Biochimica et Biophysica Acta, 455 (1976) 937–946

BBA 77528

MOLYBDATE AND TUNGSTATE TRANSFER BY RAT ILEUM COMPETITIVE INHIBITION BY SULPHATE

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(Received May 3rd, 1976)

SUMMARY

For both MoO_4^{2-} and WO_4^{2-} the maximum rate of uptake by the small intestine of the rat (studied in vitro using the everted sac technique) occurs in the lower ileum. Kinetic constants, derived by a least squares procedure, are compared with those previously obtained for SO_4^{2-} transport. For both V and K_a, $SO_4^{2-} > MoO_4^{2-} > WO_4^{2-}$, with only small differences between sacs IV and V. Mutual inhibition of MoO_4^{2-} and WO_4^{2-} transport and inhibition of both by SO_4^{2-} are competitive processes. This is shown by the generally good agreement between K_a values and derived K_i values and by V values in the presence and absence of the inhibiting species. The three ions SO_4^{2-}, MoO_4^{2-} and WO_4^{2-} are probably transferred across the intestine by a common carrier system. Implications for the sulphate-molybdenum interaction in molybdosis are discussed.

INTRODUCTION

In our previous paper [1], we described SO_4^{2-} transport studies by everted sacs of rat ileum, and showed a competitive inhibition of SO_4^{2-} transport by MoO_4^{2-}. Our interest in the MoO_4^{2-}/SO_4^{2-} interaction in transport systems arises from the existence of a molybdenum/sulphur antagonism in experimental molybdenum toxicity [2, 3]. Symptoms of molybdenum toxicity in animals can in some circumstances be alleviated by feeding SO_4^{2-}, and competition by the two anions for transport at the intestinal or renal level has been proposed as a major factor in the antagonism [4, 5].

Molybdenum is unique among the essential trace metals in existing as the simple oxyanion MoO_4^{2-} at normal physiological pH [6]. The metal therefore is unlikely to show competitive binding interactions with other trace metal cations for enzymic and transport processes, but might be expected to compete for anion binding sites appropriate to its charge and tetrahedral stereochemistry. Reduced forms of molybdenum [Mo (V) and Mo (IV)] occur in molybdoenzymes [7], but because of the large reduction potentials involved are unlikely to be formed in transport across the intestine. There are two brief previous reports [3, 8] describing intestinal absorption of MoO_4^{2-}, studied in vivo. Factors affecting molybdenum uptake have never

been examined. The main aim of the present work was to study the intestinal uptake of MoO_4^{2-} and the influence of a variety of factors including SO_4^{2-}, WO_4^{2-} and Cu (which alleviates molybdenosis in some circumstances [2, 3]).

The transport of the best known molybdenum antagonist, tungsten, as WO_4^{2-} [9] was also examined, to establish whether the three anions SO_4^{2-}, MoO_4^{2-} and WO_4^{2-} are transported by a common transport system in the small intestine. WO_4^{2-} transport has never been previously studied.

EXPERIMENTAL

Everted sacs of rat small intestine were prepared and incubated as previously described [1]. All media were SO_4^{2-}-free unless stated, as before. $Na^{99}MoO_4$ or $Na^{185}WO_4$, (both obtained from the Radiochemical Centre, Amersham, England), were used as tracers. Both were essentially carrier-free, and the concentrations of MoO_4^{2-} and WO_4^{2-} used in the experiments were obtained using Na_2MoO_4 and Na_2WO_4.

Samples containing ^{99}Mo were counted 40 h after each experiment as ^{99}Tc on a Nuclear Chicago γ-counter. Biological discrimination in favour of MoO_4^{2-} was very evident in samples counted before reaching radioactive equilibrium. Aliquots of serosal and mucosal fluid were counted, but gut sacs themselves were counted whole.

Samples containing ^{185}W were counted on a Packard Tri-Carb scintillation counter as 1 ml samples in 10 ml of toluene/Triton X/PPO scintillant [10]. Aliquots of serosal and mucosal fluid were again counted. None of the standard homogenisation and deproteinisation techniques gave a good recovery of added ^{185}W standards from gut tissue, presumably due to precipitation of the tungsten. 1 ml aliquots of gut homogenate were therefore treated with 1 ml of "Protosol" tissue solubiliser (New England Nuclear) and counted after digestion using the same scintillation fluid. Little quenching in these samples was detected by the channels ratio method, and it was found that recovery of label from the three fractions combined was normally better than 99 % of label added initially.

