Tungstate as a competitive inhibitor of molybdate in nitrate assimilation and in N_2 fixation by *Azotobacter**

The stimulatory effect of molybdenum on N_2 fixation by *Azotobacter*, first reported by BORTELS[1], has since been demonstrated by a number of other investigators[2]. Molybdenum is not uniquely specific for N_2 fixation nor for *Azotobacter*, since it also promotes the growth of other microorganisms and higher plants when nitrate is the sole nitrogen source. Its effect, however, is small or negligible with ammonia[2]. It has already been shown that molybdenum is required in the nutrient medium for the enzymic reduction of nitrate[3] and that it is the metal component of nitrate reductase from *Neurospora*[4,5], from soy bean leaves[6,7], and probably from *Escherichia coli*[8]. Recently HIGGINS, RICHERT AND WESTERFELD[9,10] reported that tungstateis a dietary antagonist of molybdate in animal nutrition, and a competitive inhibitor of molybdate in *Aspergillus niger* when nitrate is the sole nitrogen source. The experiments reported in the present paper demonstrate that there is a similar type of competitive inhibition of tungstate with molybdate in nitrate assimilation and in N_2 fixation by *Azotobacter*. On the other hand, vanadium, which has been reported by a number of workers[11] to serve in place of molybdenum in N_2 fixation, failed to show a competitive antagonism with tungstate or molybdate in nitrate assimilation and in N_2 fixation.

Azotobacter vinelandii, strain Original, kindly provided by Dr. R. H. BURRIS, was grown in modified Burk's nitrogen-free medium[12] containing 2% sucrose in which the original ferrous sulfate was replaced with ferric citrate (16.9 mg/l). The organism was incubated for 13 to 24 hours in 50 ml of medium in 500 ml Erlenmeyer flasks on a shaker at 30° using a 2% inoculum (*i.e.*, 1 ml of a 24-hour culture of *Azotobacter* in basal medium). For a nitrogen source other than air (*i.e.*, 20% N_2), 1.44 g of potassium nitrate, 0.94 g of ammonium sulfate, or 2.36 g of monosodium glutamate per liter medium were added as indicated. Calcium carbonate (0.5 g/l) was always included in the ammonium sulfate-containing medium to prevent a lowering of the pH. In some experiments the medium was subjected to the copper sulfide coprecipitation method[13] before addition of the trace elements iron and molybdenum in order to remove some of the metal impurities. Growth was measured in a Klett-Summerson colorimeter with a No. 66 filter (red.) Since readings greater than 120 Klett units were not linear with cell concentration, corrections were accordingly made from a standard curve. One Klett unit represents 2.5 μg dry weight of N_2-grown *Azotobacter* per 5 ml medium. Calcium carbonate, when included in the medium, was first dissolved by adding acetic acid (2% final concentration) before measuring growth.

TABLE I

INHIBITORY EFFECT OF TUNGSTATE ON N_2 FIXATION AND NITRATE ASSIMILATION BY *Azotobacter*

Nitrogen source	Control*	plus Na_2WO_4 (M)									
		10^{-3}		$5 \cdot 10^{-4}$		10^{-4}		$5 \cdot 10^{-5}$		10^{-5}	
	growth**	growth	% inhibition	growth	% inhibition	growth	% inhibition	growth	% inhibition	growth	% inhibition
Expt. 1											
N_2	271	82	70	—	—	—	—	—	—	250	8
KNO_3	400	183	54	—	—	—	—	—	—	343	14
Expt. 2											
N_2	345	90	74	109	69	221	36	315	9	—	—
KNO_3	525	—	—	221	58	340	35	415	21	—	—

* Control media contained molybdate at $10^{-5}M$ final concentration, the amount usually included with Burk's medium.

** Growth is expressed in Klett units.

Table I shows that the addition of tungstate to the culture medium inhibited the growth of *Azotobacter* as much as 75% when N_2 or KNO_3 served as the nitrogen source. Inhibition by tungstate is almost completely overcome, however by increasing the molybdate concentration in the medium. The competitive nature of tungstate inhibition with molybdate in nitrate assimilation and in N_2 fixation was demonstrated by the typical LINEWEAVER-BURK plots[14] as shown

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in Fig. 1. The inhibition (68%) of N_2 fixation resulting from the addition to the growth medium of tungstate at a final concentration of $5 \cdot 10^{-4} M$ was almost completely offset by increasing the molybdate content to $1 \cdot 10^{-4} M$ final concentration (approximately a 5:1 ratio of tungsten to molybdenum).

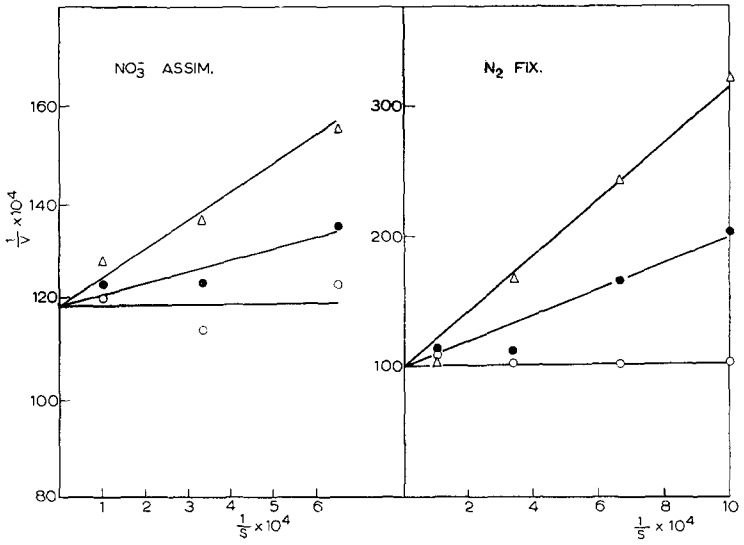

Fig. 1. Lineweaver-Burk plot showing competitive inhibition of molybdate by tungstate on growth of *Azotobacter* in a medium with nitrate or N_2 as the sole nitrogen source. V is the growth after 16 to 17 hours as expressed in Klett units. S is the molar concentration of molybdate. O——O, control; ●——●, $5 \cdot 10^{-4} M$ tungstate; △——△, $10^{-4} M$ tungstate. Control media contained molybdate at $10^{-5} M$ final concentration, the amount usually added with Burk's medium.

