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Transport of Molybdate by *Clostridium pasteurianum*

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The transport of $^{99}MoO_4^{2-}$ into dinitrogen-fixing cells of *Clostridium pasteurianum* was investigated. Transport of molybdate in this organism is energy dependent; sucrose is required in the minimal media, and the system is inhibited by the glycolysis inhibitors, NaF, iodoacetic acid, and arsenate. The cells accumulate molybdate against a concentration gradient, and the uptake shows a marked dependence on temperature (optimum 37 C) and pH (optimum 6.0). The rate of molybdate uptake with increasing molybdate concentrations shows saturation kinetics with an apparent K_m and V_{max} of 4.8×10^{-5} M and 55 nmol/g of dry cells per min, respectively. Inhibition studies with the anions SO_4^{2-}, $S_2O_3^{2-}$, WO_4^{2-}, and VO_3^{2-} show that SO_4^{2-} and WO_4^{2-} competitively inhibit MoO_4^{2-} uptake (apparent K_i $[SO_4^{2-}]$ is 3.0×10^{-5} M; apparent K_i $[WO_4^{2-}]$ is 2.4×10^{-5}), whereas $S_2O_3^{2-}$ and VO_3^{2-} have no inhibitory effect. Exchange experiments with MoO_4^{2-} show that only a small percentage of the $^{99}MoO_4^{2-}$ taken up by the cells is exchangeable. Exchange experiments with WO_4^{2-} and SO_4^{2-} indicate that once inside the cells WO_4^{2-} and SO_4^{2-} cannot substitute for MoO_4^{2-}.

The importance of MoO_4^{2-} in biological dinitrogen fixation has been demonstrated (3). Recently attempts have been made to characterize the role of molybdenum in the catalytic reactions of the nitrogenase components and its role in the regulation of the nitrogenase system (2, 4, 6). Although the antagonistic properties of tungsten have been used extensively in these studies (4, 6), there is presently little information concerning the nature of inhibition by tungsten.

We have investigated the molybdenum problem from the level of permeation. In this paper we characterize the molybdate transport system in *Clostridium pasteurianum* and show that the antagonistic effects of tungstate are at the level of molybdate permeation.

MATERIALS AND METHODS

Cell growth. A chemostat culture of *C. pasteurianum* W5, a N_2-fixing anaerobe, was grown in a medium described by Daesch and Mortenson (7) which was 0.22 mM with respect to SO_4^{2-} and 10 μM with respect to MoO_4^{2-}. For each experiment 600 ml of chemostat cells were used to inoculate 5,400 ml of fresh medium with no molybdate. When the optical density reached 1.3 (550 nm with a 1-cm light path in a Bauch & Lomb Spectronic 20 spectrophotometer), 800 ml of this culture was used to inoculate 7,200 ml of fresh medium without molybdate. The final concentration of molybdate in the second culture was 0.1 μM. In all cultures N_2 served as the sole nitrogen source.

Cells at an optical density at 550 nm of 1.3 were harvested in a Sorvall model RC-2 refrigerated centrifuge at 13,200 × *g* for 10 min. The collected cells were washed twice by suspending them in cold, degassed minimal medium, followed by anaerobic centrifugation at 0 C.

Uptake Assay. Washed cells were resuspended to 1 g (wet weight)/8 ml in degassed minimal medium (standard medium without added mineral solution), under 1 atm argon. The standard reaction mixture consisted of 1 ml of cell suspension, 8.9 ml of degassed minimal medium, 0.5% sucrose, and 200 μg of chloramphenicol per ml to prevent protein synthesis. The cell concentration in the final mixture was approximately 10^8 cells/ml. The mixture was equilibrated for 30 min at 37 C under anaerobic conditions (1 atm argon), and then the reaction was started by adding $^{99}MoO_4^{2-}$ (specific activity, 1.21 mCi/g) to a final concentration of 10 μM. Each reaction was terminated by removing a 0.25-ml sample of reaction mixture by syringe directly to a membrane filter (Millipore Corp., 25 mm in diameter, 0.45 μm pore size) and immediately filtering and washing with 5 ml of the same medium. The filters bearing the cells were removed immediately from the suction apparatus, dried, and transferred to vials containing 5 ml of scintillation solution, and the radioactivity was counted for 10 min with a Beckman LS-150 liquid scintillation counter. The scintillation solution, containing 5 g of 2,5-diphenyloxazole per liter and 100 g of naphthalene per liter, was made to 1 liter with dioxane.