Parameters used, methods of calculation and the system of weighing used to determine fluid transport were as previously described [1].

RESULTS AND CONCLUSIONS

Site of MoO_4^{2-} and WO_4^{2-} uptake

Both MoO_4^{2-} and WO_4^{2-} are maximally absorbed in the lower ileum under the conditions used (Fig. 1). This pattern resembles that previously observed for SO_4^{2-} uptake [1], though the gradient along the intestine is not so steep. A larger fraction of the total uptake, relative to SO_4^{2-}, is found in the gut, and a smaller fraction in the serosal fluid. This pattern is observed more with WO_4^{2-} than MoO_4^{2-}. Serosal transfer for the three anions still shows the same overall trends as total mucosal transfer, however (Fig. 2). Although sacs other than IV and V have been used in several experiments, patterns of inhibition or other behaviour observed were always similar, and the following account deals only with the ileum, sacs IV and V.

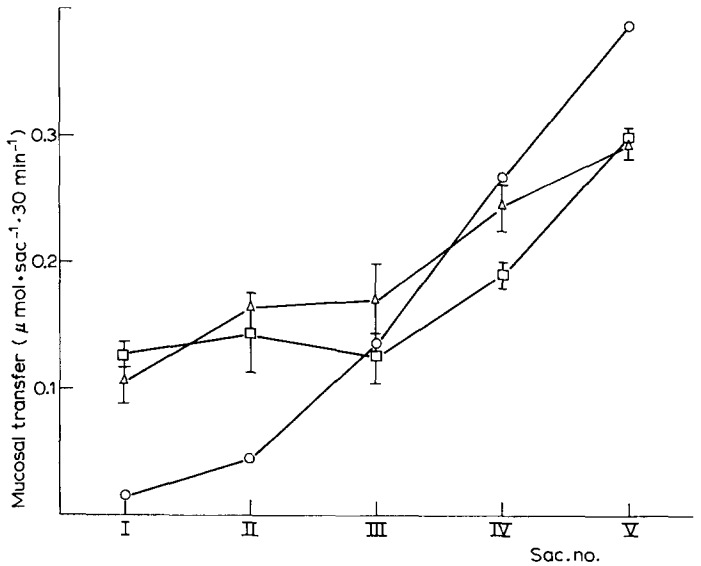

Fig. 1. MoO_4^{2-} and WO_4^{2-} transport by different parts of rat small intestine. Incubation is as described under Methods. Each point is the mean of two or more determinations, with range as shown. Mucosal transfer is expressed as μmol accumulated in 30 min, using an initial mucosal concentration of 0.05 mM (1.25 μmol per incubation), and is defined as the sum of serosal and gut transfers. Values for SO_4^{2-} at 0.04 mM are plotted for comparison [1]. □, WO_4^{2-}; △, MoO_4^{2-}; ○, SO_4^{2-}.

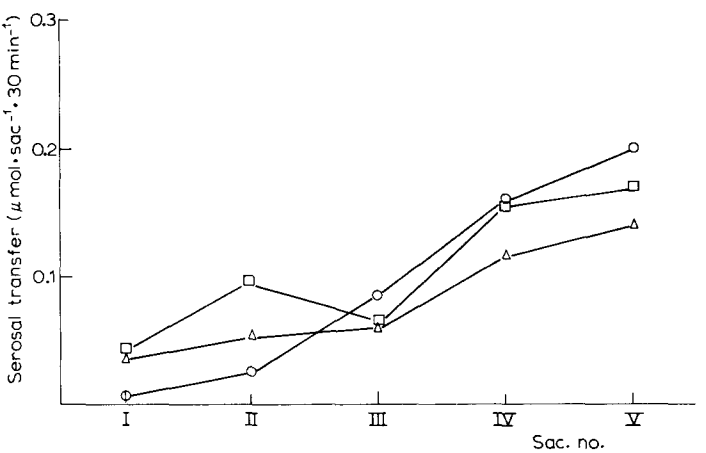

Fig. 2. Serosal MoO_4^{2-} and WO_4^{2-} transfer. This parameter is measured directly. Conditions as in Fig. 1. △, MoO_4^{2-}; ○, SO_4^{2-}; □, WO_4^{2-}.

