Copper–Molybdenum Interactions with the Sulfate-Reducing System in Rumen Microorganisms[1] (36177)

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An interaction between copper, molybdenum, and sulfate has been known for several decades (1). In ruminants, this interaction is much more sensitive to low levels of molybdenum than in nonruminants (2, 3). Studies in this laboratory indicated that a "Cu–Mo" complex is formed which is absorbed, transported, and excreted as a unit *in vivo*, and in which the copper is biologically less available (4, 5). The role of sulfate in this interaction, however, remains unclear. The precipitate resulting from mixing copper sulfate and sodium molybdate solutions has been chemically characterized *in vitro* (4, 6) and has been shown to contain no sulfur.

Since it is known that ruminants can reduce sulfate (7, 8), it is proposed that an intermediate or product of the sulfate reducing pathway, rather than sulfate itself, interacts with copper and molybdenum (9). This proposal is supported by studies showing that molybdate inhibits sulfate reduction in *Desufovibrio desulfuricans* (10).

This paper describes experiments investigating the interaction of molybdate with the sulfate-reducing system in sheep fed purified diets and the effect copper has on the system in the presence of molybdate.

Materials and Methods. Rumen samples were taken from a fistulated sheep on a sulfur-adequate (1.27% sodium sulfate) dietary regimen (11). The sheep were fed for 1 hr in the evening and again the morning. One hour following the morning feeding, 400 ml of rumen fluid were removed by use of a syringe.

Washed suspensions of rumen microorganisms were prepared according to the method of Sijpesteijn and Elsden (12) with the exception that sodium sulfide was excluded from the washing solution. All solutions were deoxygenated by using distilled water which was freshly boiled, cooled, and gassed with an oxygen-free mixture of 95% N_2–5% CO_2. The washed cells were then suspended in the designated buffer such that a 1:100 dilution of the suspension had an absorbance of 0.6 to 0.7 at 420 nm.

Reactions were conducted in an atmosphere of 95% N_2–5% CO_2 at 37° in a shaking water bath using Warburg flasks. The main compartment contained the washed ruminal suspension. The substrate (sulfate or sulfite), hydrogen donor (glucose), and inhibitor solutions were placed in one side bulb and the other contained 0.2 ml of 20 *N* H_3PO_4. Zinc acetate-impregnated filter paper accordions were placed in the center well to quantitatively precipitate hydrogen sulfide generated during the incubation.

Prior to starting the reaction, 0.1 μCi of $^{35}SO_4^{2-}$ in 0.1 *N* HCl (International Chemical and Nuclear Corp.), or where indicated, ^{35}S-sodium sulfite (Amersham/Searle Corp.) in 0.025 ml, was added to the side bulb containing the reactants. The flasks were gassed with the N_2–CO_2 mixture and then

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TABLE I. Effect of Molybdate on Sulfate Reduction.[a]

Conc $\times 10^{4+}$ M MoO_4^{2-}	Recovery of ^{35}S (%)				Inhibition of sulfate reduction (%)
	S^{2-}	SO_4^{2-}	Protein	Total	
0.0	42.7	53.0	5.0	101	0
1.7	24.4	70.9	7.9	103	43
3.3	6.0	83.2	6.3	96	86
6.7	0.6	95.4	6.6	103	99

[a] Each flask contained 2.0 ml of washed cell suspension, 30 μmoles of Na_2SO_4 (containing 0.1 μCi of $^{35}SO_4^{2-}$), 25 μmoles of glucose, indicated final concentrations of Na_2MoO_4 and 0.05 M sodium phosphate buffer (pH 6.8) in a total volume of 3.0 ml. Percentage recovery values were calculated after correcting for background counts, machine efficiency (62%), and differences in counting method (sulfide 69.2%, sulfate 79.4%, and protein 92.9%). Each value represents the mean of two separate determinations. Each flask was incubated for 4 hr at 37°.

stoppered securely. The reactions were initiated by tipping the contents of the side bulb containing the reactants into the main compartment and placing the flasks in the water bath. After 4 hr of incubation, the reaction was stopped and any hydrogen sulfide remaining in solution was liberated by tipping the phosphoric acid into the main compartment. The flasks were allowed to stand at least 15 min before removing the stoppers. The filter papers were then removed and dried in a drying oven.