A control experiment was run at 0 C under identical conditions to determine the nonspecific binding of $^{99}MoO_4^{2-}$, and this small correction was applied to all

experimental values. The results are averages of at least three determinations. All values have been corrected for the decay of ^{99}Mo over the time required to count all vials for a single experiment.

Kinetics of transport of $^{99}MoO_4^{2-}$. The initial rates of $^{99}MoO_4^{2-}$ uptake were determined by filtration of samples after 1 min of incubation.

Calculation of the intracellular $^{99}MoO_4^{2-}$. Cell water as determined by the method of Winkler and Wilson (21) averaged 2 μl of cell water per 1 mg (dry weight) of cells.

Chemicals. $Na_2{}^{99}MoO_4$ (specific activity, 105 Ci/g) was purchased from New England Nuclear Corp. Na_2MoO_4, Na_2WO_4, Na_2SO_4, $Na_2S_2O_3$, Na_2VO_3, chloramphenicol, iodoacetic acid (IAA), NaF, K_3AsO_3, pyruvate, 2,3-diphosphoglyceric acid, 3-phosphoglyceraldehyde, glucose 6-phosphate, phosphoenol pyruvate, lactic acid, acetic acid, adenosine 5'-triphosphate, nicotinamide adenine dinucleotide phosphate, and glyceraldehyde 3-phosphate were purchased from Sigma Chemical Co. (St. Louis, Mo.) and were of the highest purity available.

RESULTS

Effects of energy source. Figure 1 shows a typical uptake curve for MoO_4^{2-} transport under standard conditions. *C. pasteurianum* cells showed slow uptake of $^{99}MoO_4^{2-}$ in the presence of 0.5% sucrose, and the uptake reached a steady state level in 90 min. Higher sucrose concentrations did not further enhance the rate of uptake or the steady state level of MoO_4^{2-}. In the absence of sucrose there was no MoO_4^{2-} uptake.

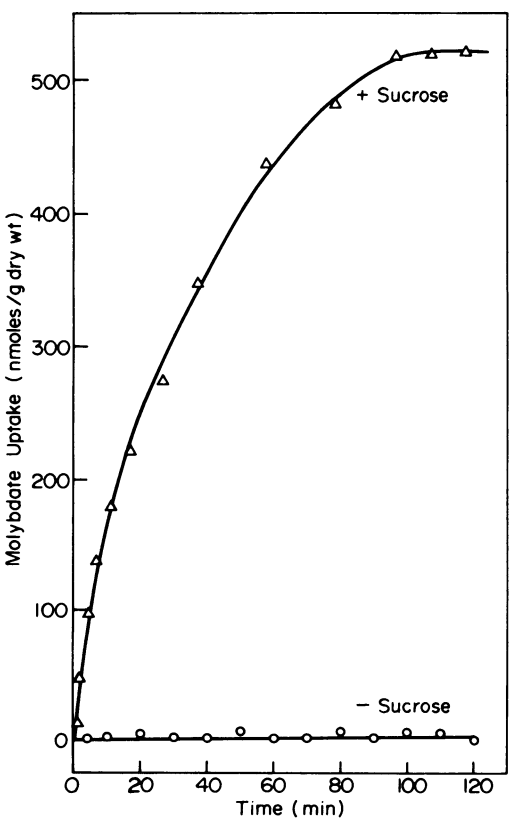

FIG. 1. *Molybdate uptake by whole cells of C. pasteurianum in the presence and absence of 0.05% sucrose.*

Several alternate energy sources were tested for their effect on MoO_4^{2-} uptake. Of those tried only sucrose and pyruvate were capable of supporting MoO_4^{2-} uptake to any significant extent. At concentrations of 0.5 to 5% sucrose supported the highest levels of MoO_4^{2-} uptake (394 nmol/g [dry weight] per 60 min). At 10 mM concentrations pyruvate supported uptake to 138 nmol/g (dry weight) per 60 min. At 10 mM concentrations, lactate, acetate, 2-deoxy-D-glucose, glucose 6-phosphate, 2,3-diphosphateglycerate, 3-phosphoglyceraldehyde, glyceraldehyde-3-phosphate, phosphoenol pyruvate, adenosine 5'-triphosphate, and nicotinamide adenine dinucleotide phosphate were not capable of supporting MoO_4^{2-} uptake. The failure of the phosphorylated intermediates of glycolysis to support uptake may be because of the inability of *C. pasteurianum* to transport these compounds.

