OXIDATIVE ACTIVITY AND NITROGEN FIXATION IN CELL-FREE PREPARATIONS FROM *AZOTOBACTER VINELANDII*¹

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Ammonia is generally conceded to be the "key intermediate" in biological nitrogen fixation—that is, the compound which reacts to form the first stable organic compound, glutamic acid (Wilson and Burris, 1953). However, there is virtually no information concerning the reactions functioning in the conversion of N₂ to ammonia. Reproducible nitrogen fixation by a cell-free preparation from bacteria would be most helpful in elucidating the enzymatic mechanism of the intermediate steps.

Bach *et al.* (1934) reported fixation by cell-free preparations from *Azotobacter vinelandii*, but these results could be repeated neither in Bach's laboratory nor elsewhere. Wilson and Roberts (1954) have presented a brief account of their negative results with cell-free preparations. Hamilton *et al.* (1953) used N₂¹⁵ as a tracer and reported the detection of limited fixation of nitrogen by cell-free preparations from *A. vinelandii*.

As nitrogen fixation requires energy, it is important to study the respiration of the azotobacter as a potential source of that energy. Lee *et al.* (1942) investigated cell-free preparations of *A. vinelandii* and found succinic, lactic and malic dehydrogenases and hydrogenase by coupling the reactions through methylene blue. They also demonstrated oxalacetic and α -ketoglutaric acid decarboxylases.

For a time it was believed that the citric acid cycle was inoperative in these organisms, because they utilized citrate very slowly and exhibited a marked lag in the oxidation of succinate and malate. Recently, Stone and Wilson (1952a, 1952b, 1952c) have shown an active citric acid

cycle in cell-free preparations of the azotobacter. The most satisfactory methods for cell breakage were grinding in a bacterial mill, grinding in the presence of alumina powder, or breaking by sonic vibration.

Mortenson and Wilson (1954) have demonstrated the operation of an alternate pathway for glucose oxidation in *A. vinelandii* preparations. One enzyme of the glycolytic scheme was not found (phosphohexokinase), so these organisms may oxidize glucose to pyruvate entirely by the shunt mechanism.

Attempts to obtain cell-free nitrogen fixation in the present experiments were made with preparations capable of vigorous oxidation of normal substrates; it was hoped that the proper high energy compounds and reduced coenzymes thus would be kept available. Runs also were made under anaerobic conditions, which assured maintenance of reduced pyridine nucleotides.

In common with other nitrogen fixing organisms, the azotobacter require molybdenum when they are fixing nitrogen (Bortels, 1930). The level is higher than that necessary for growth on combined nitrogen, including nitrate (nitrate reductase is known to be a molybdoflavoprotein in *Neurospora crassa* (Nason and Evans, 1953; Nicholas and Nason, 1954)). In light of this special requirement, the distribution of molybdenum in cell-free preparations was investigated with cells grown on Mo⁹⁹.

MATERIALS AND METHODS

Cells were grown on Burk's salts plus: sucrose, 3 per cent; Fe, 3 ppm; Mo, 1 ppm; in 3 and 9 L aerated bottles from a 10 per cent inoculum. Vigorous aeration was provided, and the cells were harvested while still in the log phase of growth. In a number of runs, the culture was chilled to 0 to 5 C by submerging the aerated culture in an ice-salt bath at the end of the growth period. The cells were recovered by centrifugation in a Sharples supercentrifuge or