The $H_2{}^{35}S$ trapped as ZnS was determined by placing the dried filter papers into 10 ml of toluene scintillation fluid (13) and counting with a liquid scintillation spectrometer. The cell suspension remaining in the main compartment was transferred to a test tube and placed in boiling water for 3 min to precipitate the protein. After centrifugation for 30 min at 12,000g, the supernatant was removed for sulfate determination. The protein precipitate was washed, recentrifuged, and this supernatant was combined with the first supernatant solution. The washed protein precipitate was transferred to a counting vial and oxidized with a modified Pirie's reagent (13). The white residue was dissolved in 1 ml of 0.05 M HCl, and toluene–Triton (Packard, scintillation grade) 2:1 mixture (14) was added and mixed, resulting in the formation of a clear emulsion suitable for liquid scintillation counting.

Sulfate present in the supernatant from the protein precipitation was precipitated as $BaSO_4$ and collected on a Millipore filter (5 μ) and washed. This filter was then dried and placed in a vial containing 10 ml of the toluene scintillation fluid for counting. The remaining supernatant, after removal of sulfate and protein, was concentrated and counted using the toluene–Triton 2:1 mixture.

Each experiment was conducted with a single source of rumen microorganisms and all treatment comparisons were run at the same time. Each value represents the mean of either two or three determinations as noted.

Results. Distribution of ^{35}S. Washed rumen cells were incubated anaerobically with 30 μmoles of $Na_2{}^{35}SO_4$ as described in Table I; and, subsequently, the amount of ^{35}S recovered as H_2S, protein, SO_4^{2-}, and supernatant sulfur was determined. The results shown in Table I do not include supernatant sulfur since this was negligible in every case. Using this procedure, it was possible to recover 100 ± 4% of the total counts initially introduced into each flask after correcting for machine and counting method efficiency.

Effect of molybdate on sulfate reduction. In the presence of Na_2MoO_4, the amount of H_2S produced was greatly reduced (Table I). The ^{35}S recovered in the protein fraction remained nearly constant, and that recovered

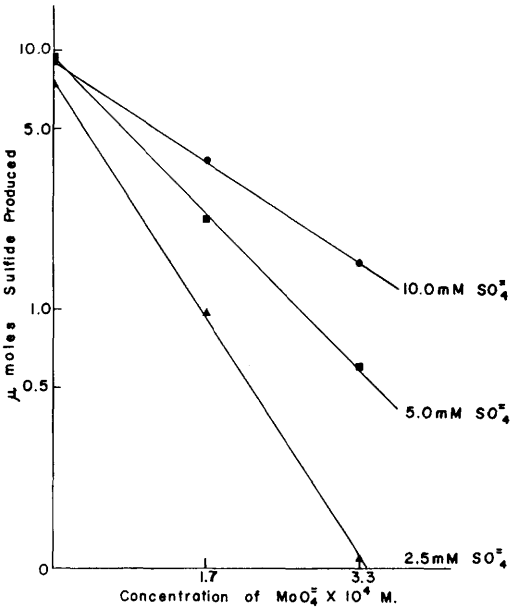

FIG. 1. The effect of molybdate on sulfide production under different concentrations of sulfate: Each flask contained 2 ml of washed cell suspension, 25 μmoles of glucose, indicated amounts of Na_2MoO_4 and Na_2SO_4 (each containing 0.1 μCi of ^{35}S-H_2SO_4) and 0.05 *M* sodium phosphate buffer (pH 6.8) in a total volume of 3.0 ml. Each datum represents the mean of two separate determinations. Each flask was incubated for 4 hr at 37°.

as sulfate increased in proportion to the decrease in sulfide production. In the presence of 10 m*M* Na_2SO_4, concentrations of molybdate as low as 6.7×10^{-4} *M* caused 99% inhibition. As the concentration of sulfate is decreased (Fig. 1), molybdate inhibition increases such that in the presence of 2.5 m*M* Na_2SO_4, concentrations of molybdate as low as 3.3×10^{-4} *M* caused 99% inhibition. Therefore, in the presence of low levels of substrate (limiting), sulfate reduction is much more sensitive to molybdate inhibition than at high levels of substrate.

Effect of molybdate on sulfite reduction. Initial experiments indicated that $^{35}SO_4^{2-}$ not reduced to sulfide remained as sulfate. This suggested that molybdate inhibited an early step of sulfate reduction prior to the possible formation of sulfite. If this were the case, then molybdate would have no effect on sulfite reduction. The results shown in Table

TABLE II. Comparison of the Effect of Molybdate on Sulfate and Sulfite Reduction.[a]

Conc × 10^{-6} *M* MoO_4^{2-}	Inhibition of reduction (%)	
	SO_4^{2-}	SO_3^{2-}
3.3	52.6	7.7

[a] Each flask contained 2 ml of washed cell suspension, 5 μmoles of substrate (Na_2SO_4 with ^{35}S–H_2SO_4 or Na_2SO_3 with ^{35}S–Na_2SO_3), 25 μmoles of glucose, indicated final concentration of Na_2MoO_4, and 3.7×10^{-3} *M* sodium bicarbonate–CO_2 buffer (pH 6.8) in a total volume of 3.0 ml. Each value represents the mean of three separate determinations. Each flask was incubated for 4 hr at 37°.