The effects of inhibitors on transport can be seen in Table 1. The metabolic inhibitors used were NaF and IAA, which inhibit enolase and glyceraldehyde 3-phosphate dehydrogenase, respectively, and arsenate, which prevents energy production from the oxidation of glyceraldehyde 3-phosphate. All inhibited MoO_4^{2-} transport by 75% at 10 mM concentration and 90 to 95% at higher concentrations.

Oxygen inhibits MoO_4^{2-} uptake completely

TABLE 1. *Effect of inhibitors of glycolysis on $^{99}MoO_4^{2-}$ uptake*[a]

Addition to reaction mixture	Concn (mM)	Uptake (%)
O_2		0
None		100
NaF	10	25
NaF	100	9
K_3AsO_3	10	25
K_3AsO_3	100	8
IAA	10	23
IAA	100	5

[a] Uptake assays were carried out under standard conditions with the additions of NaF, K_3AsO_3, or IAA with 0.5% sucrose. Data represent uptake after 60 min. Aerobic assays were carried out in a 10-ml reaction mixture in a 125-ml flask in a 37 C water bath shaker. 100% corresponds to 375 nmol of MoO_4^{2-}/g (dry weight) of cells.

in *C. pasteurianum*, but the reason for this inhibition is unknown.

Effect of temperature and pH. The effects of temperature on MoO_4^{2-} uptake are shown in Fig. 2. The optimum temperature for MoO_4^{2-} transport was 37 C. No uptake occurred at 0 C. As the temperature increased from 0 to 37 C, both the maximal rate of entry and the maximal concentration ability increased. At 55 C the ability of *C. pasteurianum* to concentrate MoO_4^{2-} dropped to 15% that of 37 C. The reduction in transport rate at low temperatures was reversible, whereas after incubation at 55 C there was no recovery of the ability to transport MoO_4^{2-}. The molybdate uptake was also markedly influenced by the pH of the uptake medium (Fig. 3), with an optimal value of accumulation at pH 6.0.

Kinetics of MoO_4^{2-} uptake. The effect of various external MoO_4^{2-} concentrations on the rate of molybdate uptake is shown in the form of a Lineweaver-Burk plot (Fig. 4). The molybdate uptake system displayed saturation kinetics, exhibiting a V_{max} of 55 nmol/g (dry weight) per min and an apparent K_m of 4.8×10^{-5} M for MoO_4^{2-}.

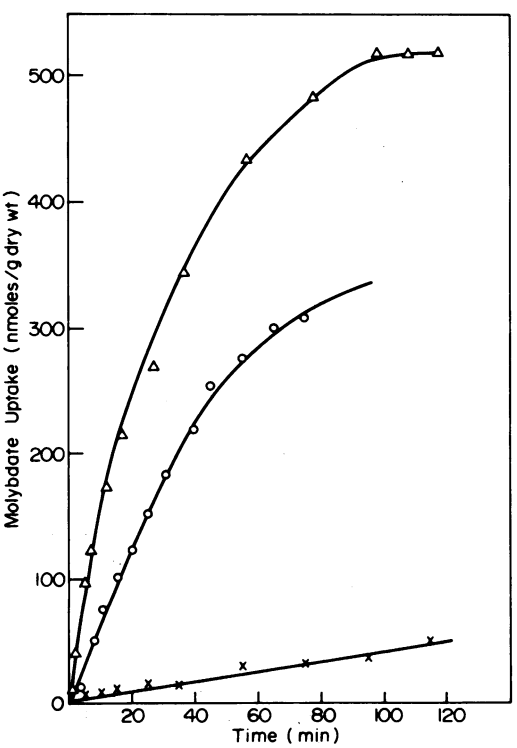

FIG. 2. *Effect of temperature on MoO_4^{2-} transport by C. pasteurianum. Symbols: × 15 C; O, 25 C; Δ, 37 C.*

