Br. J. Nutr. (1980), 43, 329

The influence of molybdenum on the copper metabolism of the rat at different Cu levels of the diet

By H. NEDERBRAGT*

Zootechnical Institute, Department of Animal Nutrition, Faculty of Veterinary Medicine, State University, Utrecht, The Netherlands

(*Received* 13 *June* 1979 – *Accepted* 29 *August* 1979)

1. Male WAG/Cpb inbred rats fed on rations with approximately 1·5 mg copper/kg (deficient), 6·0 mg Cu/kg (adequate) and 25·0 mg Cu/kg (excess) were supplemented with varying amounts of molybdenum (0, 50, 150 and 500 mg/kg diet) and the effect on the Cu concentration of blood, plasma, liver and kidney, the caeruloplasmin activity of plasma and the Mo concentration of liver and kidney were studied.

2. Mo increased the Cu concentration of blood, plasma, liver and kidney and the Mo concentration of liver and kidney.

3. In the plasma of Mo-supplemented rats the presence of a Cu-containing fraction was demonstrated, the Cu of which did not react with dithiocarbamate and was not related to caeruloplasmin. The Cu in this fraction was not able to increase the caeruloplasmin activity in the plasma of Cu-deficient Mo-supplemented rats. The Cu concentration of the erythrocytes did not seem to have been increased by the Mo treatment.

4. When compared to Cu-adequate rats the effect of Mo on the Cu distribution was reduced both by Cu deficiency and Cu excess. This decreased effect of Mo was explained by reduced uptake or retention of Mo in the body as observed in the liver and kidney.

In both ruminants and single-stomached animals (non-ruminants) molybdenum alone or in combination with sulphate interacts with copper metabolism. In ruminants Mo decreases the Cu concentration of blood and liver, an effect that is enhanced by SO_4. In non-ruminants the Cu concentration of blood and liver is increased by Mo, an effect that is counteracted by SO_4. In ruminants the Cu–Mo interaction may be explained by the dominating role of microbial processes in the rumen at the Mo levels commonly used in ruminant diets; it is suggested that the microbial-mediated formation of Cu-thiomolybdate complexes in the rumen prevents Cu absorption (Suttle, 1974; Dick *et al.* 1975).

However, in sheep it has been observed that Mo has some effects comparable to those in non-ruminants. These effects are: an increase in the free plasma Cu concentration and the presence of a 'tightly' bound, non-caeruloplasmin Cu fraction (Smith *et al.* 1968; Suttle & Field, 1968; Smith & Wright, 1975*a*, *b*; Bremner & Young, 1978), a reduced transport of ^{64}Cu from the blood to the liver after intravenous injection (Smith *et al.* 1968; Marcilese *et al.* 1969) and a continuously increased plasma Cu concentration when high doses of Mo are given orally (Bingley, 1974). The same observations have been made as a consequence of Mo feeding in non-ruminants: an increased plasma Cu concentration in rats (Miller *et al.* 1956), a plasma Cu fraction in guinea-pigs that is insoluble in trichloric acid (Suttle, 1973; Smith & Wright, 1975 *b*) and a reduced transport of ^{64}Cu from blood to liver in rat (Compère *et al.* 1965), rabbit (Gaballah, Abood, Caleed *et al.* 1965) and pig (Standish *et al.* 1975). This resemblance in influence of Mo on systemic Cu in ruminants and non-ruminants suggests that the effect of Mo feeding in ruminants may be the result of two interactive processes of which the first may be found in the rumen and the second (comparable to that in non-ruminants) in the tissues. It was the purpose of this study to investigate the systemic effect of Mo on Cu metabolism in more detail, using rats as an animal model.

Very little information is available about the Cu–Mo interaction in the gastro-intestinal

* Present address: Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

0007-1145/80/3357-2908 $01.00 © 1980 The Nutrition Society

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

tract of non-ruminants. Although it cannot be excluded that microbial processes in the gastro-intestinal tract of rats may have some influence, in particular when sulphur compounds are involved, it was assumed that they play a minor role compared to those in the rumen; supportive evidence for this assumption is given by the finding that additional dietary Cu, Mo or S has no influence on the absorption of ^{99}Mo in sheep when this is administered via the duodenum (Mason *et al.* 1978).

