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FRACTIONATION OF SOLUBLE MOLYBDENUM-BINDING PROTEINS FROM LIVER, KIDNEY, PLASMA AND ERYTHROCYTES FROM SHEEP SUPPLEMENTED WITH MOLYBDENUM*

By

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NORHEIM, GUNNAR, NILS E. SØLI, ARNE FRØSLIE and MILICA MJØR-GRIMSRUD: *Fractionation of soluble molybdenum-binding proteins from liver, kidney, plasma and erythrocytes from sheep supplemented with molybdenum.* Acta vet. scand. 1980, *21*, 428—437. — Five ewes were given a daily dose of 1.4 mg Mo/kg body weight as ammonium molybdate for 21 to 55 days. Plasma molybdenum concentrations increased considerably following the dosage, but no systematic changes were seen in the plasma copper concentrations. About 80 % of the molybdenum present in blood was found in plasma. With gel filtration it was shown that practically all of the molybdenum present in plasma and red cell haemolyzates was found in a fraction with molecular weight less than 1,500. Soluble proteins from liver and kidney cortex were separated into one high molecular weight fraction with molecular weight above 200,000 and a non-protein fraction with molecular weight less than 1,500. Significant correlations were found between the amounts of molybdenum present in the high molecular weight fraction and the molybdenum concentrations in liver and kidney.

molybdenum; sheep; liver; kidney; plasma; erythrocytes; protein binding; molybdenum supplementation.

The interaction between molybdenum and copper has been studied carefully since the early observations that molybdenum counteracts chronic copper poisoning in sheep and that copper

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overcomes the toxic effect of high molybdenum diets in cattle. The mechanisms of these phenomena are dependent upon the presence of inorganic sulphate (*Underwood* 1977).

Interactions between these elements have been found in the rumen (*Dick et al.* 1975, *Suttle* 1975, *Mason et al.* 1978, *Grace & Suttle* 1979), in plasma (*Suttle & Field* 1968, *Smith & Wright* 1975a, b, *Bremner & Young* 1978, *Grace & Suttle*), in the liver (*Smith et al.* 1968, *Marcilese et al.* 1969) and in the kidney as well (*Smith et al., Marcilese et al.* 1970, *Bremner & Young*). These interactions have recently been reviewed by *Mason* (1978).

The aim of the present investigation was to study the distribution of molybdenum among soluble proteins from liver, kidney, plasma and erythrocytes of sheep with high copper levels which received supplemental molybdenum.

MATERIALS AND METHODS

Animal dosage

Five two-years-old ewes (Nos. 1—5) of Norwegian mixed breed, originating from a herd with high copper status, were dosed with ammonium molybdate. $(NH_4)_6Mo_7O_{24}4H_2O$ (p.a. Merck) was dissolved in distilled water at a concentration of 0.1 %, and the doses were as follows: 1.4 mg Mo/kg body weight was given daily as a drench for 21—55 days. Sheep Nos. 1—4 were killed 24 h after the last dose and sheep No. 5 was killed 20 days after the last dose. The animals were fed hay and water and kept in indoor pens. The hay and water contained 3.6 μg Cu/g dry weight and 0.28 μg Cu/ml, and 0.22 μg Mo/g dry weight and 0.001 μg Mo/ml, respectively, as determined by neutron activation analysis. The sulphur content in the hay was 0.13 % S, as determined by x-ray diffraction (Institutt for atomenergi, Kjeller, Norway). No additional sulphur was given. Summarized information on the experiment is given in Table 1. Blood samples were collected before and at weekly intervals after the start of the dosing and 24 h after the last dose. The red cells were separated by centrifugation and washed twice with 0.9 % saline. All samples were stored at —20°C.

Analytical methods

The gel filtrations of cytosol of tissue cells, red cell haemolyzates and plasma were performed on Sephadex G-100 superfine

Table 1. Body weight and dosing programme of the experimental sheep.

Sheep No.	Body weight (kg)	Length of dosing (days)	Total dosage of molybdenum (g Mo)	Remarks
1	34	30	1.4	Killed 24 h after the last dose
2	42	55	3.2	„
3	74	21	2.2	„
4	75	21	2.2	„
5	35	36	1.8	Killed 20 days after the last dose