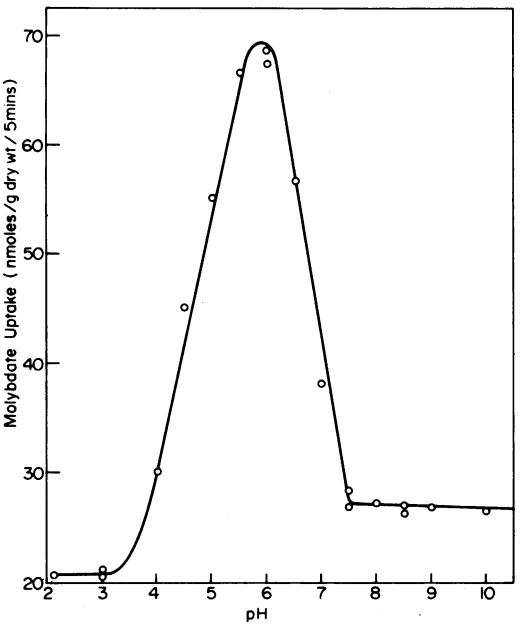

FIG. 3. *pH dependence of MoO_4^{2-} transport by C. pasteurianum. The buffers used were: pH 2 to 3, 0.05 M glycine-hydrochloride; pH 3 to 6, 0.05 M citrate; pH 6 to 7, 0.05 M phosphate; pH 7.5 to 8.5, 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.5 to 10.0, 0.05 M glycine-NaOH.*

Effect of tungstate, sulfate, thiosulfate, and vanadate on molybdate uptake. Figure 5 shows the effect of various WO_4^{2-} concentrations on the uptake of MoO_4^{2-}. A Dixon plot (Fig. 5) shows that the extrapolated V_{max} for MoO_4^{2-} uptake was not altered, but the slope is increased, indicating that WO_4^{2-} inhibition of MoO_4^{2-} uptake was competitive. The apparent K_i was found to be 24 μM. The competitive inhibition by WO_4^{2-} of MoO_4^{2-} uptake suggests that WO_4^{2-} was transported by the MoO_4^{2-} uptake system.

Figure 6 shows the effect of SO_4^{2-} on the MoO_4^{2-} uptake system. Inhibition of MoO_4^{2-} uptake by SO_4^{2-} also was competitive. The apparent K_i was found to be 30 μM.

Similar experiments were done with $S_2O_3^{2-}$ and VO_3^{2-}. These anions did not inhibit MoO_4^{2-} uptake.

Uptake and exchange studies. The ability of externally added unlabeled MoO_4^{2-} to exchange with the internal $^{99}MoO_4^{2-}$ accumulated in the cells is shown in Fig. 7. After 20 min only 9% of the radioactivity taken up by the cells was "chased" by adding a 100-fold excess of unlabeled MoO_4^{2-}. As the time of exposure to $^{99}MoO_4^{2-}$ increased, the amount of labeled MoO_4^{2-} that could not be chased increased. The

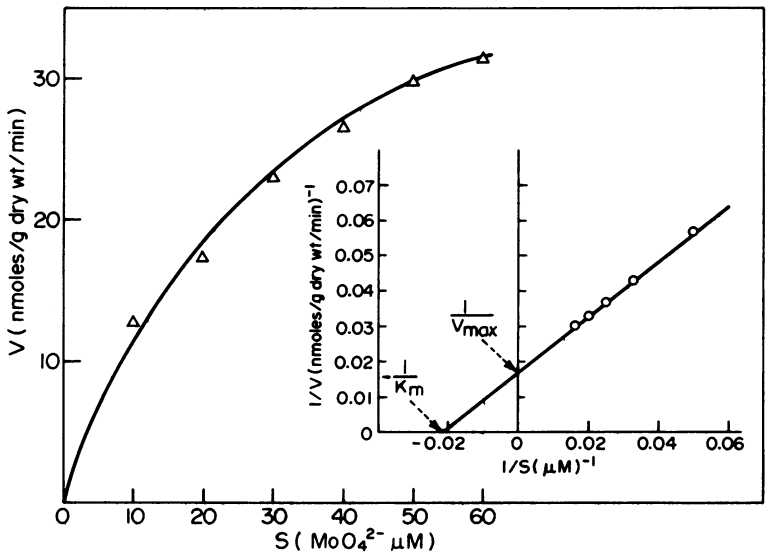

FIG. 4. *Uptake of MoO_4^{2-} by C. pasteurianum over the external concentration range of 10 μM to 60 μM. Insert, Lineweaver-Burk plot used to determine the K_m and V_{max}. Points represent samples taken at 1 min.*

