moting activity of myristic acid since purified commercial myristic acid (recrystallized) was essentially inactive. Activity of the latter could be potentiated by traces of α-hydroxymyristic acid, but this substance did not appear to be present in the isolated material.

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Trace Metal Requirements of *Aerobacter aerogenes* for Assimilation of Molecular Nitrogen.* (24653)

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One of the surprising developments in biological nitrogen fixation during the past few years has been the discovery that many well-known bacteria previously unsuspected to possess the ability to use molecular nitrogen may do so when cultivated under appropriate conditions(1). These discoveries provide new tools for investigating the biochemistry of nitrogen fixation—studies that previously have been largely confined to the traditional agents of fixation: the azotobacter, the clostridia and the symbiotic system. For example, in spite of research extending over a quarter century, doubt still exists regarding the role of minerals (specifically, calcium, molybdenum and iron) in biological nitrogen fixation(2,3). Perhaps, some of the current disagreements in this respect can be resolved if attention were directed to the requirements of the new agents rather than concentration on the old. With this in mind, we have investigated the response of *Aerobacter aerogenes* strain 5al to these 3 minerals in the fixation of N_2.

Materials and methods. The growth medium and analytical procedures were essentially those previously described(2,4) altered as indicated for specific studies. The test medium for studies on the iron requirement was the same except that demineralized water prepared by passing distilled water through a Barstead "Bantamatic" demineralizer was used in all media, and iron was added separately rather than with the molybdenum solution. Solutions of the phosphate salts were run through 1 x 10 cm columns of Dowex 50 resin charged with sodium or potassium ions; a 5% solution of the corresponding chloride salt was used to charge the columns. Before passing the phosphate solutions through, the columns were washed with demineralized water until the effluent pH was near 7. A 10% solution of sucrose was purified by passing it through a similar column charged with hydrogen ions. The following solutions were prepared: Solution A, 12.5 g Na_2HPO_4 (Mallinckrodt, A. R. grade or comparable purity for all salts) in 250 ml; B, 1.5 g KH_2PO_4 in 250 ml water; C, 10 g Bacto-sucrose in 100 ml water; D, $MgSO_4 \cdot 7H_2O$ in 250 ml slurried with several grams of Dowex 50 charged with Mg ions; E. 0.02 g, $CaCl_2$ in 10 ml water; F. 2.5 μg molybdenum/ml as Na_2MoO_4; G. 2.5 μg iron/ml as $FeCl_3$. Combination of the solutions in the following quantities made up the media in the test flasks: 6.25 ml A, 6.25 ml B, 5.0 ml C, 2.5 ml D, 1 drop E, 1.0 ml F,

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and amounts of G to give 0.5, 0.1, 0.08, 0.06, 0.04, 0.02, 0.01, and 0.00 parts per million of iron in the series of flasks. Water was added to bring the volume of the medium to 25 ml in the 125 ml Erlenmeyer flasks. The flasks were covered with 50 ml beakers and autoclaved at 15 lb. pressure for 20 min. A 0.5% inoculum of *Aerobacter aerogenes* strain M5al from an iron deficient culture was used. When growth was followed turbidimetrically, flasks with colorimeter tubes fused to them were used(4). All cultures were grown on a Brunswick rotary shaker at 30°C, unless otherwise indicated. Nitrogen fixed was determined by a semi-micro Kjeldahl method (5). For the studies with carbon monoxide, growth of the cultures was followed by placing 50 ml of a one liter culture of the organism just entering the exponential phase of growth into each of several 500 ml Erlenmeyer flasks with pyrex glass tubes of a size to fit the Klett-Summerson colorimeter fused into the side. The desired atmospheres were placed in the flasks by evacuation with a water pump and refilling with the required gas mixture. Growth was followed turbidimetrically for 3 to 4 hours. For determination of molybdenum requirement, the medium was freed of this element by the method employed by Nicholas and Fielding(6) based on coprecipitation of the molybdate ion with a ferric quinolate complex from the macro-nutrients after which the excess quinolate is extracted from the solution with chloroform and ether. Adequate iron is then added to the medium together with the desired quantities of sodium molybdate solution. Inoculation was made from a molybdenum deficient culture, and after 3 to 4 days of incubation, the cultures were harvested and analyzed as has been described for the experiments on iron. Control flasks containing 150 or 200 μg of ammonium acetate-nitrogen were included in each experiment. The supply of ammonium acetate was checked for molybdenum by ashing a sample of the salt and adding the "residue" to control flasks. No fixation occurred in these flasks. For determination of the calcium requirement the 2 phosphate salts and the $MgSO_4$ were recrystallized from ion free water and the balance of the chemicals used were Analytical Reagent grade (Mallinckrodt). Inoculation, incubation and harvesting of the cultures were employed as has been described. Helium served as the atmosphere in the fixed nitrogen controls.

