Copper and molybdenum in subcellular fractions of rat liver

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1. Black-hooded weanling rats were given a copper-deficient diet or diets providing 3 ppm Cu with or without supplements containing combinations of molybdate, sulphate and sulphide salts to provide 35 ppm molybdenum and 2 μg atoms sulphur/g. Changes in weight and blood haemoglobin concentration were studied during 48 d of treatment. The subcellular distribution of Cu and Mo in the liver was subsequently determined.

2. Rats fed on the Cu-deficient diet had a lower growth rate than animals receiving 3 ppm Cu and suffered a decline in blood haemoglobin concentration; Mo supplementation of the diet providing 3 ppm Cu produced similar adverse effects on growth but not on Hb. Effects of Mo on growth were exacerbated by a sulphide supplement which also decreased the rate of gain in Hb concentration. This concentration of dietary sulphide was without effect when Mo was omitted from the diet.

3. The Cu-deficient diet decreased both the Cu concentration and proportion of total liver Cu in mitochondria + microsome and supernatant fractions of liver.

4. Mo-supplemented diets greatly increased both the Cu and Mo contents of all liver fractions. This phenomenon is considered in relation to previous suggestions that an unavailable Cu–Mo complex can form in tissues as a response to Mo accumulation.

Growth retardation and, in some instances, anaemia have been shown to result from the inclusion of supplements of molybdate salts in the diets of chicks, rats and rabbits. These effects are largely overcome if the level of copper in the diet is subsequently increased, a result which suggests that molybdenum restricts the physiological utilization of dietary Cu. It is accordingly somewhat surprising that in the rat it has been shown that Mo may increase four- or five-fold the quantity of Cu stored in the liver (Miller, Price & Engel, 1956; Mills, 1960).

From studies on the co-precipitation of Cu and Mo salts in vitro, Dowdy & Matrone (1968*a*) suggested that a Cu–Mo complex having an atomic ratio of Cu:Mo of approximately 4:3 can form in aqueous solutions at or near neutral pH. They suggested, on the basis of studies of the influence of Mo on Cu utilization by sheep, chicks and pigs, that the formation of this complex may be the mechanism responsible for the well-established antagonistic effect of Mo on the utilization of Cu by animal tissues. In contrast, Brinkman, Miller & Engel (1961), during studies on the influence of dietary Mo on the subcellular distribution of Cu in the livers of rats, were unable to find any evidence for the formation of a Cu–Mo complex.

In the course of investigations into the nature of the Cu–Mo interaction in the rat we have examined the subcellular distribution of these elements in the livers of rats made Cu-deficient by omission of Cu from a semi-synthetic diet and in rats fed on

Cu-supplemented diets containing Mo and sulphate or sulphide salts to modify the effects of Mo.

The objectives of the experiment were to determine whether Cu deficiency resulted in a decrease in the content of soluble Cu in the supernatant fractions of high-speed centrifugates of liver homogenates and whether the induction of Cu deficiency by feeding with Mo resulted in a similar fall in the content of soluble Cu. It had been previously found (Mills, 1960) that the administration of sulphate reduced the antagonistic effects of Mo on Cu utilization in the rat, while the inclusion of traces of sulphide salts in diets containing Mo exacerbated the effects of Mo. Accordingly the influence of these sulphur supplements on Mo and Cu distribution was also studied and the results were examined in relation to the possible existence of a Cu–Mo complex in individual liver fractions.

EXPERIMENTAL

Sixty young rats of the Rowett Institute strain of black-hooded Lister were reared under conditions in which they were denied access to the stock colony cube diet given to their dams. They were weaned at 22 d of age and fed for the next 2 weeks on a semi-synthetic diet (Mills & Murray, 1960) supplemented with Cu to give 3 μg Cu/g dry matter. Previous experience had shown that this procedure produces animals with a mild Cu-deficiency anaemia and which are sensitive to changes in the availability of dietary Cu. At this stage, when the mean weight was 83 g and the mean blood haemoglobin concentration was 8·49 g/100 ml, animals were randomized into six groups of ten and given the following diets:

(A) (−Cu) Cu-deficient basal diet providing 0·4 μg Cu/g dry matter (0·006 μg atom/g);

(B) (Cu) as (A) but containing $CuSO_4.5H_2O$ to give 3 μg Cu/g (0·047 μg atom/g);

(C) (Cu + Mo) as (B) but containing $(NH_4)_2MoO_4$ to give 35 μg MoO_4^{2-}/g (0·218 μmol/g);

(D) (Cu + Mo + S^{2-}) as (C) but including CaS to give 2 μg atoms S^{2-}/g;

(E) (Cu + Mo + SO_4^{2-}) as (C) but including $Na_2SO_4.7H_2O$ to give 2 μmol SO_4^{2-}/g;

(F) (Cu + S^{2-}) as (B) but with CaS to give 2 μg atoms S^{2-}/g.

