EFFECT OF TUNGSTATE ON THE UPTAKE AND FUNCTION OF MOLYBDATE IN *AZOTOBACTER AGILIS*

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ABSTRACT

BULEN, WILLIAM A. (Charles F. Kettering Foundation, Yellow Springs, Ohio). Effect of tungstate on the uptake and function of molybdate in *Azotobacter agilis*. J. Bacteriol. **82**:130–134. 1961.—The reported competitive inhibition of molybdate by tungstate was investigated in an effort to elucidate molybdenum functions associated with nitrogen fixation by *Azotobacter agilis* (*A. vinelandii*). Growth, respiration, and N_2^{15}-incorporation experiments with normal and molybdenum-deficient cells indicated that tungstate inhibits the uptake of molybdate but does not compete with the metabolically functional molybdenum of cells metabolizing N_2. Neither a molybdenum requirement nor a tungstate inhibition was observed with cells metabolizing urea.

Several recent reports have described tungstate as an antagonist of molybdate. Keeler and Varner (1957) and Takahashi and Nason (1957) reported a competitive inhibition of molybdenum by tungsten in the growth of Azotobacter. The former authors also investigated the effect of tungsten on the uptake of Mo^{99} and studied the incorporation of Mo^{99} and W^{185} into *Azotobacter agilis* (*A. vinelandii*) (Keeler and Varner, 1958). Higgins, Richert, and Westerfeld (1956*a*, *b*) reported that tungstate is a dietary antagonist of molybdate in animal nutrition and a competitive inhibitor of molybdate in *Aspergillus niger* cultured on nitrate.

Several enzymes are now known to contain or require molybdenum especially when one-electron acceptors are involved: nitrate reductase (Nicholas and Nason, 1954), xanthine oxidase (Richert and Westerfeld, 1953; Totter et al., 1953), aldehyde oxidase (Mahler et al., 1954), and hydrogenase (Shug et al., 1954). A molybdenum requirement for Azotobacter growing on atmospheric nitrogen is well documented. Some of the earlier studies have been reevaluated and confirmed by Esposito and Wilson (1956) using specially purified media. The enzymatic function of molybdenum in nitrogen fixation is unknown.

The reported competitive inhibition of molybdate by tungstate suggests the usefulness of tungsten as a tool for studying the metabolic roles of molybdenum. A specific competition of tungsten with functional molybdenum would thus make possible the inhibition of the molybdenum enzymes and obviate the necessity for depleting the cells of molybdenum and the introduction of secondary effects resulting from growth under deficiency conditions. Such an approach woud be useful in a search for metabolic changes associated with the molybdenum requirement for nitrogen fixation. This paper describes some growth, O_2-uptake and N_2^{15}-incorporation experiments with both normal and molybdenum-deficient Azotobacter indicating that tungstate is a competitive inhibitor of molybdate only when the observed function depends upon the cellular uptake of molybdenum.

MATERIALS AND METHODS

The *A. agilis* used in these experiments was the strain O originally obtained from Wisconsin with the cooperation of R. H. Burris. Cells were cultured in Burk's nitrogen-free mineral salts medium (Burk and Lineweaver, 1930) with 2% sucrose in 500-ml Erlenmeyer flasks containing 150 ml of medium. Cultures were incubated at 32 C on a rotary shaker at 500 rev/min and maintained by daily transfer of a 5% inoculum. Purity checks have been made periodically in the sugar-free peptone medium recommended by Burk and Burris (1941) and by checking the pH of molybdenum-deficient cultures as discussed later.

Molybdenum-deficient cells were obtained by transferring a 5% inoculum to medium to which no molybdenum was added. The only precautions required in preparation of low-molybdenum medium were the use of reagent grade sucrose

and demineralized water. Two transfers were sufficient to establish obvious deficiency. Cultures obtained 16 hr after the second transfer contained slightly less than one-half the cell nitrogen of normal controls and were designated as −Mo #2 cultures. Under conditions of molybdenum deficiency, the pH of impure cultures was found to drop sharply below normal as the cultures became deficient. This proved a sensitive test for purity observable even when microscopic examination of the inoculum failed to show the presence of contaminating organisms.

Growth experiments were conducted in colorimeter tubes (20 by 180 mm) containing a total volume of 12 ml and incubated at 32 C on the rotary shaker at 500 rev/min. Growth was measured turbidimetrically with an Evelyn colorimeter using a 660 mμ filter. In general an inoculum of 0.75 ml of a 16 hr normal or 1.5 ml of a 16 hr −Mo #2 culture containing 0.16 to 0.18 mg cell N was used. Growth has been plotted as 2+ log optical density (OD) vs. a sliding time scale in which the first point of a given curve represents time zero and additional points the growth at 1-hr intervals. To prevent cell aggregation on the precipitate formed during autoclaving, the iron was omitted during autoclaving and added as a sterile solution just before use. Reagent grade tungstate and molybdate were added as the sodium salts.