Concentration dependence of MoO_4^{2-} and WO_4^{2-} uptakes

For both MoO_4^{2-} and WO_4^{2-} the concentration-dependence of mucosal transfer shows saturability in both sacs IV and V (Fig. 3)*. Kinetic constants have

* As the pattern for both Mo and W is very similar, the data for W have been omitted.

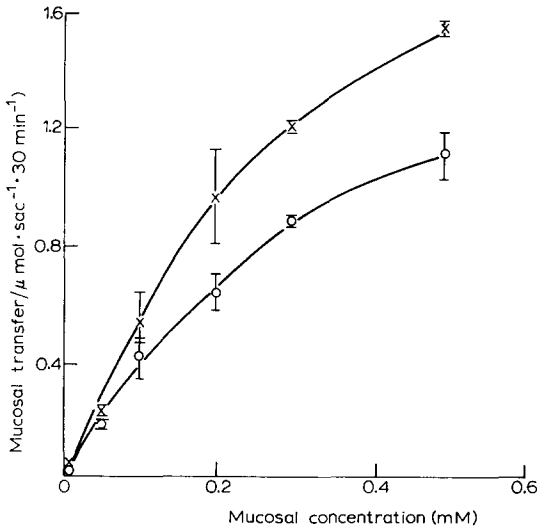

Fig. 3. Concentration dependence of MoO_4^{2-} transfer by rat ileum. Each point is the mean of at least two determinations with range as shown. The range at lower concentrations is normally too small to be visible. Mucosal transfer is expressed as μmol accumulated per sac in 30 min. ○, Sac IV; ×, Sac V.

been derived from the data by linear regression of S/V upon S[11], but visual inspection of the S/V over S plots show that for MoO_4^{2-} the plot is not linear for very low values of S (< 0.01 mM). These points have been omitted from the analysis. The deviation is probably a consequence of significant depletion of the mucosal fluid during incubation, and an artefact of the procedure. The derived kinetic constants are presented in Table I, and the values previously derived [1] for SO_4^{2-} transport included for comparison. As expected, the differences between sacs IV and V are smaller than for SO_4^{2-}, and are not significant in terms of derived standard errors. Both K_a and V values decrease in the order $SO_4^{2-} > MoO_4^{2-} > WO_4^{2-}$, conventionally interpreted as giving WO_4^{2-} the highest affinity for the binding site but the

TABLE I

KINETIC CONSTANTS FOR MOLYBDATE AND TUNGSTATE TRANSFER BY RAT ILEUM

Results are expressed in ±S.E., with the number of runs in brackets. Sac IV and sac V are as defined previously [1]. Constants are obtained by linear regression analysis as described in the text.

	Sac IV		Sac V	
	V (μmol sac^{-1} · 30 min^{-1})	K_a (mM)	V (μmol sac^{-1} · 30 min^{-1})	K_a (mM)
SO_4^{2-}	3.7±0.2	0.44±0.08	6.8±0.2	0.60±0.05
MoO_4^{2-}	2.9±0.22 (19)	0.51±0.71 (19)	3.5±0.31 (24)	0.41±0.07 (24)
WO_4^{2-}	1.0±0.08 (12)	0.22±0.03 (12)	1.7±0.16 (12)	0.28±0.04 (12)

lowest rate of transfer. To eliminate the possibility that this higher affinity of WO_4^{2-} was an artefact due to simple external binding of WO_4^{2-}, experiments using 1 min incubation times were performed. In all cases a negligible gut uptake was observed, together with negligible WO_4^{2-} loss from the external incubation medium. Additionally, examination of the concentration ratio plots (Figs. 4 and 5) shows that, at all