Gray & Daniel (1964) suggested that the Cu status of the rat might be of importance when the influence of SO_4 on the Cu–Mo interaction is investigated. More evidence for a possible role of the Cu status in the Cu–Mo interaction is given by the observation that in Mo-fed rabbits the increased plasma Cu concentration can be decreased by raising the dietary Cu content while maintaining the dietary Mo supply (Gaballah, Abood, Kapsalis *et al.* 1965). In the present experiments, therefore, the influence of Mo on Cu metabolism was investigated by determining Cu distribution in blood, liver and kidney of rats fed on diets which had deficient, adequate and excess Cu contents.

MATERIALS AND METHODS

Male rats of the inbred strain WAG/Cbp (Centraal Proefdieren Bedrijf TNO, Zeist, The Netherlands), varying in weight from 150 to 200 g, were housed in plastic cages with stainless steel wire floors and lids. Immediately after arrival they were fed with the Cu-adequate diet (6·0 mg Cu/kg diet) and after 8 d they were allocated to the different experimental dietary groups.

To each of three different levels of dietary Cu (mg/kg; deficient 1·5, adequate 6·0, excess 25·0) Mo was added at four different dietary concentrations (mg/kg): 0, 50, 150 and 500, giving twelve dietary groups. Each dietary group consisted of six rats.

In the first experiment the influence of Mo was studied using rats given diets containing adequate or excess amounts of Cu and the Cu concentration of blood, plasma, liver and kidney was determined. The method used in this experiment gave values for blood and plasma Cu which were not reliable. In the second experiment the Cu concentration of blood and plasma was determined according to Henkin (1971); this method will be described later.

The Mo concentration of liver and kidney was determined also in this second experiment, as well as the influence of Mo on rats fed on Cu-deficient diets. The results from both experiments for the Cu concentrations of liver and kidney of the Cu-adequate and Cu-excess dietary groups were taken together and the mean values were therefore based on twelve observations; all other means were based on six observations.

The Cu-deficient diet used was a commercial one (Muracon ssp tox., without copper sulphate; Trouw en Co., Putten, The Netherlands) which had a Cu content that varied from 1·0 to 1·9 mg Cu/kg with a mean value of 1·4 mg Cu/kg during the whole experimental period. Sulphur or sulphate contents of the diet were not determined but were kept constant throughout this study. Cu was added as $Cu(NO_3)_2.2H_2O$ and Mo as $(NH_4)_6Mo_7O_{24}$. The diet was given *ad lib.* in glass jars, and distilled water in glass bottles with glass cannulas.

The rats were maintained on the experimental diets for 6 weeks after which they were killed, three rats from each group on each of 2 d between 08.30 and 10.00 hours. An exception was made for the rats on the diet with (/kg) 6·0 mg Cu and 500 mg Mo; they were maintained on the diet for 5 weeks because fluctuations were observed in the Cu concentrations of the plasma that started between the fifth and sixth week of Mo feeding.

Blood was taken by heart puncture under diethyl ether anaesthesia; the rats were killed by an overdose of diethyl ether and liver and kidneys were removed. Blood was analysed on the day of sampling, organs were frozen until required for analysis. To obtain plasma the blood was centrifuged for 15 min at 3000 *g*.

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

Chemical analysis

Glassware was made Cu-free by soaking it in nitric acid (100 ml/l) for at least 12 h and then rinsing with distilled water and double-distilled water.

Cu. The Cu concentration of total blood and plasma was determined according to Henkin (1971) by mixing 0·3 ml blood or plasma with 2·7 ml butanol (60 ml/l) and aspirating the samples directly into an air–acetylene flame of an atomic absorption spectrophotometer (Perkin Elmer 103); standards and blanks, dissolved in sulphuric acid (50 ml/l) and saline (9 g sodium chloride/l) were mixed with butanol also and determined simultaneously.

Free plasma Cu was determined according to Blomfield & MacMahon (1969) by adding 0·3 ml ammonium pyrrolidine dithiocarbamate (1 g/l; DTC) and 2·0 ml 4-methyl-2 pentanon (methyl-isobutyl ketone; MIBK) to a 0·3 ml sample of plasma; the mixture was shaken for 5 min and after centrifuging the MIBK layer was removed and aspirated into a reduced-acetylene flame of the atomic absorption spectrophotometer. Standards and blanks were determined simultaneously with the same method.

After thawing, organs were cleaned of fat, connective tissue and blood and dried for at least 24 h at 100°. After weighing the dried organs were wet-ashed with H_2SO_4–HNO_3 until a colourless solution was obtained. Samples of a known volume of these solutions were dissolved in blank and standard solutions to prevent erroneous results caused by unknown differences in H_2SO_4 concentration of the samples, and the Cu concentrations were determined by atomic absorption spectrophotometry.