Animals were housed in heavily galvanized cages and offered distilled water *ad lib.* Dietary treatments were continued for a period of 48 d, when animals were killed by decapitation. Livers were perfused with Cu- and Mo-free ice-cold 0·25 M-sucrose solution. The procedure for the fractionation of subcellular components was essentially that of Thiers & Vallee (1957). The contents of Cu and Mo in the pooled fractions from two groups of five rats in each treatment were determined by spectrographic analysis (Mitchell, 1964) of the ashed material using d.c. arc excitation, with preliminary concentration for the Mo determination.

RESULTS AND DISCUSSION

Compared with the growth obtained on diet B (3 ppm Cu), rats fed on diets A (Cu-deficient), C (Cu+Mo) and D (Cu+Mo+S) had a significantly lower rate of weight gain ($P < 0.01$ in each instance) (Table 1). As was expected from previous work, the adverse effects of Mo were less marked in diet E, which contained sulphate. The inclusion of sulphide in diet F (Cu+S) had no adverse effect on growth. Diet A produced a very significant decline in blood haemoglobin concentration during the course of the experiment. Diet D (Cu+Mo+S) produced a significantly lower rate of haemoglobin increase during the experiment than diets B, C, E and F ($P < 0.01$). These results agree closely with those obtained in previous work in which it was further

Table 1. *Copper and molybdenum content of connective tissue and subcellular fractions of the livers of rats given a Cu-deficient diet alone or together with Cu-, Mo- and S-containing supplements*

Dietary supplement	Change during experiment: Weight (g)	Change during experiment: Haemoglobin (g/100 ml blood)	Content in fraction (μg/g dry matter): Connective tissue Cu	Connective tissue Mo	Nuclei + debris Cu	Nuclei + debris Mo	Mitochondria + microsomes Cu	Mitochondria + microsomes Mo	Supernatant fraction Cu	Supernatant fraction Mo
A (−Cu)	+59	−3·7	9·1	1·3	4·1	1·3	4·6	1·4	2·2	1·7
B (+Cu)	+89	+5·6	13·7	1·7	5·9	1·7	8·4	1·7*	15·5	3·7
C (+Cu+MoO_4^{2-})	+61	+5·8	29·0	18·8	15·1	12·7	29·9	15·4	34·3	56·8
D (+Cu+MoO_4^{2-}+S^{2-})	+50	+1·9	15·9	9·5	12·5	7·6	20·2	10·3	22·5	20·9
E (+Cu+MoO_4^{2-}+SO_4^{2-})	+73	+5·7	22·2	14·6	10·9	7·2	16·7	12·9	26·3	38·0
F (+Cu+S^{2-})	+87	+5·6	10·1	3·0	6·6	1·7	9·7	2·0	17·4	3·8

To provide sufficient material for analyses, livers from the ten rats on each treatment were pooled into two groups of five before fractionation. Values reported are the means derived from the two sets of values with the exception of that marked (*) where one sample was lost. Only nine animals survived the experiment in each of groups A and D. Mean weight at randomization, 83 g; mean Hb, 8·49 g/100 ml.

shown that the adverse effects upon growth and blood haemoglobin concentration induced by a diet containing the same amounts of Mo and sulphide as in the present work could be overcome if the Cu content of the diet was increased to 25 μg/g (Mills, 1960).

The Cu and Mo concentrations of liver fractions are presented in Table 1. Dietary treatments B and F (Cu and Cu+S) yielded liver fractions that had closely similar Cu concentrations. Cu deficiency induced by diet A led to a marked reduction in the Cu content of the supernatant and mitochondria+microsome fractions of the liver compared with the values on diet B ($P < 0.001$ and < 0.05 respectively). The presence of Mo in diets C, D and E increased both the Mo and the Cu content of all liver fractions, and here the effects were greatest on diet C which included no sulphide or sulphate supplement.

Despite the fact that the inclusion of Mo (diet C) reduced the rate of growth and Mo+S (diet D) adversely affected both growth rate and haemoglobin regeneration – situations which we believe from evidence outlined earlier to be due to an induced

Cu deficiency – there was no indication that these diets had caused a reduction in the concentration of Cu in either the supernatant or mitochondria + microsome fraction or in the contribution of these fractions to the total Cu content of the liver. This situation contrasts markedly with that found in rats fed on the Cu-deficient diet, where our results agree with those of previous workers who have studied the effects of uncomplicated Cu deficiency on liver Cu distribution (Gregoriadis & Sourkes, 1967).