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on a large preparative head in an International centrifuge at 0 C. Cell breakage was accomplished by grinding the cell paste in a cold mortar with cold, washed alumina (McIlwain, 1948), or by treatment of a suspension of cells in a 10 kc Raytheon sonic generator. The chamber of the Raytheon generator was cooled with a water-alcohol mixture (-3°C), so that the temperature of the cell suspension did not rise above 0 to 5 C. Breakage time was from 5 to 20 min. The preparations were centrifuged in the cold at 1,500 to 10,000 $\times$ G to remove cellular debris and whole cells. The preparations so obtained were used immediately in tests for nitrogen fixation. Warburg flasks containing the preparation (2.0 to 4.0 ml), test substrates, and co-factors were gassed with a mixture containing: N_2^{15} , 10 per cent (N^{15} , 30 to 36 atom per cent); O_2 , 20 per cent; He, 70 per cent; and then were incubated for 1 hr at 30 C in a Warburg bath. Anaerobic runs were made with a gas mixture of N_2^{15} , 10 per cent and He, 90 per cent. Protein in each flask was precipitated with 2.0 ml of freshly prepared 20 per cent metaphosphoric acid or with 5 per cent trichloroacetic acid, and the soluble portion was analyzed for N^{15} incorporation. Free ammonia was distilled from the protein-free supernatant *in vacuo* in a number of runs and tested for N^{15} incorporation.

For the preparation of a protein fraction rich in molybdenum from *A. vinelandii*, cells were grown in 16 L of medium in a 20 L stirred jar with rapid aeration and agitation (16 L of air/min) at 34 C in the presence of 15×10^6 c/min of Mo^{99} (3 ppm). The Mo^{99} as Mo^{99}O_3 was dissolved in alkali and added to the stirred jar. The cells were harvested after 18.5 hr (total yield, 188 g cell paste), and 120 g were ground in the presence of alumina in cold mortars. All subsequent procedures were carried out at 0 to 5 C. The mixture was taken up in 250 ml of 0.05 M phosphate (pH 7) and centrifuged. Protein concentration was determined by the method of Warburg and Christian (1941) by reading absorption at 260 and 280 m μ . Radioactivity measurements were made on small aliquots with a thin window Geiger counter. Precipitates from ammonium sulfate fractionation were suspended in water, reprecipitated with excess ammonium sulfate, centrifuged, and resuspended before making radioactivity determinations, to avoid carry-over of Mo^{99} occluded with the pre-

cipitate. Each count was corrected for background and converted to activity at the beginning of the experiment (half life of Mo^{99} is 2.85 days).

Oxidase enzymes were sought in both cell suspensions and cell-free preparations. Cells from vigorously growing cultures were spun down at 0 C and resuspended in 0.05 M phosphate buffer (pH 7). Cell-free preparations were made by grinding the cells with alumina and suspending in phosphate buffer. The supernatant from 18,000 $\times$ G centrifugation was used in these determinations. Aldehyde oxidase was assayed spectrophotometrically by following the reduction of cytochrome c at 550 m μ under anaerobic conditions (Morell, 1952). Beckman cells, fitted with a Thunberg joint and side arm, were evacuated on a water aspirator, and the enzyme solution was tipped in from the side arm at zero time.

RESULTS

Tests for nitrogen fixation by cell-free preparations. Acetate (40 μ moles) plus malate (10 μ moles) frequently was used as a mixed substrate for oxidation by these preparations. Isocitrate (50 μ moles) or glucose-6-phosphate (25 μ moles) also supported a vigorous oxygen uptake. Anaerobically, the reduction of TPN^{*} or DPN (more slowly) could be observed upon the addition of glucose-6-phosphate. DPN was reduced in the presence of malate and TPN in the presence of isocitrate.

Glutamic dehydrogenase activity was detected readily by oxygen uptake when coupled with *N*-methyl phenazine sulfate dye (figure 1). The preparation was made by grinding frozen cells with alumina, suspending in 0.05 M phosphate (pH 7) and centrifuging at 20,000 $\times$ G. Dialysis for 1 hr with stirring was necessary to reduce the endogenous oxygen uptake. Incorporation of $\text{N}^{15}\text{H}_4^+$ by the preparations plus α -ketoglutarate also demonstrated the presence of glutamic dehydrogenase (table 1).

Table 2 shows a few typical results from a large number of tests for cell-free nitrogen fixa-

* The following abbreviations are used: Coenzyme A (CoA), diphosphopyridine nucleotide (DPN), ethylenediaminetetraacetate (EDTA), flavinadenine dinucleotide (FAD), α -ketoglutarate (α -KG), triphosphopyridine nucleotide (TPN).

Figure 1. The oxidation of glutamate by a dialyzed, cell-free preparation from *Azotobacter vinelandii*.