Mo. This was determined on the same wet-ashed samples according to the method of Sandell (1958), based on Mo reduction by stannic chloride, complexing Mo(VI) with thiocyanate and extracting the complex in a mixture of carbon tetrachloride and 3-methyl 1-butanol (isoamyl alcohol); its colour intensity is a measure of the Mo concentration and was determined spectrophotometrically at 466 nm. Cu and Mo concentrations of the organs are expressed as μg/g dry matter (DM).

Caeruloplasmin (ferroxidase; EC 1.16.3.1). This was determined according to its *p*-phenylene diamine (PPD)-oxidase activity. To 0·05 ml plasma 2·0 ml 0·1 M-acetate buffer (pH 5·6) was added; a sample of the same plasma with 0·1 ml sodium azide (10 g/l) served as blank. To both samples 1·0 ml PPD (1 g/l) in acetate buffer was added and the mixtures were incubated for 1 h at 37°. The reaction was stopped by adding 1·0 ml sodium azide and the colour intensity was determined spectrophotometrically (model 25, Beckman) at 525 nm. Enzyme activity is given in extinction units (E_{525})/ml per h.

RESULTS

Blood and plasma

In blood and plasma of Mo-supplemented rats the Cu concentration became very high; when the dietary Mo concentration was 500 mg/kg the Cu concentration of blood reached 4·0 μg/ml (Fig. 1) and the plasma Cu concentration 7·2 μg/ml (Fig. 2). However, a considerable variation in the Cu concentration was observed; in some rats the Cu concentration was 6·5 μg/ml in blood and 12·0 μg/ml in plasma. The Cu concentration of plasma increased more than that of blood.

In the Cu-adequate rats the erythrocyte volume was found to be approximately 0·45; assuming that the erythrocyte volume had not been changed by the Mo treatment it could be calculated that the Cu concentration of the erythrocytes of the Mo-supplemented rats was as high as or lower than that of the Cu-adequate rats. The increase in plasma Cu concentration caused by Mo feeding was highest in rats given a Cu-adequate diet. Cu-deficient rats without Mo had a markedly depressed plasma Cu concentration; a dietary

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

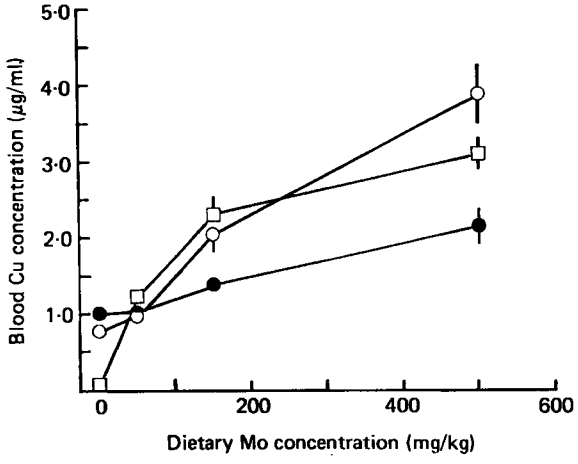

Fig. 1. Blood copper concentration (μg/ml) of male rats receiving different dietary molybdenum and Cu levels. The Cu levels of the diet were deficient (1·5 mg/kg; □—□), adequate (6·0 mg/kg; ○—○), or excess (25·0 mg/kg; ●—●). Each group consisted of six rats. The points are mean values with their standard errors indicated by vertical bars.