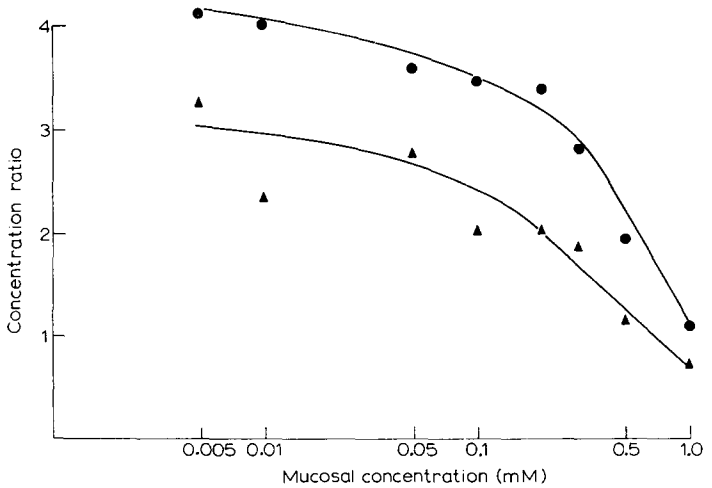

Fig. 4. Dependence on mucosal MoO_4^{2-} concentration of the degree of concentration of MoO_4^{2-} by rat lower ileum (sac V). Final serosal/mucosal (●) and gut/mucosal (▲) concentration ratios developed are plotted against initial mucosal MoO_4^{2-} concentration. The values plotted are derived from the same experimental data used in deriving the kinetic constants for sac V. Final serosal, gut and mucosal volumes are calculated from the system of weighings used [13]. The fluid content of the gut was assumed to be 80 % of initial wet weight. Final mucosal volume was obtained by difference. The logarithmic scale of the plot is purely for convenience and implies nothing about the form of the relationship between the variables.

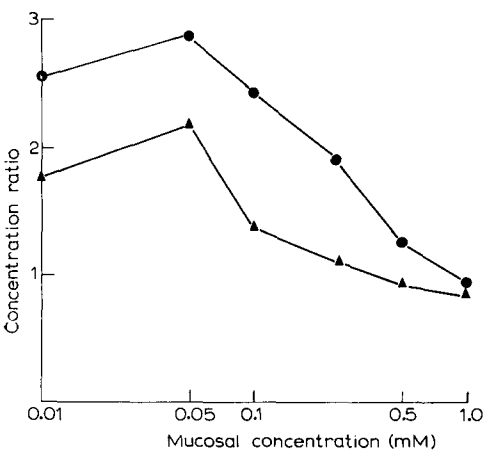

Fig. 5. Dependence on mucosal WO_4^{2-} concentration of the degree of concentration of WO_4^{2-} by rat lower ileum (sac V). Ratios calculated as described in the legend to Fig. 6. ●, serosal/mucosal; ▲, gut/mucosal.

concentrations, for both MoO_4^{2-} and WO_4^{2-}, the serosal/mucosal concentration ratio is consistently higher than the gut/mucosal ratio. If binding unrelated to net transport occurred to a significant extent, relatively lower serosal concentrations would be expected. The concentration ratios developed during the 30 min incubation are lower than those for SO_4^{2-} [1], but nevertheless clearly demonstrate concentration by the intestine against a concentration gradient, for both ions*. With both these ions the assumption of negligible gut concentrations at the start of the experiment is an extremely good one; mean levels of Mo in human ileum (no data published for the normal rat) are about 0.03 $\mu g \cdot g^{-1}$ of wet tissue [12] corresponding, even if all this were free MoO_4^{2-}, to about 0.004 mM.

Inhibition studies

MoO_4^{2-} inhibition of WO_4^{2-} transfer was studied by redetermining the concentration dependence of WO_4^{2-} transfer in the presence of 1.0 mM MoO_4^{2-} (Fig. 6). Inhibition of MoO_4^{2-} transfer by WO_4^{2-} and SO_4^{2-} was studied in less detail,