Nitrogen fixation was determined isotopically with N^{15}. Washed cell suspensions in a total volume of 3 ml in Warburg flasks were incubated at 30 C with shaking, first for 15 min in an atmosphere of 20% O_2 and 80% He and then for 1 hr in an atmosphere of 10% N_2, containing 98% N^{15}, 20% O_2, and 70% He. To augment recovery of any partially reduced nitrogen compounds, cultures were transferred to pressure tubes for a reduction pretreatment with 3 ml 57% HI for 8 hr at 300 C. Following removal of I_2, samples were subjected to Kjeldahl digestion (1 hr) and distillation. Ammonia nitrogen was converted to N_2 by hypobromite oxidation and the isotope content determined by mass analysis.

RESULTS

Tungstate inhibition of growth on N_2. Under the conditions described for growth, normal cells enter the log phase after approximately 1 hr. Neither the lag period nor the logarithmic rate of normal cells in regular medium containing 1

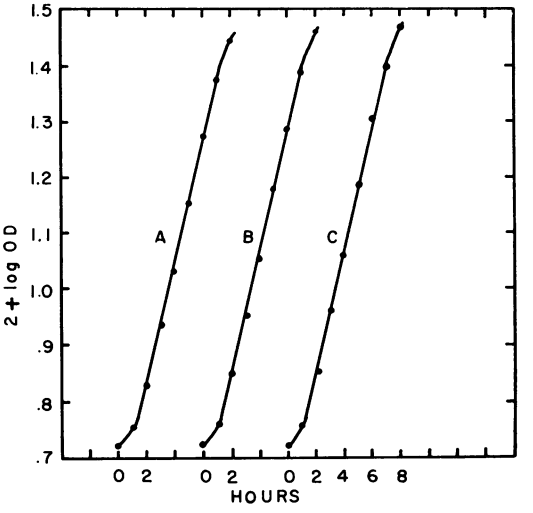

FIG. 1. *Effect of tungstate on growth of normal cells. All tubes contained 10^{-5} M Mo and the following tungstate concentrations: A, 0; B, 6 × 10^{-3} M; C, 10^{-2} M. Inoculum 0.5 ml of normal 16 hr culture. Identical curves obtained with intermediate concentrations and in duplicate experiments.*

ppm molybdenum (1.04×10^{-5} M) is affected by tungstate at concentrations up to 10^{-2} M, i.e., a W to Mo ratio in the medium of 1,000:1 (Fig. 1).

Keeler and Varner (1957) have shown that *A. agilis* strain O incorporates molybdenum in excess of that required for immediate growth. To check the possibility that during prolonged incubation in liquid culture our normal cells had accumulated sufficient excess molybdenum to significantly effect the W to Mo ratio, 15 ml of a 24-hr molybdenum-deficient (−Mo #2) culture were used to inoculate 150 ml of regular medium. After 24 hr incubation, 7.5 ml were used to inoculate 150 ml of regular medium. After 16 hr growth, 0.75 ml of the latter culture (+Mo #2) was used to inoculate growth tubes. Considering the molybdenum content of the original deficient inoculum as insignificant relative to the demonstration of a deficiency and assuming that in subsequent transfers all molybdenum was taken up by the cells (Keeler and Varner, 1957) the molybdenum content of the cells in the growth tube inocula would be only about 7.8 mμ moles. Growth curves essentially identical to those of Fig. 1 were obtained in low-molybdenum medium both with and without

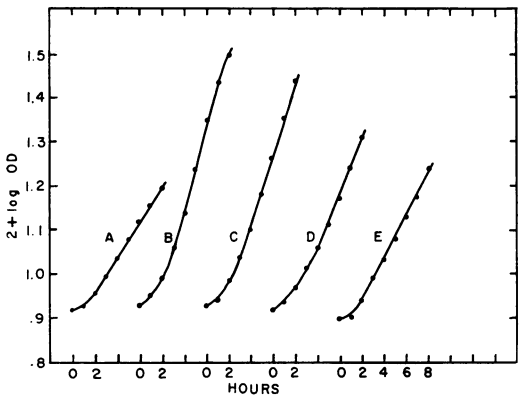

FIG. 2. *Tungstate inhibition of the molybdenum dependent growth of deficient cells. Low-molybdenum medium. Additions: A, none; B, 10^{-5} M Mo; C, 10^{-5} M Mo plus 5 × 10^{-4} M W; D, 10^{-5} M Mo plus 10^{-3} M W; E, 10^{-5} M Mo plus 1.5 × 10^{-3} M W. Inoculum 1.5 ml of −Mo #2 culture.*

the addition of up to 10^{-3} M tungstate. Thus tungstate at W to Mo ratios up to 1,500:1 was not inhibitory.