A. Oxidation in the presence of N-methyl phenazine sulfate. 1, Complete system; 2, complete system minus N-methyl phenazine sulfate; 3, complete system minus glutamate.

Complete system: 0.50 ml dialyzed preparation, 0.30 ml 3 M glutamate (or 0.30 ml water), 0.3 mg diphosphopyridine nucleotide (DPN), 75 μ g N-methyl phenazine sulfate (or 0.05 ml water), 0.25 ml 0.05 M phosphate, pH 7.0.

B. The effect of malonate on the oxidation of glutamate. 1, 0.5 ml preparation, no malonate; 2, 0.25 ml preparation, no malonate; 3, 0.5 ml preparation, plus malonate; 4, 0.25 ml preparation, plus malonate; 5, 0.5 ml preparation, no glutamate.

Complete system: 1.0 ml dialyzed preparation (or dilution), 0.25 ml 3 M glutamate (or 0.25 ml water), 0.1 mg DPN, 50 μ g N-methyl phenazine sulfate, 0.05 ml 0.015 M malonate (or 0.05 ml water).

tion together with the treatment of the preparation. Values of 0.015 atom per cent excess N^{15} and higher may be taken as evidence of positive fixation. Although the positive experiments are encouraging, the results are not reproducible, for undefined factors still affect the fixation.

The non-reproducibility of cell-free fixation suggested the examination of a variety of factors which might account for the irregularity. These

TABLE 1
Utilization of $N^{15}H_4^+$ by preparations from *Azotobacter vinelandii*

Additions	Atom Per Cent Excess N^{15}
Glucose-6-phosphate	0.088
Glucose-6-phosphate, triphosphopyridine nucleotide (TPN)	0.078
Malate, diphosphopyridine nucleotide (DPN)	0.057
Glucose-6-phosphate; heated to 95 C for 2 min	0.008

Complete system: 2.0 ml cell-free preparation, 50 μ moles α -ketoglutarate. Where noted: 50 μ moles malate, 25 μ moles glucose-6-phosphate, 0.2 mg DPN or TPN. Flasks incubated 1 hr anaerobically. 0.5 mg N as $(N^{15}H_4)_2SO_4$ (7.4 atom per cent excess N^{15}) added to each flask. Residual labeled ammonia distilled off before analysis of the protein-free supernatant.

investigations ancillary to the problem of cell-free nitrogen fixation not only are of interest to this problem, but they also shed light on the general respiratory potentialities and molybdenum metabolism of the azotobacter.

The preparation of a protein fraction rich in Mo^{99} . Because Mo has been implicated in biological nitrogen fixation, it was hoped that supplementation of cell-free preparations with the fraction of the cells carrying most of the Mo would enhance the nitrogen fixation. A hundred ml of a 16 hr culture grown with Mo^{99} was centrifuged. The cells (1.9 g paste) were resuspended, counted (1.94×10^6 c/min total), and recentrifuged. The cell paste, broken by grinding with alumina, was taken up in phosphate buffer and centrifuged at $1,000 \times G$ (1.06×10^6 c/min total in the supernatant) and at $20,000 \times G$ (1.11×10^6 c/min total in the supernatant). Although the recovery of radioactivity was incomplete, it was judged satisfactory for the large scale preparation. The final preparation did not lose radioactivity during dialysis for 1 hr. Several protein precipitation methods were investigated in attempts to obtain a purified Mo^{99} fraction. Nucleic acids could be precipitated by the stepwise addition of a 0.2 per cent protamine sulfate solution (Nutritional Biochemicals Corp.) as described by Chou and Lipmann (1952), and ammonium sulfate provided a sharp fractionation between

