RELATIONSHIP OF NITRITE AND HYDROXYLAMINE REDUCTASES TO NITRATE ASSIMILATION AND NITROGEN FIXATION IN *AZOTOBACTER AGILE*[1]

DONALD SPENCER,[2] HAJIME TAKAHASHI,[3] AND ALVIN NASON

McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

Received for publication October 25, 1956

The nitrogen fixation process has been recently reviewed by Wilson (1952), Burris (1956), Shug *et al.* (1956), and Gest *et al.* (1956). Although the characteristics of the over-all process *in vivo* are well known, neither the pathway nor the enzymatic steps has been established. On the other hand, a number of enzymes from bacteria, fungi and higher plants have been characterized which together can catalyze the reduction of nitrate to ammonia by way of nitrite and hydroxylamine. The properties of these systems and their possible role in the main pathway of nitrate assimilation have also been recently reviewed (Verhoeven, 1956; Taniguchi *et al.*, 1956; Nason, 1956; McElroy and Spencer, 1956). That nitrogen fixation and nitrate assimilation might have a common pathway has been suggested by a number of workers.

The present paper reports the presence in *Azotobacter agile* (*A. vinelandii*) of pyridine nucleotide nitrite and hydroxylamine reductases with properties similar to those already described from *Neurospora* and soybean leaves (Nason *et al.*, 1954; Zucker and Nason, 1955). These systems have been characterized, and some indication of their physiological significance in nitrogen fixation and nitrate assimilation has been obtained from adaptation experiments.

[1] Contribution No. 170 of the McCollum-Pratt Institute. This investigation was supported in part by research grants from the National Science Foundation and the U. S. Public Health Service (R.G.-2332). A preliminary report of this work was given at the Federation meetings in April, 1956 (Spencer, D., Takahashi, H., and Nason, A., Federation Proc., **15**, 358).

[2] Post-doctoral fellow of the McCollum-Pratt Institute. Present address: Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T., Australia.

[3] Post-doctoral fellow of the McCollum-Pratt Institute on leave from the Tokyo University, Tokyo, Japan.

METHODS AND MATERIALS

Culture methods. A. agile, American Type Culture Collection No. 9104, was grown in Burk's medium (Newton *et al.*, 1953) to which was added 1.44 g KNO_3 per L. Three-liter cultures were grown at 35 C for 18 to 24 hr with vigorous forced aeration. Cells were harvested by centrifugation at approximately 2,000 × G, and washed once or twice with 0.2 per cent KCl. This removed nitrite which had accumulated in the growth medium in considerable quantity. The yield of cells under these conditions was 10 to 20 g wet weight per 3 L. Cells not used immediately for the preparation of cell free extracts were stored at −15 C. Such cells could be stored for several weeks under these conditions without affecting the activity of the extracts subsequently prepared from them. Cells grown on nitrogen gas, glutamate, and ammonium sulfate were obtained essentially in the same manner as described above except that the nitrogen source was air (i. e., 80 per cent nitrogen gas), monosodium glutamate (2.36 g per L), or ammonium sulfate (0.94 g per L) respectively instead of potassium nitrate. When ammonium sulfate was used, calcium carbonate (0.5 g per L) was added to avoid a lowering of the pH of the medium.

Preparation of cell free extracts. Azotobacter cells were suspended in three times their weight of a solution of tris-(hydroxymethyl) aminomethane (tris) (0.05 M, pH 7.1) and glutathione (10^{-3} M). The suspended cells were subjected to sonic oscillation for 5 min in a Raytheon 10-kcy oscillator at 0.94-amp. output and centrifuged at 20,000 to 25,000 × G for 20 to 30 min. The supernatant solution was used as the cell free extract. Extraction of the cells by grinding with alumina powder (Alcoa A-301) also yielded active extracts.

Cofactors. Triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN)

were obtained from Pabst Laboratories. Reduced TPN was prepared by enzymatic reduction, using isocitric dehydrogenase and isocitrate as described by Nason and Evans (1953). An excess of isocitrate was used so that the resultant solution of reduced triphosphopyridine nucleotide (TPNH) would contain this compound. Chemically reduced TPNH was prepared according to the method of Kaplan *et al.* (1952).

Flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN) were obtained from the Sigma Chemical Company. Boiled pig heart extract was prepared as previously described (Nason and Evans, 1953) from the acetone powder of pig heart. Sodium DL-isocitrate and barium glucose-6-phosphate were obtained from the H. and M. Chemical Company, Santa Monica, California, and Sigma Chemical Company, respectively. The glucose-6-phosphate was used as a sodium salt solution.

