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Received September 13, 1956. P.S.E.B.M., 1956, v93.

Trace Metal Requirements of Azotobacter.* (22820)

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Although metallic ion requirements for fixation of atmospheric nitrogen by the azotobacter have been studied for a number of years(1,2,3) an extension and reevaluation of the earlier work was thought worthwhile in light of recent advances in methodology of trace metal studies. These earlier studies established marked differences in response of both strains and species of the organism. Therefore, an added reason was to determine trace metal requirements of our strain *Azotobacter vinelandii* O which is used in many biochemical and bacteriological laboratories.

Materials and methods. *Azotobacter vinelandii* O was maintained and studied in Burk's sucrose mineral salts medium(4) in 500 ml Erlenmeyer shake flasks. The cultures were incubated at 30°C in Brunswick rotary shaker at 400 rpm. Flasks were covered with 50 ml pyrex beakers instead of cotton plugs. All glassware was cleaned by washing with detergent followed by 4 rinses in distilled water. It was then filled with 0.5% solution of tetra sodium salt of ethylene diamine tetraacetic acid (Sequestrene Na 4, Alrose Chemical Co.) and autoclaved at 15 lb. pressure for 30 minutes. After autoclaving, glassware was rinsed 5 times with ion-free water obtained by passing distilled water through a Barnstead "Bantamac" demineralizer mode Bd-1. The effluent from the column contained less than .05 ppm total ionizable solids calculated as sodium ion equivalent. The 8-OH quinoline coprecipitation technic of Nicholas(5) was used to remove traces of iron and molybdenum from growth medium. This method was not suitable for investigation of calcium, so recrystallized analytical reagent chemicals were used. Nitrogen was determined by Kjeldahl semi-micro procedure(4). Turbidities were measured with Klett-Summerson colorimeter using the 660 mμ filter.

TABLE I. Effect of Nitrogen Source on Molybdenum Requirement of *Azotobacter vinelandii* O.

	μg N_2 fixed/ml*					
Exp.	N_2 .1 ppm Mo	N_2 –Mo	N_2 .2 ppm V	NH_4^+ .1 ppm Mo	NH_4^+ –Mo	Ashed NH_4^+
I				204	186	
II	205	50		201	200	
III		50			211	86
IV		56			211	
V	212	58		279	301	88
VI	262		83			
VII	166		62			

* In all tables, the ammonia grown cells harvested and washed with water, then resuspended in water and assayed for nitrogen. In all tables, "ashed NH_4^+" was prepared by heating ammonium acetate overnight in a porcelain crucible at 150°C. Residue was dissolved in dilute acid, neutralized and added to culture flasks.

Results. In agreement with previous studies with other strains, molybdenum and iron are both specifically required for nitrogen fixation process in *Azotobacter vinelandii* O. When ammonium ion is supplied as a nitrogen source the requirement is either eliminated, as with molybdenum or spared as with iron (Tables I and II). Vanadium has been reported to replace molybdenum in certain *Azotobacter* species(6) but this does not appear to be true with strain O.

* Supported by grants from Rockefeller Foundation and Research Committee of Graduate School from funds of Wisconsin Alumni Research Foundation.

TABLE II. Effect of Nitrogen Source on Iron Requirement of *Azotobacter vinelandii* O.

	% maximum growth					
	N_2		NH_4^+		Ashed NH_4^+	
Added Fe^{+++} (ppm)	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
1.0	100	100	100	100		
.5	92	83	100	99		
.1	59	51	86	77		
0	17	14	16	14	16	10

Species of *Azotobacter* grown on N_2 possess an active hydrogenase capable of carrying out the knallgas reaction(7), and H_2 has been shown to be competitive inhibitor of the fixation of nitrogen(8). The effect of various levels of iron and molybdenum on hydrogen inhibition of nitrogen fixation by *Azotobacter vinelandii* O was studied. The effect was studied in a sealed system employing a KOH trap to remove respiratory carbon dioxide output. Any net gas uptake in the growth flask was replaced with pure oxygen. The flasks were equipped with colorimeter tube sidearms so that Klett-Summerson readings could be made without disturbing the gas phase. The results of a typical experiment with iron are shown in Fig. 1. As can be seen the growth constant is drastically reduced in presence of H_2, independent of the level of iron (1-10 ppm) in the medium. However, an increase in iron content of the medium caused a large increase in the apparent oxygen uptake of the culture. Sucrose utilization and CO_2 evolution values were not sufficient to account for the increase in oxygen uptake, indicating that excess gas uptake was a product of a stimulated knallgas reaction. The effect of molybdenum was also studied over a range of 0.001 to 1.0 ppm and always a large increase in apparent oxygen uptake was obtained when hydrogen was present. The amount of increased oxygen uptake, however, was the same for the entire range of molybdenum concentrations tested.