TABLE 2
Nitrogen fixation by cell-free preparations from Azotobacter vinelandii

Exp.	Cells Broken by	Centrifugation	QO ₂ (N)	Additions	Atom Per Cent N ¹⁵ Excess
Aerobic nitrogen fixation					
1	Raytheon (5 min)	1,500 × G	373	—	0.108
6	Raytheon (5 min)	1,800 × G	435	—	0.065*
7	Raytheon (6 min)	1,500 × G	—	—	0.002†
9	Alumina grind	2,000 × G	751	—	0.001
			432	2% sucrose	0.103
10	Alumina grind	1,500 × G	261	—	0.017
			321	5 × 10 ⁻⁵ Methylene-diaminetetra-acetate (EDTA)	0.002
16	Alumina grind	1,500 × G	354	pH 7	0.004
			410	pH 6.4	0.011
17	Alumina grind	1,500 × G	—	0.3 mg pyridoxal	0.003
			433	pH 6.5, Armour's coenzyme A (CoA)	0.007
			482	Purified CoA, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN)	0.005
			347	No CoA	0.039
23	Powdered glass	1,500 × G	243	—	0.009
	Carborundum	1,500 × G	256	—	0.009
Anaerobic nitrogen fixation					
18	Alumina grind	1,500 × G	—	—	0.007
			—	DPN, glucose-6-phosphate	0.013
19	Alumina grind	1,500 × G	—	Glucose-6-phosphate	0.009
			—	Glucose-6-phosphate, TPN	0.016
			—	Glucose-6-phosphate, TPN, (α-keto-glutarate α-KG)	0.008
			—	Malate, DPN, α-KG	0.005

* Ammonia distilled directly from the solution and analyzed.

† Residue (protein free) after ammonia distillation.

Complete system: 2.0 ml enzyme solution pH 7 except when noted, 40 μ mole acetate, 10 μ moles malate, 2 mg CoA, runs 1-17 (Armour's, except as noted in run 17). 1.0 mg DPN or TPN where noted, 50 μ-moles glucose-6-phosphate or α-ketoglutarate where noted. One hour exposure to N₂¹⁵ (30-36 atom per cent N¹⁵ excess); only protein free portion analyzed for N¹⁵ incorporation.

TABLE 3
Purification of a protein fraction rich in Mo⁹⁹

Fraction	Volume	Total Protein	Total	Specific Activity*
	ml	mg	c/min	
Crude.....	50	1950	15.38 × 10 ⁵	0.0079
1st (NH ₄) ₂ SO ₄ precipitate (1.4 to 1.9 M)....	16	494	15.0 × 10 ⁵	0.030
After protamine sulfate treatment.....	33	—	10.1 × 10 ⁵	—
After calcium phosphate gel treatment.....	36	395	9.64 × 10 ⁵	0.024
2nd (NH ₄) ₂ SO ₄ precipitate.....	15	118	5.70 × 10 ⁵	0.049
3rd (NH ₄) ₂ SO ₄ precipitate.....	2	1.85	2.15 × 10 ⁵	1.17

* Counts/min/mg protein; thin window counter.

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1.4 and 1.9 M. Some impurities were adsorbed by the addition of calcium phosphate gel (18 mg solids/ml, Sigma Chemical Co.). Neither pH precipitation with 0.1 M, pH 4 acetate, nor ethyl alcohol fractionation proved satisfactory.

Table 3 shows the purification obtained with 50.0 ml of the crude, dialyzed preparation. The precipitate was saved between 1.4 and 1.9 M ammonium sulfate and resuspended in 16.0 ml total volume. Twenty ml of 0.2 per cent protamine sulfate solution was added, but no precipitate resulted, as the nucleic acid already had been removed in the first step. Calcium phosphate gel was added in 2.0 ml portions and centrifuged; after 6.0 ml had been added some radioactivity was carried down. The second

Figure 2. The relation between the uptake of Mo^{99} and adaptation of *Azotobacter vinelandii* to nitrate.

Two 3 L cultures aerated in series with no fixed nitrogen on Mo^{99} (9 ppm). At 13 hr 1.0 g $KN^{15}O_3$ was added to one of the cultures. Solid points indicate analyses made on the N_2 grown culture; open symbols, the analyses made on the $N^{15}O_3^-$ culture.

A. Growth of the 2 cultures (mg N/L on a log plot), and uptake of N^{15} from $N^{15}O_3^-$.

B. Radioactivity from Mo^{99} in the cells (c/min/mg dry wt), and hydrogenase activity with methylene blue as the hydrogen acceptor ($\mu L H_2$ uptake/hr/mg N).