Assay methods. Nitrite reductase activity was measured by the following assay procedure. The reaction mixture consisted of boiled extract of acetone powder of pig heart, 0.05 ml; or 1.2×10^{-4} M FAD, 0.03 ml; 2×10^{-4} M $NaNO_2$, 0.15 ml; 2×10^{-3} M TPNH, 0.03 ml; 0.02 to 0.06 ml, enzyme and tris buffer (0.1 M, pH 7.1, to give a final volume of 0.5 ml. After incubation for 10 to 15 min at room temperature, 1.5 ml of H_2O and 0.5 ml of sulfanilamide reagent (1 per cent in 3 N HCl) were added to stop the reaction, followed by 0.5 ml of N-1-napthylethylenediamine hydrochloride (0.02 per cent aqueous solution) to develop the color. After 10 min the optical density of the resultant pink solution was measured in a Klett-Summerson colorimeter using a no. 54 filter (green). A similar reaction mixture at zero time or from which TPNH had been omitted was used as the control.

Hydroxylamine reductase activity was assayed by the following procedure. The reaction mixture consisted of 10^{-4} M FMN or 2×10^{-4} M FAD, 0.01 ml; 4×10^{-4} M hydroxylamine hydrochloride, 0.15 ml; 2×10^{-3} M TPNH, 0.02 ml; 0.2 M glucose-6-phosphate, 0.02 ml; 0.02 M $MnCl_2$, 0.02 ml; enzyme, 0.02 to 0.06 ml; and tris buffer (0.1 M, pH 7.1) to give a final volume of 0.5 ml. After incubation at room temperature for 10 to 20 min the remaining hydroxylamine was estimated by the method of Czáky (1948). This involves the oxidation of hydroxylamine by iodine to nitrite.

In every case, the preparation contained isocitric and glucose-6-phosphate dehydrogenases which were able to maintain almost all the added pyridine nucleotide in the reduced state in the presence of their respective electron donors. Some assays of both enzyme activities were carried out, using the oxidized pyridine nucleotides instead of the reduced forms in the presence of either DL-isocitrate (3 μmoles) or glucose-6-phosphate (4 μmoles).

Ammonia was determined essentially as described by Zucker and Nason (1955), except that the ammonia in the center well of the Conway dish was assayed with Nessler's reagent (Vanselow, 1940). For the ammonia determination a 3.0-ml reaction mixture with components in the same proportions as above for the nitrite reductase assay was used, and the incubation carried on for approximately 1 hr. One-tenth ml of sodium DL-isocitrate (0.4 M) was included in these reaction mixtures. Endogenous and non-enzymatically formed ammonia was measured in parallel assays in which enzyme and nitrite were omitted singly. For each incubated reaction mixture a corresponding zero time control was also assayed. The ammonia liberated due to nitrite reductase activity was then given by the total increase in ammonia during incubation, minus the sum of the increase in the absence of nitrite and the increase in the absence of enzyme.

A unit of nitrite reductase or hydroxylamine reductase is defined as that amount of enzyme which results in the disappearance of one mμmole of nitrite or hydroxylamine under the above conditions of assay for ten min.

The specific activities of nitrite and hydroxylamine reductases were expressed as the decrease in nitrite or hydroxylamine in units (or mμmoles) per mg of protein per 10 min, respectively.

Protein was estimated by the method of Lowry *et al.* (1951). For most of these studies 300 to 600 μg of nitrite reductase or hydroxylamine reductase were used.

RESULTS

Proportionality between enzyme concentration and nitrite or hydroxylamine disappearance. The proportionality between enzyme concentration and nitrite or hydroxylamine disappearance under the described assay conditions are shown in figure 1. There is a slight non-enzymatic loss of nitrite and hydroxylamine in the presence of TPNH,

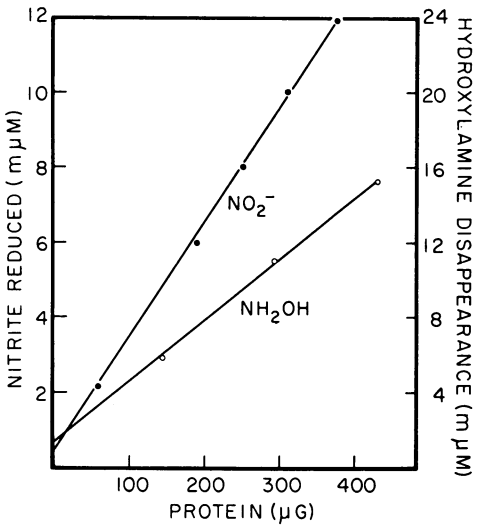

Figure 1. Effect of concentration of nitrite or hydroxylamine reductase on the disappearance of nitrite and hydroxylamine, respectively.

and hence the plot does not pass through the origin in both cases.