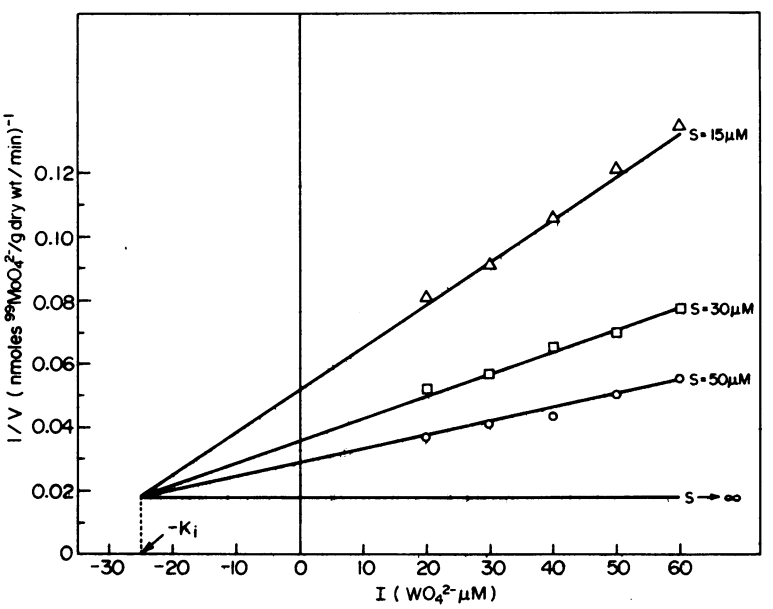

FIG. 5. *Dependence of the rate of MoO_4^{2-} uptake upon concentration of WO_4^{2-}. Plot of 1/V versus I, where V is the rate of MoO_4^{2-} uptake (nmol/g dry weight per min), I is the concentration of the inhibitor, WO_4^{2-}, and S is the concentration of MoO_4^{2-}. Uptake assays were carried out under standard conditions as described except that the appropriate concentration of WO_4^{2-} were added at zero time with $^{99}MoO_4^{2-}$. Points represent samples taken at 1 min.*

amount of $^{99}MoO_4^{2-}$ that remained in the cells after the initial exchange remained the same throughout the secondary incubation period.

The ability of SO_4^{2-} to exchange for radioactive MoO_4^{2-} present in the cells at various times during uptake is shown in Fig. 8 The initial exchange reaction was similar to that seen in the MoO_4^{2-} chase experiment. After the initial exchange, however, $^{99}MoO_4^{2-}$ uptake resumed but at a slower rate than the initial uptake in the absence of SO_4^{2-}. Identical chase experiments were done using WO_4^{2-} to ex-

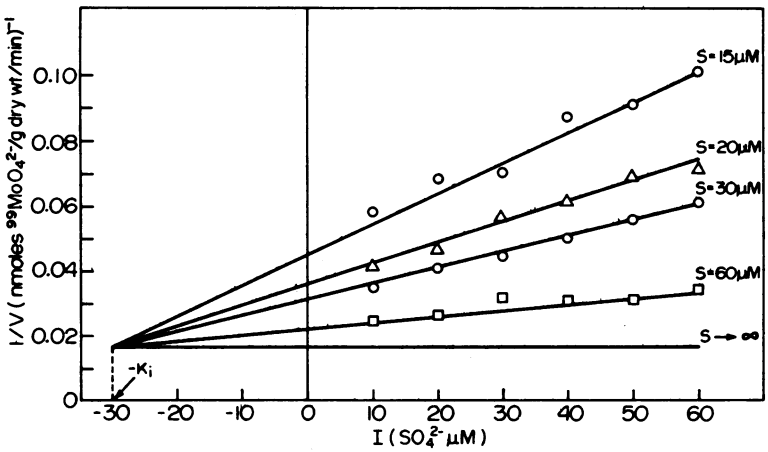

FIG. 6. *Effect of the rate of MoO_4^{2-} uptake upon the external concentration of SO_4^{2-}. Dixon plot of 1/V versus I, where V is the rate of MoO_4^{2-} uptake, I is the concentration of the inhibitor, SO_4^{2-}, and S is the concentration of MoO_4^{2-}. Assays were carried out under the conditions described in the legend to Fig. 5.*

change for radioactive MoO_4^{2-}. The results were typical of those shown in Fig. 8 for SO_4^{2-}.