Figure 3. The oxidation of xanthine and acetaldehyde by cell suspensions and cell-free preparations from *Azotobacter vinelandii*.

A. The oxidation of xanthine and acetaldehyde by cell suspensions.

Complete system: 1.0 ml cell suspension (0.9–1.2 mg N/ml) in 0.05 M phosphate (pH 7), 0.2 ml of M acetaldehyde or 0.2 ml of 0.05 M xanthine in 0.05 M NaOH, 0.003 M CN^- where noted, volume to 2.5 ml with phosphate buffer. Endogenous O_2 uptake was subtracted from each curve.

B. The oxidation of acetaldehyde alanine and glucose by cell-free preparations.

Complete system: 1.0 ml cell-free preparation in 0.05 M phosphate (pH 7), 0.2 ml of M acetaldehyde, or 0.2 ml of 0.2 M DL-alanine or 0.4 ml of 2 per cent glucose, 0.005 M CN^- where noted, volume to 1.45 ml with phosphate buffer. Endogenous O_2 uptake was subtracted from each curve.

Figure 4. The coupling of the oxidation of acetaldehyde or pyridoxal to the reduction of cytochrome c with a cell-free system from *Azotobacter vinelandii*.

Complete system: 0.1 ml of enzyme solution or dilution (1:2, 1:4, 1:10) in the side arm, 0.1 ml of M acetaldehyde or 0.1 ml of 0.1 M pyridoxal, 0.1 ml cytochrome c solution (1.0 mg) and phosphate buffer (pH 7) to 3.5 ml. Vessel evacuated, tipped, and the reduction of cytochrome c measured at 550 $m\mu$ against a blank containing complete system minus substrate.

Insert: Standard curve obtained from the change in optical density between 2.0 and 0.5 min with the original preparation taken as 10 units; pyridoxal as substrate.

ammonium sulfate fractionation was performed by the addition of reagent until incipient turbidity resulted. The suspension was centrifuged, and more ammonium sulfate was added until turbidity once more resulted. This was repeated several times, and the three precipitates containing the most radioactivity were combined. This process was repeated for the third ammonium sulfate fractionation. The yield of Mo^{99} in the third ammonium sulfate precipitate was 14.1 per cent in terms of original radioactivity, and the total purification was 148 fold per mg protein over the original crude preparation.

One of the active fractions was tested for its loss of radioactivity upon dialysis against 0.05

M phosphate buffer (pH 7) and against buffer containing $5 \times 10^{-3} M$ CN^- . After 7.5 hr of dialysis (with stirring), 83.0 per cent of the radioactivity remained when dialyzed against buffer, but only 13.5 per cent remained after dialysis against the cyanide.

Addition of the fraction rich in Mo^{99} did not enhance nitrogen fixation by cell-free preparations, so there is no evidence that this Mo -rich fraction functions in nitrogen fixation. It, of course, is possible that inactivation occurred during processing.

The Mo^{99} uptake of *A. vinelandii* grown on N_2 and $N^{15}O_3^-$. Cultures of the azotobacter grown on N_2 do not utilize nitrate without a period of adaptation. As nitrate reductase from some organisms contains Mo , it seemed pertinent in the general study of Mo metabolism to determine whether uptake of Mo is altered during a period of adaptation of *A. vinelandii* to nitrate.

Two cultures of *A. vinelandii* were grown in series in aerated, 3 L bottles at 30 C on 9 ppm Mo^{99} with no fixed nitrogen. One g $KN^{15}O_3$ (7 atom per cent excess N^{15}) was added to one of the bottles after 13 hr of incubation. Growth was measured turbidimetrically, and adaptation to nitrate was followed by determination of N^{15} in the washed cells. Mo^{99} uptake was measured by counting dried, weighed aliquots of washed cell suspensions. The results are shown in figure 2.