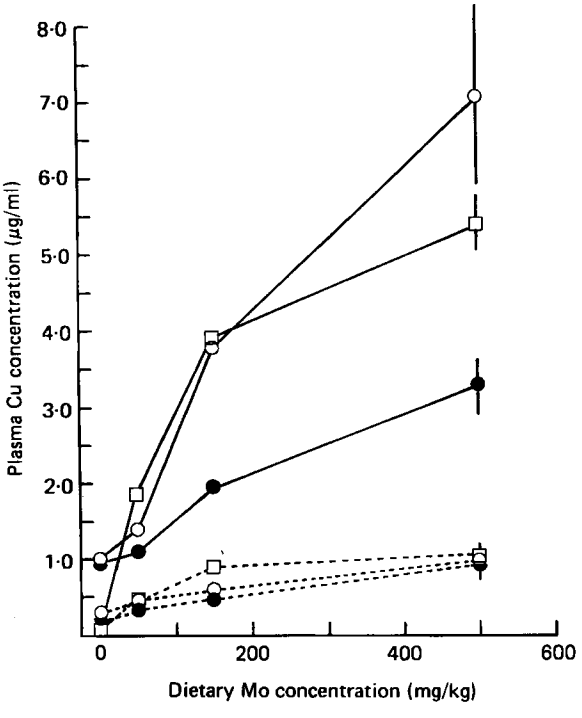

Fig. 2. Total (——) and 'free' (– – – –) plasma copper concentration (μg/ml) of male rats receiving different dietary molybdenum and Cu levels. The Cu levels of the diet were deficient (1·5 mg/kg; □—□), adequate (6·0 mg/kg; ○—○), or excess (25·0 mg/kg; ●—●). Each group consisted of six rats. The points are mean values with their standard errors indicated by vertical bars.

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

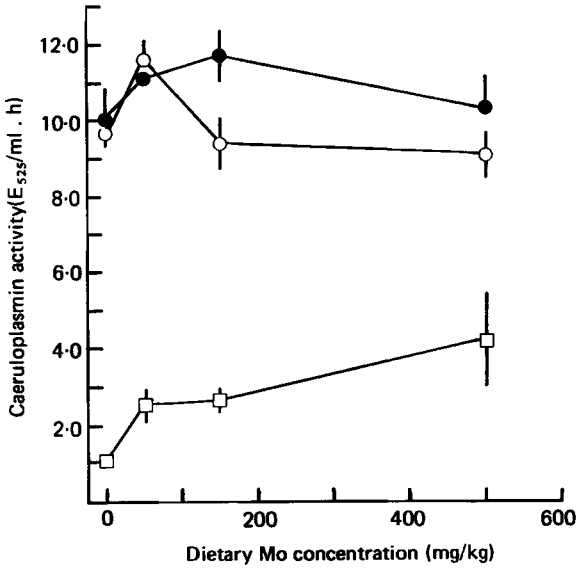

Fig. 3. Caeruloplasmin activity (extinction units (E_{525})/ml per h) in the plasma of male rats receiving different dietary molybdenum and copper levels. The Cu levels of the diet were deficient (1·5 mg/kg; □—□), adequate (6·0 mg/kg; ○—○), or excess (25·0 mg/kg; ●—●). Each group consisted of six rats. The points are mean values with their standard errors indicated by vertical bars.

Mo level of 150 mg/kg caused an increase in plasma Cu equivalent to that in Cu-adequate rats supplemented with the same amount of Mo, but still higher levels of dietary Mo only had a small additional effect. In combination with excess dietary Cu dietary Mo was much less effective in increasing the plasma Cu concentration than in the Cu-adequate and Cu-deficient groups.

Not only the total plasma Cu concentration but also the 'free' Cu, reacting with DTC, was determined (Fig. 2). The concentration of this Cu fraction was raised by Mo in all three groups to the same extent, approximately 1·0 μg/ml at a dietary Mo concentration of 500 mg/kg. This suggests that the greater part of the plasma Cu does not react with DTC and must be considered to be 'tightly' bound. The proportion of this 'tightly' bound fraction can be calculated from the difference between the total and 'free' plasma Cu concentration, and the influence of Mo on this fraction at the three basal levels of Cu appeared to be comparable to the influence of Mo on total plasma Cu (Fig. 2). In non-Mo-fed rats the Cu-containing protein caeruloplasmin is supposed to represent the 'tightly' bound Cu fraction in plasma. It was therefore necessary to determine the influence of Mo on caeruloplasmin, for which an assay of its oxidase activity was used (Fig. 3). No difference in caeruloplasmin activity could be demonstrated between the Cu-adequate and Cu-excess rats whereas the caeruloplasmin activity of the Cu-deficient rats was lowered considerably. Although a small increase in caeruloplasmin activity could be observed in Mo-supplemented Cu-deficient rats, Mo did not seem to have a distinct effect on this activity, as demonstrated by the lack of correlation between the changes in caeruloplasmin activity (Fig. 3) and the increase in the 'tightly' bound Cu fraction in the plasma (Fig. 2).