Following two transfers in low-molybdenum medium, growth is strictly dependent upon the uptake of molybdate and this uptake-dependent growth is inhibited by tungstate. Figure 2 shows the increase in growth rate when 1 ppm Mo is added to the low-molybdenum medium inoculated with deficient cells and the inhibitory effect of tungstate.

Effect of tungstate on growth on urea. For tungsten to be useful in studying the role of molybdenum in nitrogen fixation, it seems imperative to know whether its presence inhibits the normal utilization of ammonia. Esposito and Wilson (1956) using purified media have shown that the molybdenum requirement is eliminated when ammonium ion is supplied as a nitrogen source. Recent reports are contradictory concerning the inhibitory effect of tungstate on the growth of *A. agilis* utilizing ammonia as the nitrogen source. Takahashi and Nason (1957) report a 53% inhibition when 10^{-3} M tungstate was added to control medium containing 10^{-5} M molybdate. Keeler and Varner (1957) report no inhibition of ammonia grown cells.

To offset the pH changes associated with the uptake of ammonium ions and eliminate the inclusion of either excess calcium carbonate, potassium phosphate or a metabolizable anion, nitrogen at the ammonia level was added as urea. Deficient cells exhibit identical growth rates when supplied urea in the presence or absence of added molybdenum (Fig. 3) and no inhibition was observed when either normal cells in regular medium or molybdenum-deficient cells in low-molybdenum medium were supplied tungstate at concentrations up to 10^{-2} M.

Effect of tungstate on O_2 uptake. The effect of tungstate on O_2 uptake was examined by conventional Warburg techniques using molybdenum-deficient cells resuspended in fresh medium. Over a 1-hr period the O_2 uptake by deficient cells was the same in low-molybdenum medium, in regular medium (10^{-5} M Mo), and in regular medium plus 10^{-3} M tungstate. In a typical experiment, observed values were 151, 154, and 158 μl O_2 uptake, respectively. $Q_{O_2}^{N_2}$ values of deficient cells were slightly higher (14 to 16 thousand μl O_2:mg N:hr) than those routinely found with normal cells.

Only when measurements were extended until the increment increase in O_2 uptake due to growth became significant was a tungstate inhibition evident. Although a normal increment increase was observed in medium containing 10^{-5} M molybdate, O_2 uptake in medium to which both molybdate and tungstate (10^{-3} M) were added paralleled that of cells in low molybdenum medium.

Tungstate inhibition of N_2^{15} incorporation. Although its participation as a constituent of N_2-metabolizing enzymes has not been demonstrated, one established role of molybdenum is in the nitrogen fixation process. The effect of tungstate on N_2^{15} incorporation by both normal and molybdenum-deficient cells was, therefore, examined in the absence of added molybdate and in the presence of added molybdate both with and without added tungstate. Results of the experiments (Table 1) demonstrate a tungstate inhibition only when N_2^{15} incorporation is dependent upon the uptake of molybdate. Tungstate at 10^{-3} M in medium to which no molybdate is added has no inhibitory effect on normal cells, whereas the same concentration drastically reduces that N_2^{15} incorporation by deficient cells which is dependent upon the uptake of molybdenum.

The marked dependence of N_2^{15} incorporation by deficient cells on the presence of molybdenum is also evident in these 1-hr exposures to N_2^{15}. Under identical conditions, i.e., with a 15-min

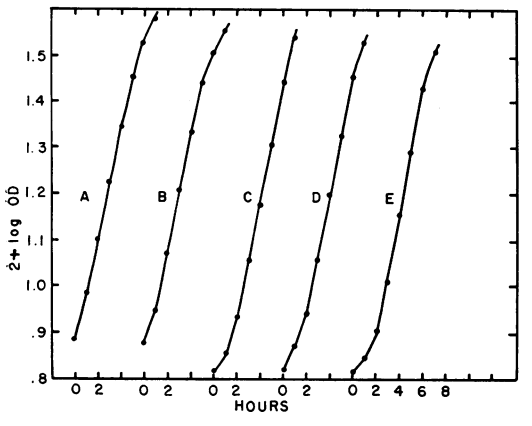

FIG. 3. *Effect of tungstate on normal and deficient cells supplied 300 ppm urea N. Curves A and B, 0.75 ml normal 16-hr culture in regular medium plus 10^{-2} M W in B. Curves C, D, and E, 1.5 ml of −Mo #2 culture in low-molybdenum medium plus 10^{-3} M W in D and 10^{-2} M W in E.*