Oxidase enzymes from *A. vinelandii*. A final approach to the investigation of the Mo metabolism of the azotobacter, and its possible relation to nitrogen fixation, was to seek enzymes from the azotobacter which had been demonstrated to contain Mo when isolated from other sources. A search for enzymes which would likely be flavoproteins indicated that whole cells grown on sucrose oxidized only xanthine, fumarate and acetaldehyde at appreciable rates in the presence of 0.003 M CN^- . Leucine, glucose, alanine, acetaldehyde, and xanthine were oxidized by whole cells in the absence of CN^- , and very low activity was observed with aspartate and glycine. Glycolate was not oxidized. The results are shown in figure 3A. Only acetaldehyde, and to a lesser extent xanthine (not shown), supported oxygen uptake by cell-free preparations in the presence of 0.005 M CN^- (figure 3B). Coupling the oxidation of acetaldehyde with cytochrome c reduction under anaerobic conditions provided a convenient assay for the

aldehyde oxidase. The results with acetaldehyde or pyridoxal as a substrate are shown in figure 4. Anaerobic conditions were necessary to prevent the enzymatic reoxidation of cytochrome c. DPN and TPN were not reduced with acetaldehyde as a substrate.

Dialysis of the cell-free preparation for 3 hr with stirring against 0.1 M pH 7 phosphate resulted in little or no loss of enzymatic activity. One third of the activity was lost by centrifugation of the dialyzed preparation for 20 min at $18,000 \times G$, which may indicate that at least a portion of the enzyme was particulate.

DISCUSSION

The failure by a large number of workers to provide any convincing data for the intermediates in nitrogen fixation which lie between N_2 and NH_3 supports the conclusion that these compounds either have a very transitory existence or else are very firmly bound. The breakage of the $N \equiv N$ bond and the reduction of the nitrogen to ammonia pose an energy requirement which may limit the growth of *A. vinelandii*, for the organism grows more rapidly on ammonia than on N_2 . However, the respiration of the azotobacter is the highest known for any cells or tissue, so it should be quite adequate to supply the energy requirements for nitrogen fixation.

In the sequence of reactions from N_2 to NH_3 , a source of reducing power is necessary. This may come from hydrogenase, reduced pyridine nucleotides, reduced flavins or other sources. Zucker and Nason (1954) demonstrated hydroxylamine reductase in *Neurospora crassa*, but only when the organisms had been utilizing nitrate. The enzyme appears to be flavin linked, and requires either TPN or DPN. This enzyme also could function in nitrogen fixation if hydroxylamine is an intermediate. Our limited search for the enzyme in *A. vinelandii* preparations has not been successful, although Suzuki and Suzuki (1954) have reported finding the enzyme in this organism grown on nitrate.

The values for nitrogen fixation listed in table 2 are typical of those observed in a large number of runs. The cause for variation in the fixation is not known, but the system is very labile and thus far has been largely destroyed by cell breakage. A more gentle (and yet complete) method for cell rupture would be highly desirable, and it would be valuable to test cellular

fragments more extensively for their ability to fix nitrogen, as the enzyme system may be closely associated with the cellular membrane or other subcellular particles. With the present methods of breakage, centrifugation is essential to insure removal of whole cells. Several facts disclaim that the observed fixation can be attributed to the presence of whole cells. The cell count must be rather high to support detectable fixation during the 1 hr experiments (conservatively, several million cells/ml). We have run viable cell counts on several preparations, and one alumina ground preparation centrifuged at low speed gave a bacterial count of several billion per ml without exhibiting measurable isotopic enrichment (0.000 atom per cent N^{15} excess). A preparation broken 10 min in the Raytheon sonic oscillator showed fewer than 10,000 viable organisms per ml.

Low concentrations of EDTA gave a stimulation in oxygen uptake by cell-free preparations on acetate plus malate; at the optimal concentration of 5×10^{-4} M there was 19 per cent stimulation. There was little if any effect on nitrogen fixation, however. A mixture of metals (10^{-4} M Mo, Mg and B) appeared to stimulate fixation by a small amount in several runs. Alpha-ketoglutarate did not enhance cell-free fixation.