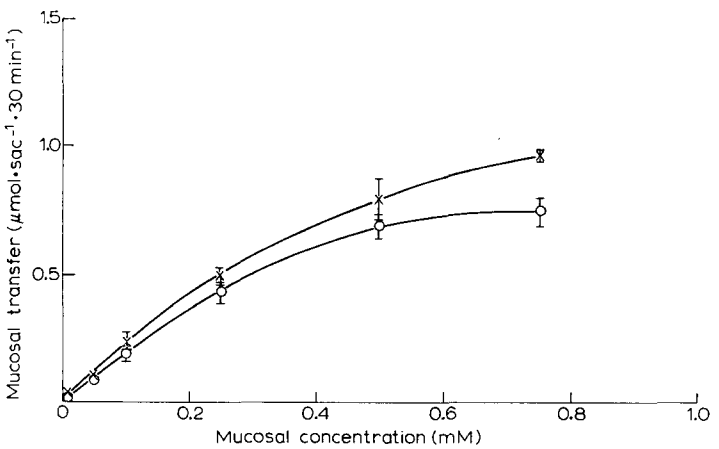

Fig. 6. WO_4^{2-} transfer by rat ileum in the presence of 1.0 mM MoO_4^{2-}. Other conditions as Fig. 4. ○, sac IV; ×, sac V.

chiefly because of the short half-life of ^{99}Mo ($t_{\frac{1}{2}}$ 68 h). K_i values were derived algebraically by taking means of pairs of duplicate runs at varying substrate concentrations (i.e. 4 runs for each K_i value). Derived kinetic constants are presented with other results for comparison in Table II. The derived constants for MoO_4^{2-} inhibition of WO_4^{2-} transport show a V for WO_4^{2-} unaffected by 1.0 mM MoO_4^{2-}, and K_i similar to K_a for MoO_4^{2-} alone. The previously derived K_i for MoO_4^{2-} inhibition of SO_4^{2-} transport is now seen to be in good agreement with these new values, particularly for sac V. Agreement of derived K_i values for SO_4^{2-} inhibition of MoO_4^{2-} transport with K_a values for these substrates is again satisfactory but better for sac V.

* It has been suggested that it would be of interest to know the magnitude of the trans-epithelial potential. As yet we have made no measurements of this potential.

TABLE II

KINETIC CONSTANTS DERIVED FROM INHIBITION STUDIES

Values are quoted ±S.E. with the number of runs in brackets. K_I values marked with (+) have been determined algebraically (see text).

Substrate		Inhibitor		
		SO_4^{2-}	MoO_4^{2-}	WO_4^{2-}
Sac IV				
SO_4^{2-}	V	3.7 ±0.2 (24)	5.9 ±0.5 (14)	—
	K_a	0.44±0.08	2.6 ±0.5	—
	K_I	—	0.32	0.53 (+)
MoO_4^{2-}	V	—	2.9 ±0.22 (19)	—
	K_a	—	0.47±0.07	—
	K_I	0.83 (+)	—	0.29 (+)
WO_4^{2-}	V	—	1.4 ±0.27 (14)	1.0 ±0.08 (12)
	K_a	—	0.64±0.19	0.22±0.03
	K_I	0.46 (+)	0.52	—
Sac V				
SO_4^{2-}	V	6.8 ±0.2 (23)	7.2 ±0.7 (14)	—
	K_a	0.60±0.05	2.5 ±0.5	—
	K_I	—	0.31	0.35 (+)
MoO_4^{2-}	V	—	3.3 ±0.09 (24)	—
	K_a	—	0.35±0.02	—
	K_I	0.52 (+)	—	0.30 (+)
WO_4^{2-}	V	—	2.2 ±0.6 (14)	1.7 ±0.16 (12)
	K_a	—	0.82±0.35	0.28±0.05
	K_I	0.53 (+)	0.36	—

Fluid transport

Mucosal fluid transport [13] taken as the sum of serosal and gut fluid uptakes, was determined in all experiments. Mean values for three series of experiments are presented in Table III. 1.0 mM MoO_4^{2-} previously caused a small but significant decrease in fluid transport [1]. The presence of WO_4^{2-} causes a small further decrease, whereas SO_4^{2-} is without effect. Further, the effects of MoO_4^{2-} and WO_4^{2-} appear to be additive. Incubation in the absence of glucose reduces fluid transport to a very low value (see also ref. 13) and prevents concentration of either ion.

TABLE III

FLUID TRANSPORT

Mean mucosal fluid transport was determined as described in ref. 13, using a 30 min incubation time. The data have been calculated from experiments using a range of MoO_4^{2-} and WO_4^{2-} concentrations and are ±S.E. with the number of experiments in parentheses.