(Pharmacia). In addition soluble proteins from two of the liver samples were gel filtrated on Sephadex G-200 superfine. Organ samples were homogenized in a Sorwall Omni Mixer with a 0.01 N Tris buffer (pH = 8.0 at 4°C) containing 0.05 N potassium chloride (*Norheim & Steinnes* 1975, 1976); 100 mg sodium azide/l was used to prevent bacterial growth. Plasma and red cells were diluted 1 + 2 with the same buffer. The homogenates and red cell haemolyzates were centrifuged at 50,000 × g at 4°C for 2 h. Before application on the column, the protein extracts were filtered through 3.0 μ Millipore filters. Three ml of filtrates were applied on the column (2.6 × 40 cm), a descending flow was used and 5 ml fractions were collected. The flow rates on the G-100 and G-200 columns were 12.5 and 5.0 ml/h, respectively. All gel filtrations were performed at 4°C. The approximate mean molecular weight associated with each fraction was estimated using the following five substances: Caeroplasmin (m.w. 151,000), albumin bovine (m.w. 67,000), chymotrypsinogen A (m.w. 25,000), cytochrome C (m.w. 12,400) and bacitracin (m.w. 1,411). The fractions were numbered I—IV, with approximate molecular weights > 90,000 (I), 35,000 (II), 10,000 (III) and < 1,500 (IV) (*Norheim & Søli* 1977, *Mjør-Grimsrud et al.* 1979). The molybdenum concentrations in the protein fractions were determined by the dithiol method (*Norheim & Waasjø* 1977) or by neutron activation analysis at Institutt for atomenergi, Kjeller, Norway (*Steinnes & Johansen,* personal communication), the detection limits were 0.1 μg Mo/g (ml) and 0.001 μg Mo/ml, respectively. Copper and zinc were determined by atomic absorption spectroscopy (*Frøslie & Norheim* 1976, *Norheim & Søli*).

Statistical methods

Linear regressions and *t*-tests were calculated on a Compucorp 344 Statistician.

RESULTS

None of the sheep showed any clinical effect of the molybdenum dosage, and routine necropsy revealed no pathological changes.

Plasma molybdenum concentrations increased considerably following the dosage (Table 2). Before dosage the concentrations were less than 0.02 μg Mo/ml, which was the detection limit for this sample with neutron activation analysis, while concentrations of more than 400 fold were measured already seven days after the first dosage. The concentrations varied, however, during the experimental period, but there were no systematic changes. In sheep No. 5, which lived for 20 days after the last dose, the molybdenum concentrations in plasma decreased slowly from about 10 to 0.2 μg/ml during this period. Plasma copper remained relatively stable (Table 2).

Table 2. The concentrations of copper and molybdenum in plasma (μg/ml) during the experiment in sheep Nos. 1, 2 and 5.

Sheep No.		Day										
		0	7	14	21	28	31	35	37	42	49	56
1	Cu	1.54	1.32	1.06	1.16	—	1.16					
	Mo	< 0.02	10	15	6.8	—	26					
2	Cu	1.28	1.20	1.18	1.10	1.10	—	1.18	1.02	1.30	1.10	1.52
	Mo	< 0.02	7.4	9.3	8.9	15	—	22	17	9.5	5.9	10
5	Cu	1.18	1.10	1.18	1.14	1.48	—	1.42	1.20	1.26	1.20	1.46
	Mo	< 0.02	9.4	11	7.2	9.4	—	12	12	3.6	2.3	0.2

At the time of killing, the packed red cells of sheep Nos. 1 and 5 contained 6.3 and 0.06 μg Mo/ml, respectively, while the corresponding concentrations in plasma were 26 and 0.2 μg Mo/ml. The ratio between molybdenum concentrations in plasma and red cells was 4.1 and 3.3, for the respective sheep.

Practically all of the molybdenum in plasma and red cell haemolyzates were present in a single fraction following gel filtration. This Fraction IV had molecular weight less than 1,500

(Fig. 1). Small amounts of the plasma molybdenum were found in the high molecular weight Fraction I.

Table 3 presents the total concentrations of copper, zinc and molybdenum in liver and kidney cortex, together with the levels in the samples of plasma and red cells used for gel filtration. The table also gives the percentage of molybdenum extractable from the tissue homogenates and the distribution of the molybdenum-binding proteins following gel filtration.