The development of a syndrome, as a consequence of Mo accumulation, which is responsive to an increased intake of dietary Cu (Halverson, Phifer & Monty, 1960; Mills, 1960) but which is accompanied by an increased accumulation of Cu in the liver without any major change in the gross distribution of Cu between subcellular fractions can be explained only if the effect of Mo is to reduce the functional availability of Cu. Essentially this is what has been postulated by Dowdy & Matrone (1968*a*, *b*) in suggesting that an unavailable Cu–Mo complex may form under physiological conditions.

Table 2. *Regression coefficients of copper (μg atoms) on molybdenum (μg atoms) in subcellular fractions of rat liver*

(Mean values with their standard errors, based on 4 degrees of freedom)

Fraction	Regression coefficient
Connective tissue	1·49 ± 0·22
Nuclei + cell debris	1·37 ± 0·16
Mitochondria + microsomes	2·05 ± 0·42
Supernatant	0·64 ± 0·17

Dowdy, Kunz & Sauberlich (1969), from results of their studies of the interaction of cupric and molybdate salts under mild conditions to yield an insoluble product, have suggested that this complex might be the mineral lindgrenite ($2CuMoO_4.Cu(OH)_2$).

If this complex formed in liver fractions of the rats in our experiment in response to Mo accumulation it would be expected that the ratio incremental increase in Cu (μg atoms): incremental increase in Mo (μg atoms) would be approximately 1·5 if it is assumed that this insoluble complex accounts for all the additional Cu found in liver fractions of rats given Mo-supplemented diets. Regression coefficients for Cu upon Mo concentration (μg atoms) in these fractions are presented in Table 2.

Relationships between Cu and Mo in connective tissue are in close accord with the suggestion that such a complex might account for Cu and Mo accumulation. In nuclei + cell debris and mitochondria + microsomes fractions the evidence is less convincing, while in supernatant fractions there is wide divergence from the theoretically expected atomic ratio of 1·5.

In all these fractions the closeness of fit to the theoretical value for lindgrenite of 1·5 will be influenced by the size of the pool of available Cu not so complexed. Implicit in the above analysis of our results is the assumption that, between groups, the size of this available Cu pool does not change as Cu accumulates in response to Mo uptake. Whether or not this assumption is valid cannot at the moment be determined. Because of this limitation we would merely regard our own results as indicating that

the postulate of Dowdy & Matrone (1968*a*, *b*) and Dowdy *et al.* (1969) could be acceptable if limited to Cu and Mo in particulate material derived from the connective tissue fraction. Even with the other particulate fractions from rats given Mo in our experiment, the observed Cu:Mo ratio is not very far removed from the expected value of 1·5 in comparison with Cu:Mo ratios of similar fractions derived from rats not given high supplementary levels of Mo (groups A, B and F: mean atomic ratio Cu:Mo, 7·0 ± 0·89).

The ultimate acceptance of the postulates of Dowdy & Matrone (1968*a*, *b*) and Dowdy *et al.* (1969) will undoubtedly depend upon unequivocal demonstration of the existence of their Cu–Mo complex in animal tissues but, until this difficult task is accomplished, the results of the present experiment suggest that their idea is not improbable. In making this comment we are aware that it is not in accord with the findings of Brinkman *et al.* (1961), who found no consistent relationships between Cu and Mo, and we are unable to point to a clear explanation of this discrepancy other than to emphasize that the Mo concentrations in the diets used by Brinkman *et al.* (1961) were six to twelve times higher than in our experiments.

The results of the fractionation experiment clearly indicate that the interfering effects of Mo upon Cu-dependent processes within the animal arise in a different way from those induced by a Cu-deficient diet in that the patterns of Cu distribution within the liver differ greatly between these two situations. Administration of Mo has adverse effects upon sulphide metabolism, as was shown by Mills, Monty, Ichihara & Pearson (1958) and as is again indicated by the performance of rats of group D (Cu + Mo + S) in the present experiment. Halverson *et al.* (1960) and Siegel & Monty (1961) have attributed the adverse effects of excess dietary cystine upon rats given Mo-supplemented diets to an impaired capacity to handle endogenous sulphide. All these situations are preventable by increasing the dietary Cu intake and it thus appears probable that they may be explicable in terms of a decrease in the metabolic availability of Cu within tissues as a response to Mo accumulation without, necessarily, a decrease in total tissue Cu concentration.

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