TABLE 1. *Effect of tungstate on N_2^{15} incorporation*

Cells	Additions*	N^{15} incorporated per mg cell N in 1 hr	
		Expt 1	Expt 2
		μg	μg
Normal (0.15 mg N)	None	138	120
	10^{-5} M Mo	137	130
	10^{-3} M W	142	145
	10^{-5} M Mo; 10^{-3} M W	141	138
Mo deficient (0.13 mg N)	None	30	29
	10^{-5} M Mo	84	70
	10^{-5} M Mo; 10^{-3} M W	43	39
	10^{-5} M Mo; 3×10^{-3} M W	29	29

* To low molybdenum medium prepared as described in the text.

incubation in an O_2-He atmosphere, a significant increase in N_2^{15} incorporation has been demonstrated after a 5-min exposure to the isotope.

DISCUSSION

Previous reports of a competitive inhibition of molybdenum by tungsten in the growth of Azotobacter suggest the tungstate inhibition of molybdenum functions, e.g., in nitrate assimilation and N_2 fixation (Keeler and Varner, 1957; Takahashi and Nason, 1957). The experiments reported here suggest alternatively that tungstate competitively inhibits the uptake of molybdate but does not inhibit the enzymatically functional molybdenum in intact cells.

Attempts to compare our growth experiments with those of the previous authors are complicated by the lack of rate information obtainable from terminal density values, uncertainty as to when low-molybdenum inocula were used, and difference in length of incubation periods. In our growth experiments no tungstate inhibition has been observed during logarithmic reproduction of cells not dependent upon the uptake of molybdate. Using urea as a source of ammonia nitrogen, we have observed no requirement of *A. agilis* for molybdate and no inhibition by added tungstate. These results confirm the observations made with ammonia by Keeler and Varner (1957), but are at variance with noncompetitive inhibition reported by Takahashi and Nason (1957).

Keeler (1957) has reported an increase in molybdenum uptake per unit growth by *A. agilis* with increasing aeration and with decreasing iron which, in view of similar observations by Lenhoff, Nicholas, and Kaplan (1956) with *Pseudomonas fluorescens*, suggested that in Azotobacter grown under high aeration molybdenum may function in a molybdoflavoprotein terminal oxidase. Although the high growth rates on urea and ammonia (Esposito and Wilson, 1956) under conditions of molybdenum deficiency and the inability of molybdate to increase short term (1-hr) oxygen uptake of molybdenum-deficient cells tend to minimize the possibility, if such a terminal oxidase is present in cells supplied adequate iron, it is not inhibited to a measurable extent by tungstate.

Short term (1-hr) N_2^{15}-incorporation experiments agree with the growth experiments in that tungstate inhibition is observed only when the process is dependent upon the uptake of molybdate.

The concept accepted in light of our present knowledge is that tungstate competes with molybdate in the uptake process rather than with functional molybdenum. Support is found in the Mo^{99}-uptake experiments of Keeler and Varner (1957). In these experiments with N_2 and nitrate-grown cells, the Mo^{99} uptake was almost completely inhibited by tungstate with only a

slight inhibition of growth. In addition, Takahashi and Nason (1957) have shown that partially purified nitrate reductase from Neurospora was not inhibited by tungstate at concentrations up to 10^{-3} M. Alternate explanations include the possibility that tungsten does compete for the site(s) of functional molybdenum but that, in experiments described here, involved enzymes were not rate limiting. Yet high concentrations of tungstate fail to inhibit normal growing cells fixing N_2 under conditions in which the addition of ammonia or urea significantly increases the growth rate. It is also possible that tungsten competes in the formation of molybdoproteins but does not replace molybdenum in these systems. No evidence for such a competition has been evident from growth experiments involving several generation times.

Since critical functions in normal cells not dependent upon the uptake of molybdate are not inhibited by tungstate, the tungstate inhibition of the uptake of molybdate should be useful in studying the uptake process. In this connection it seems possible that the W^{185}- and perhaps some of the Mo^{99}-proteins described by Keeler and Varner (1958) are constituents of the ion uptake mechanism.

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