	Sac IV		Sac V	
	g per sac	g per g tissue	g per sac	g per g tissue
MoO_4^{2-} alone	1.14±0.60 (23)	0.88±0.44 (23)	0.90±0.035 (22)	0.70±0.036 (22)
WO_4^{2-} alone	0.99±0.086 (17)	0.89±0.076 (17)	0.73±0.088 (17)	0.65±0.085 (17)
WO_4^{2-}+MoO_4^{2-} (1 mM)	0.87±0.048 (14)	0.74±0.041 (14)	0.61±0.052 (14)	0.50±0.042 (14)

TABLE IV

INHIBITION OF MOLYBDATE AND TUNGSTATE TRANSPORT BY GROUP VI OXYANIONS

Anion	Mucosal transfer (μmol · sac^{-1} · 30 min^{-1})			
	MoO_4^{2-} (0.1 mM)		WO_4^{2-} (0.05 mM)	
	Sac IV	Sac V	Sac IV	Sac V
Control	0.47 ±0.7 (5)	0.54±0.09 (4)	0.189	0.298
$S_2O_3^{2-}$	0.147	0.27	0.045	0.055
SO_3^{2-}	0.415	0.34	—	—
SeO_4^{2-}	0.188	0.194	0.091±0.02 (3)	0.134±0.02 (3)
1 mM substrate*	0.199	0.241	0.074	0.095

* To give directly comparable figures, the experimental value has been divided by 10 for MoO_4^{2-} and by 20 for WO_4^{2-}.

Group VI oxyanions

As well as SO_4^{2-}, $S_2O_3^{2-}$ and SeO_4^{2-} inhibit MoO_4^{2-} and WO_4^{2-} transport, SeO_4^{2-} reducing transport to a level similar to that of either substrate at comparable concentrations (Table IV). $S_2O_3^{2-}$ is, unexpectedly, a stronger inhibitor, perhaps indicating strong affinity of the binding site for *S.* However, L-cysteine had no effect on MoO_4^{2-} transfer, whereas SO_3^{2-} as shown in Table IV has a slightly inhibitory effect only on sac V. The higher level of sulphite oxidase in this part of the gut [9] probably results in partial conversion to SO_4^{2-}.

Effect of copper

As little as 0.01 mM Cu^{2+} added to the incubation medium caused a marked reduction in fluid transport in experiments using MoO_4^{2-} (see also ref. 14). A series of runs were performed, examining a variety of modifications of the everted sac technique (use of Tris buffer, preincubation with Cu^{2+}, addition of glycine (ligand for Cu) to the medium, addition of Cu^{2+} to the serosal side, omission of glucose). All these were designed to circumvent direct chemical reaction between Cu^{2+} and MoO_4^{2-}. Fluid and MoO_4^{2-} transfer varied in an irregular manner in these experiments, however, and no satisfactory technique of studying this interaction could be established.

DISCUSSION

The results presented in this paper establish that both MoO_4^{2-} and WO_4^{2-} are readily transported, chiefly in the lower ileum, by the rat by means of a saturable system capable of concentrating both ions against a gradient. We have shown [15] that MoO_4^{2-} uptake by sheets of ovine intestine displays the same characteristics. In one earlier in vivo study of MoO_4^{2-} absorption [8] using ligated loops, the standard errors were high and significant differences between the different parts of the small intestine were not established. In the second study, [3] using chick intestine, it was merely shown that SO_4^{2-} inhibited MoO_4^{2-} uptake in vivo when present in 400-fold excess. Both studies must be regarded as preliminary.

The agreement between the kinetic constants presented provides good evidence that the three ions SO_4^{2-}, MoO_4^{2-} and WO_4^{2-} are transferred across the ileum by a common transport system subject to competitive inhibition. The normal dietary level of SO_4^{2-} is several-thousand-fold higher than that of MoO_4^{2-} [2], so that under physiological conditions it is very likely that the extent of absorption of MoO_4^{2-} would be dependent on the luminal SO_4^{2-} level. It is highly probable that competition is also a factor at other transport sites such as the renal tubule.