DISCUSSION

It has long been known that molybdenum is an essential element for biological dinitrogen fixation (3). This requirement is due to the presence of molybdenum in one of the two protein components of the nitrogenase complex (5, 8, 10, 17). There are many cases in natural environments, however, where molybdenum is limited (1, 9, 15). If a N_2-fixing organism exists in an environment limited in molybdenum, it would be advantageous for the cells to actively scavange molybdenum from the surrounding environment.

The demonstration in *C. pasteurianum* of the existence of a heat-labile, energy-dependent transport mechanism indicates that molybdate transport is an active process. It also raises some interesting questions concerning molybdate accumulation.

The results presented in this paper strongly support the concept that molybdate uptake by whole cells of *C. pasteurianum* is energy dependent. Effects of the inhibitors of energy metabolism such as IAA, K_3AsO_3, and NaF indicate that energy for uptake is provided by the glycolytic pathway and the adenosine 5'-triphosphate-acetate phosphotransferase reaction. Inhibition of uptake by O_2 in this obligate anaerobe may be nonspecific or may be a result of the O_2 sensitivity of proteins involved in the uptake system. The pH optimum of 6.0 for uptake closely corresponds to the optimum pH for growth of this organism. The decrease of uptake activity below pH 6.0 may result in part from the protonation of MoO_4^{2-} to form

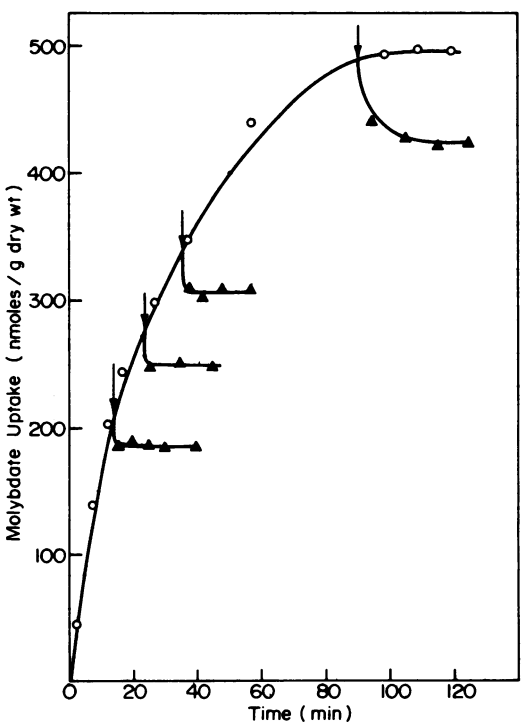

FIG. 7. *$^{99}MoO_4^{2-}$ uptake and exchange in C. pasteurianum. At zero time on the graph the standard reaction mixture was made 10 μM with respect to $^{99}MoO_4^{2-}$. At the times indicated by the arrows identical reaction mixtures (▲) were made 1 mM with respect to nonradioactive MoO_4^{2-}.*