Liver

In Cu-adequate rats an increase in liver Cu concentration could only be observed at dietary Mo levels higher than 50 mg/kg (Fig. 4). At the highest level used (500 mg Mo/kg) a liver

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

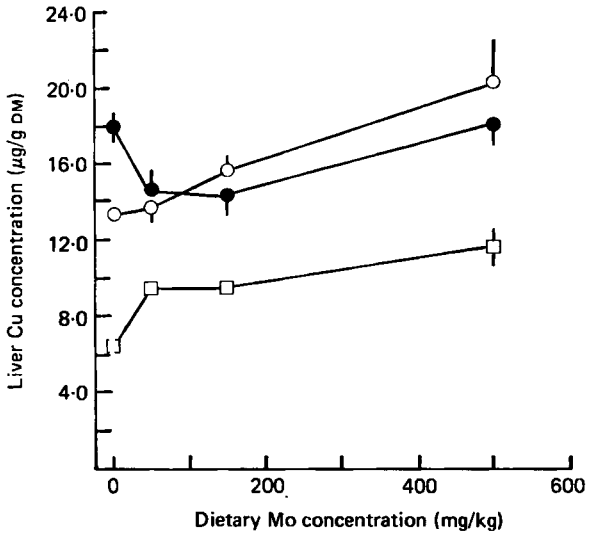

Fig. 4. Liver copper concentration (μg/g dry matter (DM)) of male rats receiving different dietary molybdenum and Cu levels. The Cu levels of the diet were deficient (1·5 mg/kg; □—□), adequate (6·0 mg/kg; ○—○), or excess (25·0 mg/kg; ●—●). The Cu-deficient groups consisted of six rats and the Cu-adequate and Cu-excess groups of twelve rats. The points are mean values with their standard errors indicated by vertical bars.

Cu concentration of 20 μg/g DM was found, giving an increase of approximately 35%. In Cu-excess rats without Mo the liver Cu concentration was higher than in control rats; however, between 0 and 150 mg Mo/kg diet the liver Cu concentration of these rats decreased, whereas between 150 and 500 mg Mo/kg it was raised again, at 500 mg Mo/kg reaching the level of that of the Cu-excess rats without Mo.

The livers of Cu-deficient rats contained a much smaller amount of Cu than those of control rats (the difference being 7·0 μg/g DM); the effect of Mo in these rats was an increase in the liver Cu concentration that is largest between 0 and 50 mg Mo/g diet. Still higher amounts of Mo only caused a slight increase in liver Cu.

Increasing dietary Mo concentrations caused increasing liver Mo concentrations (Fig. 5). This effect was most pronounced in Cu-adequate rats. In the liver of Cu-deficient rats there seemed to be a reduced uptake or retention of Mo and in the liver of Cu-excess rats this seemed to be reduced still more: in Mo-supplemented Cu-excess rats the Mo concentration of the liver was only 60% of that of the liver of Mo-supplemented Cu-adequate rats.

Kidney

The Cu concentration of the kidney of Cu-adequate rats increased (after a decrease at 50 mg Mo/kg diet) with increasing amounts of dietary Mo. At a dietary Mo concentration of 500 mg/kg a Cu level of 200 μg/g DM was reached, which is an increase of approximately 50% (Fig. 6). In rats with an excess Cu supply the Cu concentration of the kidney also increased when Mo was fed; from 0 to 150 mg Mo/kg diet this increase equalled that of control rats, but at higher dietary Mo levels no further increase in the Cu concentration in these rats could be observed. In Cu-deficient rats Mo only caused a slight increase in the kidney Cu concentration; the Mo effect was most pronounced at dietary Mo concentrations between 0 and 50 mg/kg.

Increasing amounts of dietary Mo caused an increase in the Mo concentration of the

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

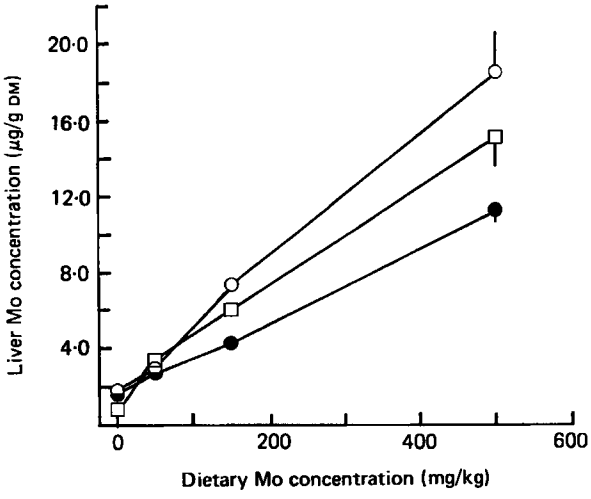

Fig. 5. Liver molybdenum concentration (μg/g dry matter (DM)) of male rats receiving different dietary molybdenum and Cu levels. The Cu levels of the diet were deficient (1·5 mg/kg; □—□), adequate (6·0 mg/kg; ○—○), or excess (25·0 mg/kg; ●—●). Each group consisted of six rats. The points are mean values with their standard errors indicated by vertical bars.