The observed inhibition of SO_4^{2-}, MoO_4^{2-} and WO_4^{2-} transport by SeO_4^{2-} and $S_2O_3^{2-}$ suggests that the anion binding site is capable of binding any anion of the same charge and stereochemistry. The effect of changing stereochemistry is shown in the lack of inhibition by SO_3^{2-} in sac IV. The K_a order $SO_4^{2-} > MoO_4^{2-} > WO_4^{2-}$, taken with the observed strong inhibition by $S_2O_3^{2-}$, presumably binding through *S*, indicate that the binding site may have a preference for the more polarizable tetrahedral anions, since one may write the ligand preferences for MX_4^{2-} as, for M, W > Mo > S, and, for X, S > O.

Tungsten is the element most chemically similar to molybdenum [7], it is the best known biological antagonist [17] and the only agent capable of producing experimental molybdenum deficiency in animals, by preventing the incorporation of molybdenum into xanthine oxidase and sulphite oxidase [9]. In the case of xanthine oxidase, inactive apoprotein is synthesised when WO_4^{2-} is fed [18], but in the case of sulphite oxidase [19], up to 35 % of the total molybdenum-free enzyme contained tungsten. The authors conclude that the systems for the absorption and transport of molybdenum, presumably as molybdate, are sufficiently non-specific to accept tungsten instead. Only in bacterial systems has tungsten incorporation given rise to active enzymes [20]. Our results show clearly that the intestinal uptake of MoO_4^{2-} is indeed not specific and that in addition to preventing molybdenum absorption, a high level of dietary tungsten will be largely absorbed by the animal. In the light of this discussion, the specific toxicity of molybdenum is puzzling. Many of the hypotheses proposed to explain the molybdenum/copper/sulphur interaction involve chemical properties of molybdenum which are well duplicated by those of tungsten. Examples are the formation of the MoS_4^{2-} anion (WS_4^{2-} is also known) and the formation of insoluble copper molybdates, also formed by tungsten [7]. An important difference between the elements, although probably not important in the present context, is that tungsten is less easily reduced to the lower oxidation states. If tungsten is well absorbed, and only distinguished from molybdenum at the point of incorporation into molybdoenzymes, it seems plausible that tungsten should itself produce toxic symptoms similar to those of molybdenum toxicity when fed in high levels. The clearest biochemical indicator of molybdenum toxicity is the elevated plasma copper level noted in sheep and guinea pigs [21] due to copper and molybdenum binding to a protein fraction of unknown origin. We have found that high levels of tungstate when fed to guinea pigs do not give rise to elevated plasma copper levels (Cardin, C. J. and Roberts, C. W., unpublished), nor to changes in the distribution of copper in plasma. We have also found that tungstate when fed to rats does not give the elevated liver copper levels resulting from molybdenum administration (Cardin, C. J., unpublished). The specificity of molybdenum toxicity must arise from interactions at sites other than the intestine. Mo (VI) and W (VI) form many analogous complexes with comparable binding constants [7] and, as in the molybdoenzymes,

only highly specific interactions can be responsible for the unique response to molybdenum of the guinea-pig. Smith and Wright [21] have shown that copper and molybdenum are bound by plasma proteins in a fixed ratio and Bremner [22] has subsequently shown that, in sheep, a single new protein is produced. This new fraction contains both acid-stable copper and most of the plasma molybdenum. It is noteworthy that both groups of workers report that the acid-stable copper (TCA insoluble) appears to be chelatable (direct-reacting), the converse of copper binding by many copper proteins such as ceruloplasmin. These properties suggest a new type of molybdenum-copper-protein interaction, as yet not established. A possible model for the kind of binding which could be involved might be the thiomolybdate complex Cu (NH_4) MoS_4 [23], which contains both MoS_4 and CuS_4 tetrahedra in the solid state. Tungsten cannot replace molybdenum in this complex, nor can nickel, cobalt or silver replace copper, which is in the Cu (I) state. There is already evidence that the new plasma protein fraction in sheep can be oxygen-sensitive [22] and therefore probably contains [Cu (I) and possibly Mo (V)]. The origin of the specificity of the molybdenum/sulphur/copper interaction remains a subject for speculation.

ACKNOWLEDGEMENTS

We thank Dr. B. Orsi for help with the kinetic analysis, and the Irish Department of Education and the Agricultural Institute (An Foras Taluntais) for financial support.

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