Uncertainty exists as to the requirement of calcium for nitrogen fixation. For example, Horner and Burk(9) demonstrated what appeared to be a specific requirement in *Azotobacter vinelandii* but later(10) stated that the requirement for calcium was unchanged whether atmospheric nitrogen or ammonium ion was used as a nitrogen source. Jensen(11) has reported that calcium is not required by *Azotobacter indicum* but is required by *Azotobacter chroococcum*. The results summarized in Table III demonstrate a specific calcium requirement for nitrogen fixation by *Azotobacter vinelandii* O. The presence of ammonium ion apparently eliminates the need for added calcium. Strontium can replace calcium at approximately the same molar concentration. During this study it was noted that levels of calcium lower than 5 μg per ml in a nitrogen free medium resulted in an ex-

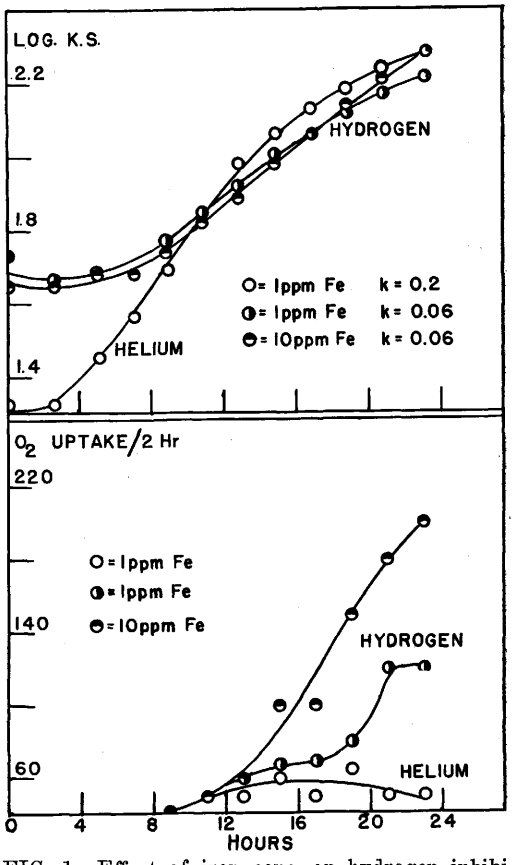

FIG. 1. Effect of iron conc. on hydrogen inhibition of nitrogen fixation by *Azotobacter vinelandii* O. k, velocity constant of growth $= \frac{2.3}{t_2 - t_1} \times \log \frac{KS_2}{KS_1}$ in which KS_1 is Klett-Summerson reading at time t_1, etc. Gas phase contained 0.2 atm O_2, 0.2 atm N_2 and 0.6 atm H_2 or He. Oxygen uptake in ml/flask/2 hr.

TABLE III. Effect of Nitrogen Source on Calcium Requirement of *Azotobacter vinelandii* O.

Added Ca^{++} (ppm)	μg N_2 fixed/ml N_2	NH_4^+	Ashed NH_4^+
25	258	337	
10	241	308	
1	203	323	
.5	170	300	
0	13	313	26

tended lag period of growth followed by an apparently normal logarithmic phase. This is illustrated in Fig. 2. Varying the magnesium to calcium concentration ratio from 10 to 1 to 1 to 1 had no effect on the lag phase. There is an apparent interrelationship between calcium and phosphorus. Fig. 3 contains the results of an experiment demonstrating this interrelationship. Curves 1 and 2 show that phosphorus concentration does not effect the lag phase in the presence of an optimum amount of calcium. Curves 3 and 4 show that a low level of phosphorus

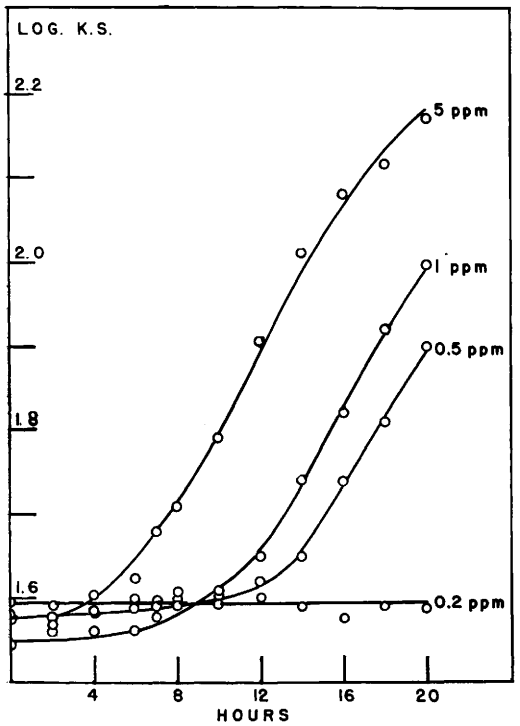

FIG. 2. Effect of calcium conc. (indicated ppm) on length of lag phase of *A. vinelandii* O fixing atmospheric nitrogen.