TABLE I. Inhibition of *Aerobacter aerogenes* by Carbon Monoxide.

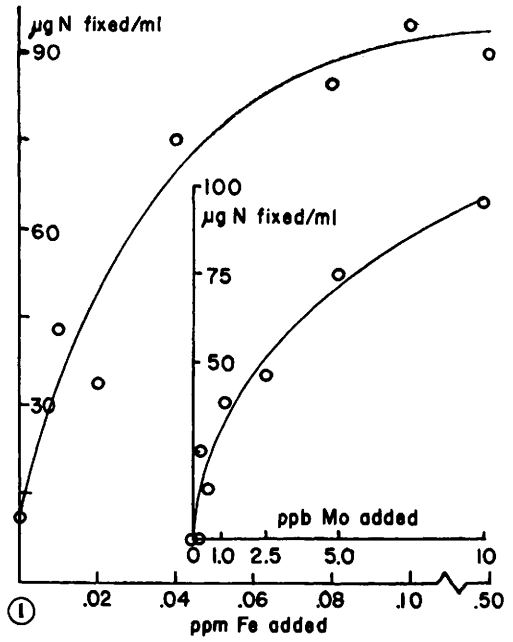
% CO in atmosphere	Ammonia		Nitrogen gas	
	Growth rate*	% inhibition	Growth rate*	% inhibition
.0	.200		.149	
.1	.202	.0	.098	34.2
1.0	.196	2.0	.057	68.5
10.0	.107	49.6	.000	100.0

* Growth rate $= \frac{2.3}{\text{Time in hr}} \log \frac{\text{Final N}}{\text{Initial N}}$.

Results. Cultures of *A. aerogenes* supplied with ammonium acetate as the nitrogen source and with an atmosphere of helium grew at the same rate and to the same extent when supplemented with 1.0, 0.5, 0.1, or 0.0 parts per million of iron, but as is shown in Fig. 1, nitrogen fixation is definitely stimulated by iron. This agrees with the finding of Esposito and Wilson (2) for *Azotobacter vinelandii* and with Carnahan and Castle(7), working with the anaerobic nitrogen fixer *Clostridium pasteurianum.*

Although growth on both free and fixed nitrogen was inhibited by carbon monoxide, assimilation of molecular nitrogen was about one hundred times as sensitive to carbon monoxide as was assimilation of ammonia nitrogen (Table I). Although carbon monoxide inhibition of a biological reaction is not *per se* proof that iron is involved in the reaction, demonstration of such an inhibiton provides support for the view that iron is involved. In comparison, Wilson and Lind(8) observed CO inhibition of nitrogen fixation by *Azotobacter vinelandii,* and Hino(9) observed CO inhibition of fixation by an organism he believed at the time to be a member of the genus *Clostridium,* but now shown to be a species of *Bacillus*(10).

The quantity of molybdenum required for maximum growth on N_2 was so small that some difficulty was encountered in obtaining a response curve but finally the curve insert in Fig. 1 was obtained. No requirement for



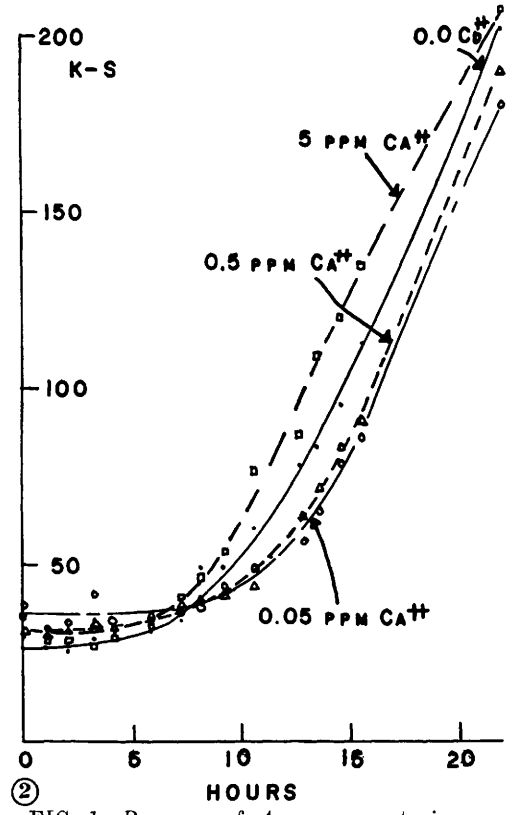


FIG. 1. Response of *A. aerogenes* to iron and molybdenum when assimilating free nitrogen.