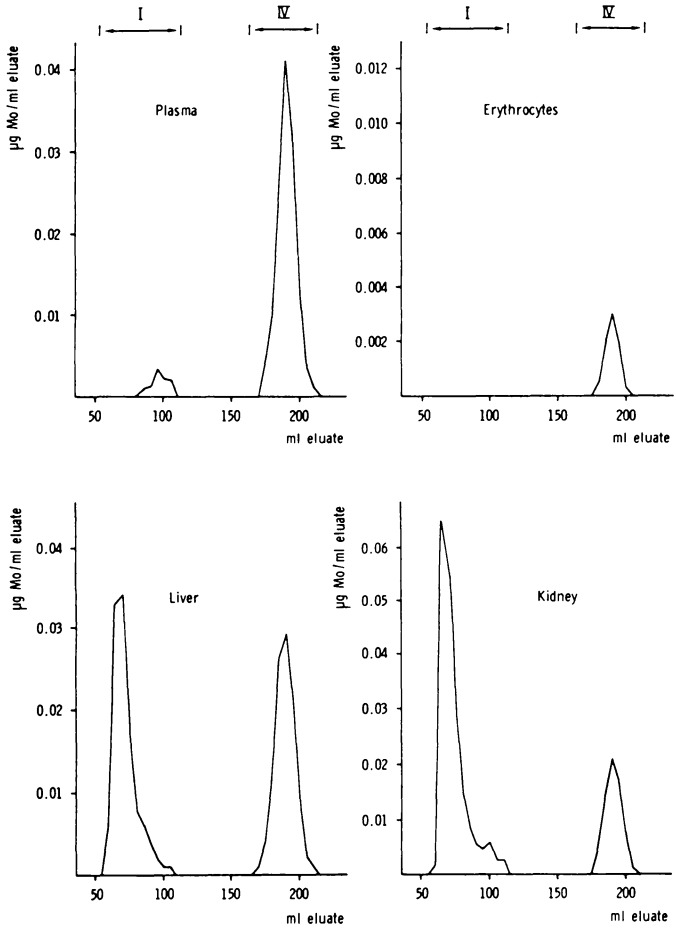

Figure 1. The distribution of soluble molybdenum-binding proteins from plasma, erythrocytes, liver and kidney from a sheep supplemented with molybdenum (sheep No. 5) after gel filtration on Sephadex G-100.

Table 3. The concentrations of copper, zinc and molybdenum in liver, kidney cortex (μg/g wet weight), plasma and red cell haemolyzate (μg/ml) in five molybdenum-supplemented sheep at the time the animals were killed. The absolute and relative amounts of molybdenum applied on the column found in Fractions I and IV after gel filtration on Sephadex G-100 are given together with the percentage of total molybdenum found in the protein extract.

Sheep No.	Organs/ body fluid	Cu (μg/g wet weight)	Zn	Mo	Extractable Mo (%)	Fraction I		Fraction IV	
						μg Mo	% Mo	μg Mo	% Mo
1	Liver	290	32	22	73	3.4	21	12.7	79
	Kidney	4	16	84	32	5.6	21	21.2	79
	Plasma	1.2		26	—	0.3	1	25.7	99
	Red cells	0.7		6.3	—	0	0	6.3	100
2	Liver	280	41	8.6	34	1.0	33	2.0	67
	Kidney	6		66	11	3.3	45	4.0	55
3	Liver	280	34	3.1	43	0.6	48	0.7	52
	Kidney	66		6.0	90	3.8	70	1.6	30
4	Liver	196		2.8	31	0.4	47	0.5	53
	Kidney	6.6	26	12	26	2.0	63	1.2	37
5	Liver	400	41	1.4	53	0.4	54	0.3	46
	Kidney	7		3.0	60	1.3	72	0.5	28
	Plasma	1.5		0.2	—	0.01	6	0.2	94
	Red cells	2.1		0.06	—	0	0	0.06	100

The extractable molybdenum in liver and kidney cortex was separated into two distinct fractions on Sephadex G-100 (Fig. 1), a high molecular weight Fraction I and a low molecular weight non-protein Fraction IV. A small part of the molybdenum present in the high molecular weight fraction was bound to a protein with approximate molecular weight as albumin bovine. Gel filtration on Sephadex G-200 gave the same results, the high molecular weight fraction was eluted with the void volume, and had thus a molecular weight above 200,000.

The absolute and relative amounts of molybdenum present in Fractions I and IV were clearly dependent on the molybdenum level both in liver and kidney. The percentage of molybdenum bound to soluble proteins decrased with increasing molybdenum level. A significant correlation was obtained between the percentage of molybdenum in Fraction I and the molybdenum concentrations in both liver ($r = -0.96$) and kidney ($r = -0.97$). In liver a significant correlation was obtained between the abso-

lute amounts of molybdenum present in Fraction I and the liver molybdenum concentration ($r = 0.99$). In kidney a significant higher percentage of the extractable molybdenum was bound to proteins compared to liver.

The soluble copper- and zinc-binding proteins were separated into three main fractions, I, II and III, with approximate molecular weights of $> 90{,}000$, 35,000 and 10,000, respectively. The protein spectra were quite similar to previous findings in normal sheep with the same copper status (*Norheim & Søli* 1977). However, in sheep Nos. 1 and 2, which had the highest molybdenum levels, there was also a small amount of copper present in the low molecular weight molybdenum-binding fraction.