A fraction was obtained from cells of *A. vinelandii* with 148-fold the Mo^{99} concentration of the original cells. This fraction (0.1 ml) was added to flasks containing 4.0 ml of a fresh cell-free preparation plus acetate and malate. No fixation of N_2^{15} was detected.

The visible and ultraviolet spectrum of the most highly purified Mo^{99} fraction showed slight humps in the region of 400 and 490 $m\mu$. The solution was boiled, and the spectrum of the soluble material showed better defined peaks in the same region; however, the peaks still were not clear enough to permit positive identification. The ultraviolet spectrum showed only a peak at 270 $m\mu$.

Hydrogenase activity did not appear to follow the Mo^{99} during fractionation. The bulk of the hydrogenase present in the crude extract was recovered in the resuspended precipitate from the first ammonium sulfate addition (0 to 1.4 M ammonium sulfate). Only a small amount of Mo^{99} was precipitated with this fraction (see table 3).

An experiment with growing organisms furnished Mo⁹⁹ was designed to answer the following questions: Does the molybdenum uptake by *A. vinelandii* change during the adaptation to nitrate utilization, and how is the hydrogenase affected during the adaptation? Molybdenum is a constituent of nitrate reductase from *Neurospora crassa* (Nicholas and Nason, 1954), and if the enzyme were formed *de novo* the molybdenum content of the cells might be expected to change during adaptation to nitrate; such a change would offer a means for studying adaptive enzyme formation. Hydrogenase is much less concentrated in *A. vinelandii* cells when they are grown on nitrate than when they are grown on N₂ (Lee and Wilson, 1943), so the hydrogenase concentration also might be expected to change during adaptation.

The results shown in figure 2B clearly indicate that there was no difference in hydrogenase between the two cultures, although the activity in each apparently increased with time. Nor was there any appreciable change in the molybdenum concentration of the cells during adaptation to nitrate; about two generation times are covered in figure 2B. This observation suggests that the molybdenum of nitrate reductase came from molybdenum already bound in the cell (assuming molybdenum is a constituent of the enzyme in these organisms). Nitrate reductase apparently is a labile enzyme in *A. vinelandii*, as a number of efforts to demonstrate its action in cell-free systems have been negative (experiments with M. M. Mozen).

The presence of an aldehyde oxidizing enzyme in cell-free preparations of *A. vinelandii* is interesting in terms of both its properties and its function in cellular processes. It had a broad pH optimum in the crude preparations; activity changed little between pH 6 and 8. The addition of FAD did not stimulate oxidation by any of the preparations. Acetone powders from cells of *A. vinelandii* showed no activity. Limited attempts at purification have so far been unsuccessful.

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SUMMARY

Cell-free preparations from *Azotobacter vinelandii* have been tested for their ability to fix

molecular nitrogen. Tests were run while vigorous oxidation of substrates was occurring. High QO₂(N) values were observed on substrates of the citric acid cycle with both sonically disintegrated and alumina ground preparations. In runs made under anaerobic conditions, the oxidation of glucose-6-phosphate or malate provided reduced pyridine nucleotides. Fixation of N₂¹⁵ in each of these systems was erratic, but each yielded occasional positive results.

Glutamic acid dehydrogenase appears to be present in the cell-free preparations, for they assimilated N¹⁵H₄⁺ in the presence of α -ketoglutarate and substrates supporting the reduction of pyridine nucleotides.

A molybdenum-protein fraction from cells of *A. vinelandii* grown on Mo⁹⁹ was purified 148-fold by repeated ammonium sulfate fractionation. The Mo⁹⁹ could be removed rapidly from the protein by dialysis against 10⁻³ M CN⁻, but was essentially stable to dialysis against phosphate buffer. Addition of this Mo-rich fraction to fresh cell-free preparations did not enhance nitrogen fixation.

Nitrate reductase and hydroxylamine reductase were not detected in cell-free preparations of *A. vinelandii*, but glutamic acid dehydrogenase and an aldehyde oxidizing enzyme were found. The aldehyde oxidase worked equally well with acetaldehyde or pyridoxal as a substrate. It exhibited a broad pH optimum and was stable to dialysis against phosphate buffer.

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