FIG. 2. Effect of calcium concentration of nitrogen fixation by *A. aerogenes*. K-S is Klett-Summerson colorimeter readings.

molybdenum could ever be demonstrated when the organism was growing on ammonium nitrogen. Esposito and Wilson(2) demonstrated a definite requirement for molybdenum by nitrogen fixing cultures of azotobacter. Their data does not allow one to decide the minimum amount required for fixation, but it appears to be somewhat lower than the iron requirement.

Although Esposito and Wilson(2) observed a definite stimulation in nitrogen fixation by *Azotobacter vinelandii* on addition of calcium, Norris and Jensen(3) claim that the requirement is not specific for fixation. The results with the strain of *Aerobacter aerogenes* used in this work likewise did not reveal a requirement for calcium (Fig. 2). Perhaps the discrepancies reported by various investigators can be explained in part by the more recent work of Esposito and Wilson(11) that revealed that the calcium requirement specific for fixation in *A. vinelandii* O can be replaced by acetate. The implication is that calcium is not involved directly in nitrogen fixation but in the production of an essential carbon compound(s). If this is the true explanation of its action, variation would be expected among different agents, even extending to strains, such as has been observed by the various investigators.

Summary. *Aerobacter aerogenes* strain M5al fixing molecular nitrogen require at least 10 times more iron than do cultures using ammonium-N. Also, the organism is about 100 times more sensitive to carbon monoxide when using free than when assimilating combined nitrogen. Although there is an absolute requirement for molybdenum for nitrogen fixation by this organism, it is considerably less than that noted for many strains of the azotobacter. No specific requirement for calcium could be demonstrated.

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Effect of Estrogen on Liver Glycogen in Adrenalectomized Rats.* (25654)

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Large doses of injected estrogen cause increased glycogen in livers of intact fasting rats but not in livers of untreated adrenalectomized rats(1,2). It was concluded that the glycogenic activity of estrogen is mediated by increased secretory activity of the adrenal cortex. The present study demonstrated an extra-adrenal effect of estrogen upon level of glycogen in livers of adrenalectomized rats treated with adrenal cortical extract (ACE).

Methods. Male rats of the Sprague-Dawley strain weighing approximately 300 g were adapted to a medium carbohydrate diet(3), tube-fed each morning and late afternoon. The temperature was 74° to 78°F. Adrenalectomies and sham adrenalectomies were done by sterile technic under ether anesthesia. The adrenalectomized rats were treated with ACE (Upjohn) having glycogenic potency of 0.1 mg of hydrocortisone/cc. Whole adrenal cortical extract was used because it represents more complete replacement therapy than any single steroid. From fifth postoperative day, estradiol cyclophentyl-propionate (Upjohn) in oil solution (1 mg/cc) was injected subcutaneously in 0.1 mg daily for 4 days. Each rat was then fasted 24 hours and, under sodium cyclopal anesthesia, the liver was rapildly excised, weighed, and dropped into hot KOH. Glycogen was determined by the anthrone method(4). Since each rat had the same initial weight and was given the same amount of food, the results are expressed as total amount of liver glycogen/rat.

Results. Five experimental groups of 60 rats each were studied in relationship to dose of ACE given the adrenalectomized rats. Each group was divided into 4 equal subgroups: adrenalectomized, no estrogen; adrenalectomized, estrogen; adrenals intact, no estrogen; and, adrenals intact, estrogen. Each subgroup was equally represented in time. The values on liver glycogen are in Table I. Livers of rats treated with estrogen averaged 21% heavier than livers of rats not given estrogen. The average amount of glycogen recovered from liver varied somewhat, but in the non-adrenalectomized rat treated with estrogen a significantly greater amount was always found. Among rats not given estrogen, the average amount of liver glycogen in adrenalectomized rats was less than in intact rats until 6 cc of ACE/rat/day were given. The injection of estrogen was associated with increased liver glycogen in each subgroup of adrenalectomized rats, the amount increasing as dose of ACE was increased. Even with a dose of 6 cc of ACE daily, the adrenalectomized rats given estrogen showed significantly smaller amounts of liver glycogen than did the non-adrenalectomized rats given estrogen.

Discussion. Estrogen has an extra-adrenal effect on amount of liver glycogen in the rat. The mechanism of this effect is unknown. It is possible than increased secretion of cortical hormones by the estrogen-treated intact rat

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