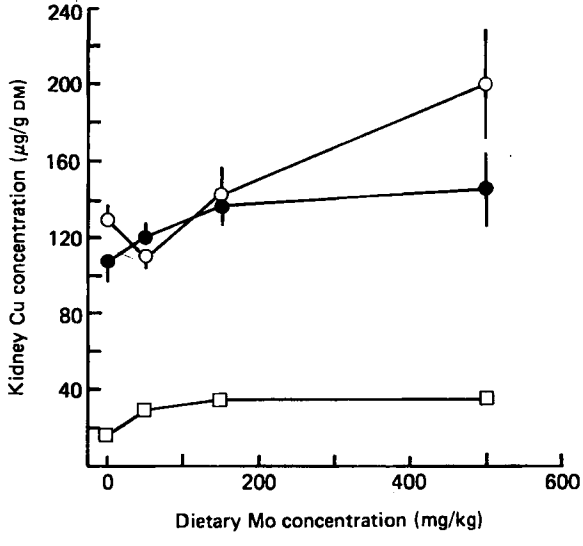

Fig. 6. Kidney copper concentration (μg/g dry matter (DM)) of male rats receiving different dietary molybdenum and Cu levels. The Cu levels of the diet were deficient (1·5 mg/kg; □—□), adequate (6·0 mg/kg; ○—○), or excess (25·0 mg/kg; ●—●). The Cu-deficient groups consisted of six rats and the Cu-adequate and Cu-excess groups of twelve rats. The points are mean values with their standard errors indicated by vertical bars.

kidney (Fig. 7). In Cu-adequate rats fed with Mo levels of 500 mg/kg diet the Mo concentration amounted to 80 μg/g DM. In Cu-deficient and Cu-excess rats the increase in the kidney Mo concentration was only approximately 30 and 40 μg/g DM respectively.

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

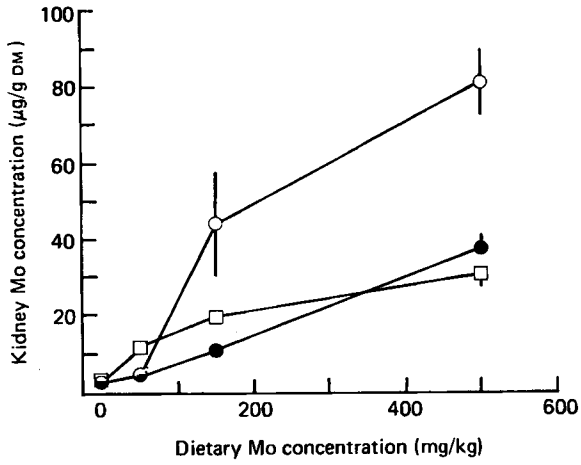

Fig. 7. Kidney molybdenum concentration (μg/g dry matter (DM)) of male rats receiving different dietary Mo and copper levels. The Cu levels of the diet were deficient (1·5 mg/kg; □—□), adequate (6 mg/kg; ○—○), or excess (25·0 mg/kg; ●—●). Each group consisted of six rats. The points are mean values with their standard errors indicated by vertical bars.

DISCUSSION

Feeding rats an Mo-supplemented diet resulted in a 'tightly' bound Cu fraction in their plasma that did not correlate to caeruloplasmin. This Cu fraction might be comparable to that demonstrated for the first time in plasma of Mo- and SO_4-supplemented sheep (Suttle & Field, 1968) and in plasma of Mo- and SO_4-treated guinea-pigs (Suttle, 1973). This Cu fraction was mainly characterized by its insolubility in trichloric acid and was demonstrated to be protein-bound and to contain Mo also (Smith & Wright, 1975*b*; Bremner & Young, 1978). The former authors also suggested that the 'tightly' bound Cu in this fraction is partly responsible for the increase of 'free' plasma Cu. Furthermore it was shown that TCA-insoluble Cu might be easily oxidated and thereby converted to a TCA-soluble form (Bremner, 1976). It can therefore not be excluded that the increase in 'free' Cu in the plasma of our Mo-supplemented rats was due to oxidation of 'tightly' bound Cu during the determination of 'free' Cu, suggesting that the 'tightly' bound Cu fraction was higher than that calculated from our results.

