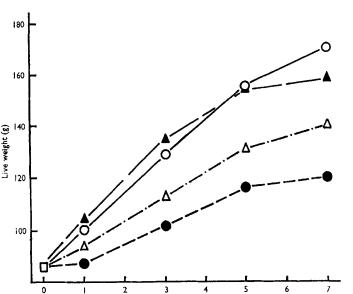
Fig. 1. Influence of dietary supplements of copper, molybdenum and sulphide on the weight increase of the rat. ○——○, 3 p.p.m. Cu + 2 μmoles sulphide/g diet; △—·—·—△, 3 p.p.m. Cu + 35 p.p.m. Mo/g diet; ●— — —●, 3 p.p.m. Cu + 35 p.p.m. Mo + 2 μmoles sulphide/g diet; ▲— — —▲, 25 p.p.m. Cu + 35 p.p.m. Mo + 2 μmoles sulphide/g diet.

In later studies the influence of supplementation of the diet with molybdenum upon the activity of a number of enzyme systems in the liver and kidney concerned with the metabolism of sulphur compounds was investigated (Mills *et al.* 1958). The most interesting finding was that the activity of the sulphide-oxidizing system of the rat liver was markedly depressed by molybdate. This finding, coupled with the results of biological trials, leads to the suggestion that the detoxication of sulphide may be restricted in tissues high in molybdenum content. Further support to this postulate is now forthcoming from the work of Halverson, Phifer & Monty (1960) who found that diets high in cystine, which would be expected to lead to excessive endogenous generation of sulphide in the liver through the activities of the cysteine-desulphydrase system, cause anaemia, diarrhoea and death if molybdenum is simultaneously given. These effects can also be prevented or reversed by increasing the copper content of the diet. The authors suggest that the signs described result from an induced copper deficiency, with this element accumulating in the liver as an insoluble sulphide. In this study the magnitude of treatment effects can once again be related to amount of liver 'sulphide oxidase' activity (Dr K. J. Monty, personal communication).

Among other enzyme systems found to be influenced by high dietary levels of molybdenum are alkaline phosphatase, the activity of which is markedly increased in the liver but depressed in the kidney (Van Reen, 1954; Mills *et al.* 1958), and liver

cytochrome oxidase which is depressed in the later stages of molybdenum intoxication. It is of interest that Gallagher, Judah & Rees (1956) found that liver cytochrome-oxidase activity was drastically reduced in the copper-deficient rat.

Wilson & Bandurski (1958) have found that the enzyme system responsible for the formation of 3′-phosphoadenosine-5′-phosphosulphate—the active intermediate of sulphate metabolism—is strongly inhibited by molybdate in vitro. They found that this overall reaction, the first step of which is to couple sulphate with adenosine triphosphate (ATP) to form adenosine 5′-phosphosulphate, is inhibited by molybdate owing to the formation of an unstable adenosine phosphomolybdate which decomposes to yield pyrophosphate and adenosine monophosphate as products. In essence this reaction would lead to a rapid reduction of tissue ATP levels, but studies by Williams & Van Reen (1956) so far suggest that ATP supplies in animals given high-molybdenum diets are normal. The significance of this mechanism in molybdenum intoxication is thus questionable.

The effects of molybdenum on sulphide metabolism in the rat prompted the examination of the possible similar relationships in the sheep. In this work one group of sheep has been fed on a grass-cube diet supplemented with sodium sulphate to give a daily intake of 10 g of the sulphate ion. A second group of animals received cubes supplemented with sulphate and with molybdate to give 50 mg molybdenum daily. The influence of these treatments on the sulphide level in the rumen and abomasum and on the soluble-copper content in the aqueous phase of the contents of these organs has been examined.

After a period of 1 month with the experimental diets to permit stabilization of rumen microflora associated with specific treatments, the sulphide content of unsieved rumen and abomasum liquor was determined at intervals throughout 24 h periods by a microdiffusion technique. The results for five such sets of 24 h readings for groups of four sheep are plotted in Fig. 2. There was a distinct trend towards
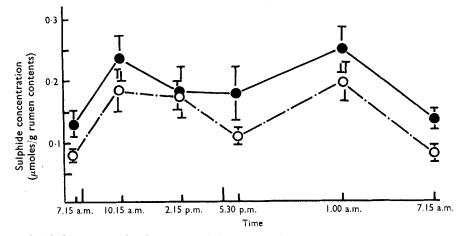

Fig. 2. Sulphide concentration in rumens of sheep given daily 1 kg grass cubes with a supplement of sulphate or molybdenum and sulphate. O—·—·—O, 10 g sulphate; ●——●, 50 mg Mo + 10 g sulphate; feeding times are indicated by the vertical lines at 7.45 a.m. and 4.30 p.m. The standard deviation for values about each point is shown.

the maintenance of higher sulphide levels in the rumen contents of sheep receiving a molybdate supplement, both during the periods of 'peak' sulphide level between 4 and 8 h after feeding and in the periods when sulphate reduction was not keeping pace with its rate of removal by microflora or by absorption into the host animal. Preliminary results obtained by Miss M. Purdom (personal communication) using washed suspensions of rumen micro-organisms from these animals suggest that the maintenance of these higher levels of sulphide may, at least in part, have been due to a more rapid rate of sulphate reduction by organisms from animals receiving molybdenum.