TABLE II

INEFFECTIVENESS OF MOLYBDATE IN OVERCOMING TUNGSTATE INHIBITION OF AMMONIA AND GLUTAMATE ASSIMILATION BY *Azotobacter*

Nitrogen source	*Control**	*Na_2WO_4 concentration*				*10^{-3} M Na_2WO_4*			*$5 \cdot 10^{-4}$ M Na_2WO_4*		
		10^{-3} M		*$5 \cdot 10^{-4}$ M*		*Na_2MoO_4 concentration(M)*			*Na_2MoO_4 concentration(M)*		
	*growth***	*growth*	*% inhibition*	*growth*	*% inhibition*	*$1.5 \cdot 10^{-5}$ growth*	*$3 \cdot 10^{-5}$ growth*	*10^{-5} growth*	*$1.5 \cdot 10^{-5}$ growth*	*$3 \cdot 10^{-5}$ growth*	*10^{-4} growth*
$(NH_4)_2SO_4$	145	69	53	98	32	64	75	62	87	92	90
Monosodium glutamate	225	150	33	143	36	150	117	121	165	160	165

* Control media contained molybdate at $10^{-5} M$ final concentration.
** Growth is expressed in Klett units.

There is also a significant inhibition of growth by tungstate when ammonia or glutamate is the nitrogen source as shown in Table II. This inhibition, although less marked than that for N_2 fixation, is not of a competitive nature, however, since it is unaffected by increasing levels of molybdenum as high as $10^{-4} M$ final concentration (Table II).

Vanadium has been reported by a number of workers to serve in place of molybdenum in N_2 fixation[11]. In view of the competitive inhibition of tungstate with molybdenum in nitrate assimilation and in N_2 fixation, the effect of vanadium was also examined. The addition of sodium metavanadate ($NaVO_3$) at $10^{-4} M$ final concentration resulted in an 85 to 100% inhibition of N_2 fixation, and a 40 to 60% inhibition when nitrate, ammonia or glutamate served as the nitrogen source. In all cases the sensitivity to vanadium is unaffected by increasing the levels of molybdate

(up to $10^{-4}M$ final concentration) in the medium. Vanadate concentrations ($1.5 \cdot 10^{-5}$ to $3 \cdot 10^{-5}M$), which cause little or no decrease in growth, failed to overcome inhibition by tungstate in N_2 fixation and in nitrate assimilation.

The data presented here point to a molybdenum system for N_2 fixation in *Azotobacter* in support of the reported stimulatory action of molybdenum observed by other investigators as indicated above. The results also suggest that a molybdenum enzyme, probably similar to that characterized in *Neurospora* and soy bean leaves, is involved in nitrate reduction in *Azotobacter*. It is of interest that tungstate has no effect (in final concentrations ranging from 10^{-6} to $10^{-3}M$) on the partially purified nitrate reductase from *Neurospora*. It is not unlikely that the *in vivo* inhibition by tungstate can be ascribed to its action in replacing molybdenum. In addition to its direct role in electron transport molybdenum apparently is necessary for the adaptive formation of nitrate reductase[4] during growth, and by extension, for the formation of the N_2-fixing enzyme system, presumably in the synthesis of the protein moiety(ies) of this system.

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The effect of thyroxine on the oxidative phosphorylation of tumour mitochondria*

Thyroxine has been shown to uncouple oxidation from the accompanying phosphorylations in the respiratory chain of liver mitochondria *in vitro*. In order to achieve this effect rather special conditions proved to be necessary. MARTIUS AND HESS[1], KLEMPERER[2] and HOCH AND LIPMANN[3] found that uncoupling took place only after preincubation of thyroxine with rat liver mitochondria. It was concluded that the hormone first had to pass the mitochondrial membrane before acting on the phosphorylating enzymes and that the rate of penetration was very slow with "intact" mitochondria. HOCH AND LIPMANN also studied mitochondria isolated from hamster liver. For thyroxine to act as an uncoupling agent, preincubation was not necessary with these mitochondria, which were considered relatively "leaky" in comparison with rat liver mitochondria. Recently TAPLEY, COOPER AND LEHNINGER[4] have shown that a 10 minute hypotonic treatment of rat liver mitochondria at 0° C is sufficient to result in a nearly complete uncoupling on subsequent incubation with thyroxine.

In the present experiments the ability of thyroxine to act as an uncoupler of tumour mitochondrial oxidative phosphorylation has been studied. The tumour mitochondria were *not* subjected to a special pretreatment intended to eliminate a possible structural barrier. This was considered unnecessary in view of (i) our earlier observations[5,6,7], which showed that the mitochondria from all the transplanted tumours studied exhibited a biochemical integrity that was less than that of liver mitochondria, and (ii) the possibility that this was due to, or at least accompanied by, a change in the mitochondrial structure and more especially in that of the membrane.