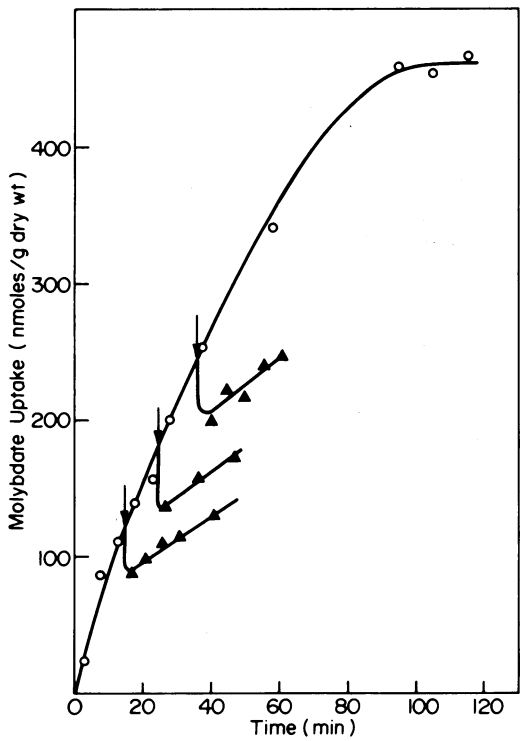

FIG. 8. *MoO_4^{2-} uptake and exchange with SO_4^{2-} in C. pasteurianum. The standard reaction mixture was made 10 μM with respect to $^{99}MoO_4^{2-}$, and at the times indicated by the arrows identical reaction mixtures (▲) were made 1 mM with respect to SO_4^{2-}.*

$HMoO_4^-$. The pH titration curve for molybdate shows a pK_a at approximately 5.3 for $MoO_4^{2-} \rightleftharpoons HMoO_4^-$ (20), and MoO_4^{2-} uptake is 60% of maximum at this pH. These results contrast with those of Tweedie and Segel (20) who report a much sharper decline in MoO_4^{2-} transport below pH 6.0 in *Aspergillus nidulans*. The temperature dependence of uptake and its heat lability is characteristic of an enzyme-like carrier-mediated process.

Recently a number of investigators have tried to elucidate the role of molybdenum in dinitrogen fixation (2, 4, 6). Tungsten was shown to have a specific antagonistic effect on N_2 fixation, and in *Azotobacter vinelandii* it seemed to be incorporated into an inactive tungsten-containing nitrogenase (2). Tungsten-nitrate reductase has been reported in *Chlorella* (19) and spinach (18), tungsten-sulfite oxidase has been isolated from rat liver (12). In contrast, *C. pasteurianum* does not synthesize a tungsten-containing nitrogenase (6). The inhibitory action of tungsten has been postulated to be at (i) the level of molybdenum permeation, (ii) molybdenum mobilization to molybdoferredoxin, or (iii) regulation of nitrogenase synthesis by molybdenum (4). Indirect evidence for the antagonistic effects of tungsten on molybdate uptake has been reported in *A. vinelandii* (13) and *C. pasteurianum* (6). The results presented here give direct evidence that inhibition of MoO_4^{2-} by WO_4^{2-} in *C. pasteurianum* is at the level of molybdate permeation.

Sulfate is known to inhibit molybdate uptake in mycelia of *Penicillium* and *Aspergillus* species (20). Results presented here indicate that sulfate is a competitive inhibitor of molybdate uptake. Experiments in this laboratory show that cells grown in high MoO_4^{2-} concentrations (0.1 mM) are repressed for MoO_4^{2-} uptake and that NH_3-grown cultures of *C. pasteurianum* are 95% repressed for molybdate uptake (B. B. Elliott and L. E. Mortenson, unpublished data). Sulfate transport via this molybdate uptake system would also be regulated by the molybdenum nutritional status of the organism and its growth on ammonia. Since this organism grows normally on low sulfate (0.2 mM) and high molybdate (0.1 mM) and under ammonia conditions with low sulfate, one would expect the cells to possess a separate sulfate permease. The antagonistic effects of high MoO_4^{2-}, SO_4^{2-}, and ammonia on the synthesis of the molybdate uptake system are currently under further investigation. Unlike Tweedie and Segel (20) we were unable to demonstrate any inhibition of molybdate transport by $S_2O_3^{2-}$.

Several researchers have investigated the presence of a factor common to all molybdenum-containing enzymes, which seems to be a low-molecular-weight compound, possibly a molybdenum-containing peptide (11, 14, 16, 22). Exchange experiments with molybdate demonstrate that only a small percentage of molybdate taken up by the cell remains free. It can be postulated that cells grown on low molybdate synthesize a molybdenum-binding component and when molybdate is later made available to the cell, it is bound to this component and cannot be easily exchanged. This molybdenum-binding component may in turn transfer its molybdenum to nitrogenase. If the affinity of this factor for WO_4^{2-} and SO_4^{2-} were less than its affinity for MoO_4^{2-} or if WO_4^{2-} and SO_4^{2-} could not bind to it at all, the resumption of MoO_4^{2-} uptake after exchange of the free pool by WO_4^{2-} or SO_4^{2-} would be explained.

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