While this work was in progress it was hoped that it would be possible to determine the effects of these dietary treatments on the proportional distribution of copper between the aqueous and solid phases of rumen and abomasum contents. It proved impracticable, owing to poor duplication of values obtained for the solid phase, a difficulty probably arising from the large differences in the copper content of microbial and plant residues which settle from suspensions at widely differing rates (Mills, 1958). Determinations of the copper content of the aqueous phase alone indicated that molybdenum and sulphate depressed the content of soluble copper in both these organs (Table 1). It appears unlikely that this effect was due to changes in the

Table 1. *Mean values with their standard errors for the copper content of the aqueous phase of rumen and abomasum contents of groups of four sheep, after centrifuging for 30 min at 27 000 g*

Group no.	Daily dietary supplement	Rumen contents		Abomasum contents	
		$\mu g/100$ ml	$\mu g/g$ dry matter	$\mu g/100$ ml	$\mu g/g$ dry matter
1	10 g sulphate	$3\cdot4 \pm 0\cdot3$	$2\cdot3 \pm 0\cdot3$	$2\cdot6 \pm 0\cdot4$	$1\cdot7 \pm 0\cdot3$
2	10 g sulphate + 50 mg molybdenum	$1\cdot7 \pm 0\cdot5$	$1\cdot2 \pm 0\cdot2$	$1\cdot5 \pm 0\cdot2$	$1\cdot2 \pm 0\cdot2$

Basal diet: 1 kg grass cubes/day (copper content 5·0 $\mu g/g$ dry matter).

pattern of water absorption since the copper content of the total dissolved solids showed the same trend, as did determinations of the distribution of ^{64}Cu in samples of abomasum liquor after admixture of radioactive copper with the diet. In this last study errors due to sedimentation during subsampling were minimized by counting large quantities of unsieved contents for ^{64}Cu γ-activity and subsequently correcting for self-absorption effects. The range of values obtained for soluble ^{64}Cu in abomasal contents (expressed as a percentage of the total in the sample) was 0·29–0·59 (mean 0·37) for four animals receiving both molybdate and sulphate and 0·54–1·51 (mean 0·91) for four animals receiving sulphate supplements only.

It would be tempting to conclude from the foregoing that this fall in the copper content of the aqueous phases of rumen and abomasum contents was associated with the precipitation of insoluble cupric sulphide—a material which has been shown to be very poorly utilized as a source of copper by the sheep (Dick, 1954). Such a

hypothesis would not be completely supported by our results since, although the soluble-copper level falls in both rumen and abomasum, the presence of appreciable quantities of sulphide in the abomasum can only be detected when rumen sulphide levels exceed 0·4 μmoles/ml, i.e. during the peak periods after feeding. In other periods abomasal sulphide levels ranged between 0·02 and 0·04 μmoles/ml irrespective of whether molybdenum was included in, or omitted from, the diet. Thus the relationship between changes in soluble-copper level and sulphide level is not yet clear. It seems probable that changes in sulphide concentration may perhaps reflect changes in the quantity or nature of other products of sulphur metabolism within the rumen such as sulphur amino acids or mercaptans, many of which have a pronounced affinity for copper and react to form stable, insoluble products.

Examination of the aqueous phase of rumen liquor by filter-paper electrophoresis after oral administration of radioactive copper did not reveal differences in the qualitative nature of organic complexes of copper present in solution that could be related to the feeding with molybdate. In no samples could the presence of free cupric ions be demonstrated (Fig. 3). Molybdenum supplementation did however result in a small overall lowering of all detectable peaks of ^{64}Cu activity.
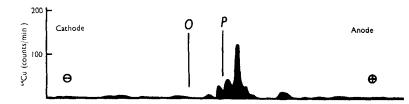

Fig. 3. Result of electrophoresis of centrifuged sheep rumen liquor sampled 10 h after oral administration of 5 mc ^{64}Cu(No$_3$)$_2$. Buffer, centrifuged rumen liquor from animal on the same diet but not receiving ^{64}Cu. P, point of application to paper; O, point of electrophoretic neutrality after correction for electro-endosmosis.