DISCUSSION

In the present investigation of molybdenum-supplemented sheep only one high molecular weight molybdenum-binding fraction was found in liver and kidney cortex, while all the molybdenum present in red cell haemolysate and mostly all in plasma were found in a non-protein fraction with molecular weight below 1,500. A certain part of the extractable molybdenum in liver and kidney was also found in this low molecular weight fraction. There seems to be a clear relationship between the amounts of molybdenum bound to proteins and tissue concentrations of molybdenum, especially in liver.

Several works deal with the protein binding of molybdenum. *Norheim & Søli* (1977) showed that in both normal and copper-poisoned sheep about half of the soluble molybdenum present in liver was protein-bound. *Smith & Wright* (1975a) found that most of the molybdenum present in plasma from molybdenum-supplemented sheep was protein-bound and that about half of this molybdenum was present in a fraction insoluble in trichloroacetic acid. Furthermore they concluded that most of the molybdenum present in plasma from molybdenum-supplemented guinea pigs was bound to proteins. This is in contrast to our findings in sheep, and also differs from the early findings of *Scaife* (1956) who reported that in sheep all plasma molybdenum was dialysable.

In sheep supplemented with both molybdenum and considerable amounts of sulphate, about half of the plasma molybdenum was protein-bound and approximately two-thirds of this molybdenum was present in a fraction with an approximate molecular

weight of 90,000 (*Bremner & Young* 1978). The remainder of the molybdenum was found in a low molecular weight fraction along with peptides and amino acids. After ultrafiltration they found that about 54 % of the plasma molybdenum was in diffusible form. They also found that the molybdenum present in the cytosol of the kidneys was detected only in the high molecular weight fraction. Thus it seems clear that sulphur intake is decisive also for the protein binding of molybdenum. *Bremner & Young* also noted a change in the copper distribution of the kidney cytosol, a change which was not seen in the present investigation.

In a recent study *Grace & Suttle* (1979) concluded that high intake of both molybdenum and sulphur may lead to the formation of thiomolybdate-type complexes. These complexes are poorly absorbed but even more poorly excreted.

In the present experiment plasma molybdenum concentrations varied considerably. The copper plasma levels were relatively high before the start of the experiment and no subsequent systematic changes were observed. However, it is well known that molybdenum supplementation alone has little effect on the copper level. We observed no clinical effects in our sheep as was reported by *Bremner & Young*. About 80 % of the molybdenum present in blood was in the plasma. A relatively high molybdenum clearance from plasma was noted.

The molybdenum concentrations in liver and kidney cortex also varied considerably, likewise the amount of molybdenum present in the cytosol. Despite these facts significant correlations were found between the amounts of molybdenum present in Fractions I or IV and the total molybdenum concentrations.

Preliminary results seem to indicate variable amounts of molybdenum in Norwegian fodder, whereas the copper and sulphur contents are more constant (*Frøslie & Norheim*, unpublished results). Nevertheless copper levels in Norwegian sheep liver vary considerably and are in most parts of the country very high (*Frøslie & Norheim* 1976, *Frøslie* 1977). Only insufficient information concerning the copper, sulphur and molybdenum content in hay and grass is available. Based on these circumstances and the present findings it may be concluded that there is a need for further investigations on the interrelationship between copper, molybdenum and sulphur in Norwegian sheep grazing on natural pastures.

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SAMMENDRAG

Fraksjonering av løslige molybdenbindende proteiner fra lever, nyre, plasma og erytrocytter fra sau gitt molybdentilskudd.

Fem søyer ble dosert daglig med 1,4 mg Mo/kg kroppsvekt, som ammonium molybdat, i 21 til 55 dager. Det ble funnet en betydelig økning av molybdenmengden i plasma etter dosering, men det ble ikke funnet noen systematisk variasjon i kobbernivået. Omlag 80 % av blodets molybdeninnhold ble funnet i plasma. Ved hjelp av gelfiltrering ble det vist at praktisk talt alt molybdenet i plasma og erytrocytter var bundet i en fraksjon med molekylvekt mindre enn 1.500. I cytosol fra lever og nyre var en del av molybdenet bundet til proteiner med molekylvekt > 200.000, mens resten ble gjenfunnet i en fraksjon med molekylvekt < 1.500. Mengden av proteinbundet molybden økte med innholdet i leveren, mens den relative mengden av proteinbundet molybden avtok med stigende molybdennivå både i lever og nyre.

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