Cu from the 'tightly' bound plasma Cu demonstrated in this study should be poorly available for Cu metabolism; this is shown by the inability to find an increase in erythrocyte Cu in rats with raised plasma Cu levels and, in addition, by the low activity of caeruloplasmin in plasma of Cu-deficient rats, despite a fivefold increase of their plasma Cu levels. However, because no growth retardation or visual signs of Cu deficiency were observed in any of the Mo-supplemented rats and because caeruloplasmin was increased more than twofold in Mo-supplemented Cu-deficient rats it must be assumed that small amounts of Cu are still available for Cu metabolism.

The ability of dietary Mo to raise the Cu and Mo concentrations of the liver of rats has been demonstrated before (Miller *et al.* 1956; Brinkman *et al.* 1961; Mills & Mitchell, 1971). An increase in the Cu and Mo concentrations of the kidney after Mo feeding has been demonstrated in guinea-pigs (Arthur, 1965) and pigs (Standish *et al.* 1975); however, in those animals the Cu concentration of the liver was either not changed or decreased following Mo supplementation. The present studies demonstrated that the effect of Mo considerably depends on the dietary Cu level. When the results for plasma, liver and kidney are

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

summarized it can be concluded that Mo was most effective when the dietary Cu supply was adequate (i.e. 6·0 mg/kg); in this instance the Cu and Mo concentrations increased with increasing dietary Mo levels. Although not effective in rats fed 50 mg Mo/kg diet, excess dietary Cu (25 mg/kg) partly counteracted the effect of higher dietary Mo concentrations: both the Cu concentrations of plasma, liver and kidney and the Mo concentrations of liver and kidney in these rats were increased to a lesser extent with Mo feeding than in Mo-fed Cu-adequate rats. Comparable observations have been made in rabbits (Gaballah, Abood, Kapsalis *et al.* 1965). The diminished increase in Mo concentrations suggested that less Mo was taken up or retained in the tissues as a consequence of excess Cu feeding.

Cu-deficient rats also showed a less effective action of Mo compared to Cu-adequate rats when given 150–500 μg Mo/kg diet; this might also be a consequence of diminished Mo uptake or retention. The reduction of uptake or retention of Mo in Cu-deficient and Cu-excess rats can be observed most clearly in the Mo concentration of the kidney (Fig. 7). On the contrary 50 μg Mo/kg diet seemed to be relatively more effective in Cu-deficient than in Cu-adequate or Cu-excess rats.

The observation that dietary Mo is less effective in raising the Cu and Mo concentrations of rat body tissues when dietary Cu supply is deficient or excessive might be well explained by the formation of metabolically inactive 'cuprimolybdate' in both tissues and gastro-intestinal tract (Huisingh *et al.* 1973). With an excess Cu supply a non-absorbable complex of Cu and Mo in the gastro-intestinal tract might reduce Mo absorption. In Cu-adequate rats sufficient Mo might be absorbed to form 'cuprimolybdate' with systemic Cu. Finally, in the instance of a deficient Cu supply even more Mo might be absorbed, but because insufficient Cu is present in plasma and tissues only a limited amount of 'cuprimolybdate' should be formed and an increase in dietary Mo is not able to raise the 'cuprimolybdate' concentration to the same level as that found in Cu-adequate rats; increased urinary excretion of Mo should explain the decreased Mo concentrations of liver and kidney in Cu-deficient rats. In agreement with the foregoing explanation are the findings of Mills *et al.* (1978) who showed that in vitro synthesized thiomolybdate when administered orally to rats changed the concentration and distribution of Cu in plasma and liver and reduced the absorption of dietary Cu, whereas dietary Cu reduced the absorption of thiomolybdate. Calculations of the Cu and Mo values in the theoretical Cu–Mo complex have been made from the Cu and Mo concentrations from liver fractions (Mills & Mitchell, 1971) and plasma (Smith & Wright, 1975*b*); however, the results presented here showed that this might give erroneous results because in liver and kidney an increase in the Mo concentration might be accompanied by a decrease in the Cu concentration.

Finally, a rather high Cu concentration of the kidney was observed in the rats used in these experiments. The Cu concentration found in the Cu-adequate and Cu-excess rats (without Mo) varied from approximately 100 to 150 μg/g DM. A Cu concentration of 5·10 μg/g wet weight was found in the kidney by Owen (1964), and assuming a DM content of approximately 200 g/kg this is in good agreement with a kidney Cu concentration of 22·3 and 24·1 μg/g DM in rats given 10 and 50 mg Cu/kg diet respectively (Alfaro & Heaton, 1973), and also with a maximum Cu concentration of 18·0 μg/g DM in the kidney of rats given Cu in their drinking water (Murthy *et al.* 1974). These differences in the kidney Cu concentration might be partly explained by differences in age (Bremner *et al.* 1978) but the influence of the composition of the experimental diet or the characteristics of the inbred rat strain used in these experiments might be responsible for this high Cu concentration in the kidney also.