The possibility that supplementation of the diet with molybdenum and sulphate might deplete tissue copper stores of the sheep by increasing copper output in faeces or urine was investigated in studies in which wethers receiving such diets were given intravenous injections of 1 mg ^{64}Cu and carrier; the subsequent urinary and faecal output of ^{64}Cu was studied over 36 h. Urinary output during this period averaged 1·3 μg copper from sheep receiving molybdate and sulphate and 2·5 μg from sheep receiving sulphate alone. Combined urinary and faecal outputs on these treatments were 10·1 and 12·2 μg copper respectively—results which do not support any suggestion that molybdenum enhances copper excretion in the ruminant.

In this review we have seen that the feeding of molybdenum to the rat results in an increased sensitivity to sulphide. Since the sulphide-oxidizing system of the rat liver has a requirement for copper as an enzyme cofactor and since increasing the dietary copper intake decreases the toxicity of orally administered sulphide, it is possible that sulphide sensitivity is an end-result of restrictions on the physiological

utilization of stored copper imposed by high concentrations of tissue molybdenum. Such an impairment of copper utilization could also account for the depression of cytochrome-oxidase activity—this enzyme containing copper as an integral component of its structure (Wainio, Wende & Shimp, 1958)—and for the failure of haemoglobin synthesis found in many studies of molybdenum toxicosis in the non-ruminant.

The possibility of molybdenum similarly restricting the use of tissue copper in the ruminant has been frequently overlooked in past discussions of these relationships. Such a mechanism would account for Marston's (1952) observation that the feeding of molybdenum to sheep maintained under conditions of inadequate copper intake decreased the rate of depletion of liver copper stores while exacerbating the signs of copper deficiency. Even though the most spectacular effect of molybdenum in the ruminant is the depletion of tissue copper stores when the diet is high in sulphate, the role of this element in restricting the utilization of these stores must also be of importance.

From the foregoing it appears that the metabolic disturbances consequent upon the feeding of molybdenum to the rat are probably the result of a failure of copper utilization within the tissues; there is certainly no evidence to indicate that molybdenum interferes with copper uptake from the digestive tract. In the ruminant the decline in tissue copper and the fall in the soluble-copper content of rumen and abomasal liquors when the diet is simultaneously supplemented with molybdate and sulphate suggest that changes within the digestive tract may restrict copper absorption and may be the underlying cause of the ensuing copper deficiency.

The significance of the increased sulphide levels in the rumen after the ingestion of molybdenum in relation to changes in soluble-copper concentration are not yet clear, but in work now being developed to study this point attention is being paid to the possibility of more extensive effects of molybdenum on sulphur metabolism within the digestive tract.

It has been suggested by Spaïs (1959) that sulphide absorbed from the rumen and circulating through the bloodstream may itself act as an inhibitor of cytochrome oxidase within the body and that such a mechanism could explain the low cytochrome-oxidase activity of nervous tissue of the lamb found in cases of the copper-deficiency disorder, swayback (Howell & Davison, 1959). This postulate is difficult to accept in view of the work, firstly of Anderson (1956) who found that, at normal levels of dietary sulphate, sulphide cannot be detected in the blood of the sheep and, secondly, that of Sörbo (1958) who convincingly demonstrated the catalytic effect of haem compounds such as haemoglobulin in the non-enzymic conversion of sulphide into thiosulphate. On the other hand, an intracellular inhibition of terminal oxidative steps by sulphide generated *in situ* from degradation of sulphur amino acids would still appear to be possible if the sulphide was not rapidly removed. In this connexion the findings relating to inhibition of enzymic sulphide oxidation in the 'conditioned' copper deficiency induced by feeding molybdenum to the rat may be of significance.

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Selenium in animal health

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Selenium is a relatively scarce element. According to Goldschmidt (1954), its mean content in the crust of the earth is about 0·09 p.p.m., making it sixty-sixth in the order of abundance. By comparison, the least abundant of the biologically important metals so far established is molybdenum, whose content is 2·3 p.p.m.

Selenium and sulphur are quite closely related crystallochemically and also geochemically. The accepted content of S in the lithosphere is 520 p.p.m., so that the mean S:Se ratio is around 6000:1. In basic plutonic rocks the S:Se ratio is 7000:1, and in shales around 4000:1. Se determinations have not been made on many rock types, but S contents are readily available and Se contents may be inferred from them.

As there are now reports of animal experiments showing that Se is biologically important, interest has become focused on the possibility that areas of soil deficiency exist. In Scotland the lowest soil concentrations may be expected in light-textured soils derived from arenaceous Old Red Sandstone or from Carboniferous sandstone rocks, or from certain but not all granites, with values of the order of 0·05 p.p.m. or less. Deficiency states in animals might be expected in these areas.

Relatively high values are found for sedimentary rocks. In sea-bottom sediments concentrations of 0·1–5 p.p.m. are reported, the amount increasing with depth of