II indicate that sulfite reduction was inhibited by molybdate to a much lesser degree than sulfate reduction. The 7.7% inhibition observed could be due to sulfate contamination.

Effect of copper on molybdate inhibition. Previous investigations (4, 5) suggested that copper could decrease molybdate inhibition of sulfate reduction through the formation of a Cu–Mo complex. Figure 2 shows that Na_2MoO_4 alone caused a much greater decrease in sulfide production than $CuSO_4$

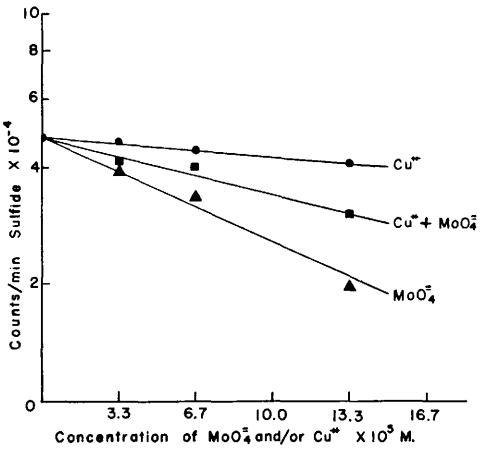

FIG. 2. The effect of copper(II) and molybdate ions on sulfide production. Each flask contained 2 ml of washed cell suspension, 30 μmoles of Na_2SO_4 (containing 0.1 μCi $H_2^{35}SO_4$), 25 μmoles of glucose, indicated amounts of Na_2MoO_4 and $CuSO_4$ and 3.7×10^{-1} *M* $NaHCO_3$ buffer (pH 6.8) in a total volume of 3.0 ml. Each datum represents the mean of two separate determinations. Each flask was incubated for four hours at 37°.

alone. In the presence of equal molar amounts of both $CuSO_4$ and Na_2MoO_4, however, the net inhibition was considerably less than the same concentration of Na_2MoO_4 alone. Therefore, in the presence of copper, molybdate could be less available for inhibition of sulfate reduction.

Discussion. In the presence of $^{35}SO_4^{2-}$, anaerobically incubated rumen microorganisms produced large quantities of $H_2{}^{35}S$. This reduction of sulfate to sulfide was shown to be inhibited by molybdate. Molybdate inhibition was decreased by increasing the concentration of sulfate, suggesting a competitive type inhibition.

Incubations in the presence of $^{35}SO_3^{2-}$ were much less sensitive to molybdate inhibition. This suggests that molybdate inhibition occurs at an initial step in the reductive pathway prior to the formation of sulfite, thereby preventing the formation of other intermediates or products. Although all of these experiments have been carried out with mixed cultures of rumen microorganisms in which the organisms primarily responsible for sulfate reduction have not been isolated (15, 16), it is known that molybdate inhibits sulfate reduction in several anaerobic sulfate-reducing bacteria, *e.g., Desulfovibrio desulfuricans* (10). The enzyme responsible for the first step in sulfate reduction, ATP-sulfurylase, has been isolated from these organisms and has been shown to be inhibited by molybdate (17). The molybdate inhibition of an initial step of sulfate reduction in ruminal microorganisms suggests that a similar pathway may exist in the rumen.

In the presence of copper, molybdate inhibition of the sulfate-reducing system was greatly decreased. This observation supports the hypothesis that copper and molybdenum interact, possibly through the formation of a Cu–Mo complex to decrease the availability of both copper and molybdenum.

The experiments described in this paper are based on the hypothesis that the sulfate-reducing system present in the ruminant is involved in the copper–molybdenum–sulfate interaction (9). The results obtained using washed rumen microorganisms support this hypothesis in showing that molybdate does interact with the sulfate-reducing system by inhibiting the formation of sulfide. Further studies are being conducted to elucidate the sulfate-reducing pathway in ruminal microorganisms, in order that the interaction of copper with intermediates of this pathway may be investigated.

Summary. Washed rumen microorganisms were investigated under conditions conducive to sulfate reduction, giving the following results: Molybdate inhibits the reduction of sulfate to hydrogen sulfide. Molybdate inhibition increases as the concentration of sulfate decreases. Molybdate has little effect on sulfite reduction. In the presence of copper, molybdate inhibition is markedly decreased.

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