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

https://doi.org/10.1079/BJN19800095 Published online by Cambridge University Press

REFERENCES

Alfaro, B. & Heaton, F. W. (1973). *Br. J. Nutr.* **29**, 73.
Arthur, D. (1965). *J. Nutr.* **87**, 69.
Bingley, J. B. (1974). *Aust. J. agric. Res.* **25**, 467.
Blomfield, J. & MacMahon, R. A. (1969). *J. clin. Path.* **22**, 136.
Bremner, I. (1976). *Proc. Nutr. Soc.* **35**, 21 A.
Bremner, I., Hoekstra, W. G., Davies, N. T. & Williams, R. B. (1978). In *Trace Element Metabolism in Man and Animals*, vol. 3, p. 44 [M. Kirchgessner, editor]. Freising-Weihenstephan: Arbeitskreis Tierernährungsforschung.
Bremner, I. & Young, B. W. (1978). *Br. J. Nutr.* **39**, 325.
Brinkman, G. A., Miller, R. F. & Engel, R. W. (1961). *Proc. Soc. exp. Biol. Med.* **107**, 666.
Compère, R., Burny, A., Riga, A., François, E. & Vanuytrecht, S. (1965). *J. Nutr.* **87**, 412.
Dick, A. T., Dewey, D. W. & Gawthorne, J. M. (1975). *J. agric. Sci., Camb.* **85**, 567.
Gaballah, S. S., Abood, L. G., Caleed, G. T. & Kapsalis, A. (1965). *Proc. Soc. exp. Biol. Med.* **120**, 733.
Gaballah, S. S., Abood, L. G., Kapsalis, A. & Sturdivant, D. (1965). *Proc. Soc. exp. Biol. Med.* **119**, 625.
Gray, L. F. & Daniel, L. J. (1964). *J. Nutr.* **84**, 31.
Henkin, R. I. (1971). In *Newer Trace Elements in Nutrition*, p. 255 [W. Mertz & W. E. Cornatzer, editors]. New York: M. Dekker.
Huisingh, J., Gomez, G. G. & Matrone, G. (1973). *Fedn Proc. Fedn Am. Socs exp. Biol.* **32**, 1921.
Marcilese, N. A., Ammerman, C. B., Valsecchi, R. M., Dunavant, B. G. & Davis, G. K. (1969). *J. Nutr.* **99**, 177.
Mason, J., Lamand, M., Tressol, J. C. & Lab, C. (1978). *Ann. Rech. Vet.* **9**, 577.
Miller, R. F., Price, N. O. & Engel, R. W. (1956). *J. Nutr.* **60**, 539.
Mills, C. F., Bremner, I., El-Gallad, T. T., Dalgarno, A. C. & Young, B. W. (1978). In *Trace Element Metabolism in Man and Animals*, vol. 3, p. 150 [M. Kirchgessner, editor]. Freising-Weihenstephan: Arbeitskreis Tierernährungsforschung.
Mills, C. F. & Mitchell, R. F. (1971). *Br. J. Nutr.* **26**, 117.
Murthy, L., Klevay, L. M. & Petering, H. G. (1974). *J. Nutr.* **104**, 1458.
Owen, C. A. (1964). *Am. J. Physiol.* **207**, 446.
Sandell, E. B. (1958). *Colorimetric Determination of Trace Metals*, 2nd ed. New York: Interscience Publishers Inc.
Smith, B. S. W., Field, A. C. & Suttle, N. F. (1968). *J. comp. Path.* **78**, 449.
Smith, B. S. W. & Wright, H. (1975*a*). *J. comp. Path.* **85**, 299.
Smith, B. S. W. & Wright, H. (1975*b*). *Clinica chim. Acta* **62**, 55.
Standish, J. F., Ammerman, C. B., Wallace, H. D. & Combs, G. E. (1975). *J. anim. Sci.* **40**, 509.
Suttle, N. F. (1973). In *Trace Substances in Environmental Health*, vol. 7, p. 245 [D. D. Hemphill, editor]. Columbia, Mo.: University of Missouri.
Suttle, N. F. (1974). *Proc. Nutr. Soc.* **33**, 299.
Suttle, N. F. & Field, A. C. (1968). *J. comp. Path.* **78**, 351.

Printed in Great Britain