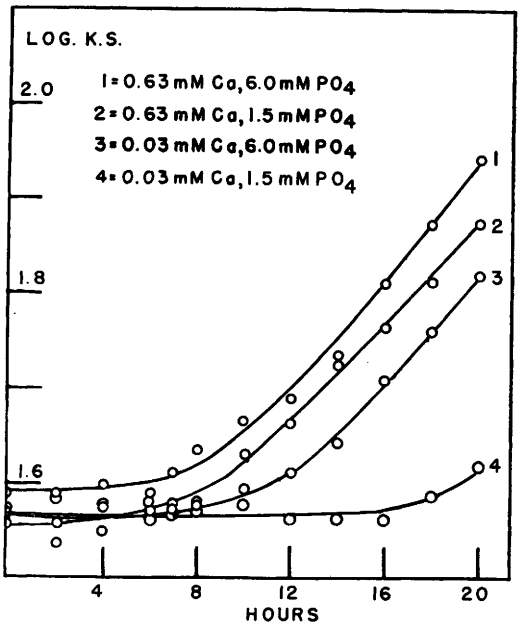

FIG. 3. Effect of varied calcium and phosphate conc. on lag phase of *A. vinelandii* O fixing atmospheric nitrogen.

results in a further lengthening of the lag phase of a culture with a suboptimal amount of calcium.

Discussion. The results of this study demonstrate that molybdenum, iron and calcium are specifically required for nitrogen fixation by *Azotobacter vinelandii* O, but the definite function of these metals in the fixation process is still to be established. However, molybdenum has been found to be a part of the hydrogenase enzyme system(12), and spectrophotometric studies of the effect of nitrogen and hydrogen on cell free preparations of nitrogen fixing organisms provide a function for iron via a hematin compound involved in the fixation process(13). The role of calcium in the system is even more obscure. The data reported here suggest that calcium functions in a reaction active during the lag phase, a reaction in which phosphate is also involved. The results with ammonium ion as a source of nitrogen indicate that the reaction or its products are not essential for the elimination of the lag phase unless the organism is actively fixing atmospheric nitrogen.

Summary. Molybdenum, iron and cal-

cium ions have been shown to be specifically required when *Azotobacter vinelandii* O is fixing atmospheric nitrogen. Ammonium ion eliminates the need for molybdenum and calcium and has a sparing action on the requirement for iron. High levels of iron stimulate the knallgas reaction of *A. vinelandii* O fixing N_2 in the presence of H_2, but molybdenum in the range tested had no effect on this reaction. Low concentrations of calcium ions in a nitrogen-fixing culture result in an extended lag phase of growth. Decrease in the concentration of phosphate in the presence of low levels of calcium brings about a further increase in the lag, but this increase is not observed if the calcium supply is adequate.

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Received September 21, 1956. P.S.E.B.M., 1956, v93.

Uricolysis in Normal and Leukemic Individuals. (22821)

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Hyperuricemia occurs with moderate frequency in leukemia and other blood dyscrasias. The hyperuricemia has been ascribed to increased nucleo-protein catabolism. In studies on the uric acid pool, Benedict(1) and her associates consistently found more urate produced than excreted. Using larger amounts of labeled uric acid, Wyngaarden (2) demonstrated that uricolysis occurs in significant amounts in humans. We attempted (3) to demonstrate the site of this uricolysis. The rate of urate disappearing from the formed elements of blood incubated with plasma was of a significant amount and could account for a large part of the degraded urate. Among our non-gouty hospitalized controls were several leukemics. These patients demonstrated a markedly lower rate of uricolysis than did the normals or even the gouty patients, the gouty patients being considerably lower than the normals. It is quite possible therefore, that elevated plasma urate levels in leukemics may be secondary, at least in part, to an impaired mechanism for uricolysis.

Methods. 50 ml of heparinized blood were obtained. A 5 ml sample was removed to serve as a whole blood specimen and the rest centrifuged. The plasma was removed and the buffy coat pipetted off. The buffy coat was centrifuged in a narrow tube in either the patient's own plasma or isotonic saline. After 3-4 separations, high concentrations of white blood cells relatively free of red blood cells were obtained. The red blood cell preparations were obtained by pipetting the red cells from the bottom of the tube and freed of white blood cells in the same manner as above. Each patient had the following samples incubated at 37° under 5% CO_2–95% O_2: 1. Whole blood. 2. Plasma. 3.White blood cells (0.5 ml) and the same patient's plasma (3. ml). 4. Red blood cells (0.5 ml) and the same patient's plasma (3. ml). Blood counts were done on preparations 3 and 4. On white cell preparations, the red counts