Purification. The cell free extract of Azotobacter cells was treated with one-half its volume of alumina C γ gel (12.1 to 16.5 mg dry weight per ml) for 15 to 30 min with intermittent stirring. The gel was collected by centrifugation at 3,000 $\times$ G for 5 min, washed twice with 5 to 10 times its volume of tris buffer (0.05 M, pH 7.1) containing glutathione (10^{-3} M), and eluted twice with potassium phosphate buffer (0.1 M, pH 7.5) containing glutathione. For each elution, carried out for 30 min with occasional stirring, the buffer volume was one-quarter of the original volume of the cell free extract. The two eluates were combined and this fraction used for subsequent enzyme studies. In some cases this fraction was recentrifuged at 144,000 $\times$ G and the active supernatant solution collected. This step was used in order to remove most of the TPNH oxidase activity which was mainly associated with the particles.

The over-all purification procedure resulted in a 2- to 4-fold increase in the specific activity of nitrite and hydroxylamine reductases as shown in table 1.

The cell free extracts possessed considerable endogenous nitrite and hydroxylamine reductase activities but, after gel absorption, washing, and elution, a definite requirement for added cofactors could be shown. All fractions possessed considerable TPNH and DPNH oxidase activities, TPN-isocitric and TPN- or DPN-glucose-6-phosphate dehydrogenases. These dehydrogenases in the presence of excess isocitrate or glucose-6-phosphate resulted in a continuous regeneration of TPNH or DPNH.

Attempts to further purify the enzyme by ammonium sulfate treatment resulted in a complete loss of nitrite reductase activity. Hydroxylamine reductase, however, was precipitated between 30 and 50 per cent ammonium sulfate saturation. A 2-fold increase in specific activity could be obtained by this treatment but with poor yield.

The location of the nitrite reducing system in the soluble, cytoplasmic fraction of the cell was suggested by the presence of considerable activity in the supernatant solution after centrifugation at 144,000 $\times$ G of a cell free extract prepared with alumina powder and buffered hypertonic sucrose solutions. This is also supported by the finding that the particulate fraction from whole sonic homogenate showed little or no activity towards hydroxylamine or nitrite.

Partially purified nitrite and hydroxylamine reductases are stable for several weeks at -15 C if stored in 0.1 M phosphate buffer, pH 7.5, containing glutathione (10^{-3} M). The enzymes will store at 0 to 2 C for approximately 1 week without marked losses in activities. The crude cell free extract was relatively unstable and all activities were lost under the above storage conditions.

Both nitrite and hydroxylamine reductases lose all their activities after 5 min at 50 C. The enzyme is stable to dialysis for 6 hr against 0.1 M K_2HPO_4 and 10^{-3} M glutathione, provided that the dialysis membrane is soaked for 30 min in the same solution prior to use.

The activity and stability of nitrite reductase were greatly enhanced when both the tris and phosphate buffers used for purification had been previously extracted with a solution of 8-hydroxyquinoline in chloroform in order to remove heavy metal contaminants. Excess 8-hydroxyquinoline was removed by repeated extraction with chloroform.

pH optimum. The effect of pH on nitrite and hydroxylamine reductase activities is shown in figure 2. Maximum activity for the former was obtained using tris buffer at pH 7.1. Hydroxyla-

TABLE 1

Summary of purification of Azotobacter nitrite and hydroxylamine reductases

Fraction	Nitrite Reductase				Hydroxylamine Reductase			
	Total	Total protein	Specific activity	Recovery	Total	Total protein	Specific activity	Recovery
	units	*mg*	*units/mg protein*	%	*units*	*mg*	*units/mg protein*	%
1. Crude extract	5,300	376	14.2		8,900	727	12.2	
2. Supernatant solution after high speed centrifugation	3,600	264	13.8	67.5				
3. Al Cγ gel eluate	4,850	114	42.6	91.0	5,300	240	22.1	59.5

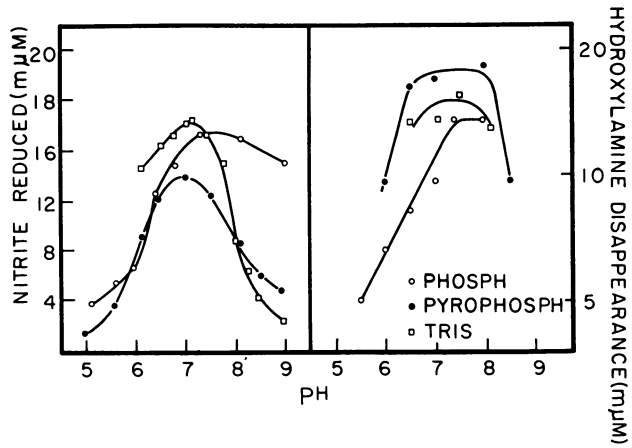

Figure 2. Effect of pH on nitrite reductase and hydroxylamine reductase. All buffers present in final concentrations of 0.05 M. Seventeen units of nitrite reductase and 20 units of hydroxylamine reductase.

mine reductase activity, however, showed a broad pH optimum in the range of pH 6.5 to 8.0.

Substrate saturation. The relationship between nitrite and hydroxylamine concentrations and reductase activities are shown in figure 3. From the reciprocal plot (Lineweaver and Burk, 1934) of these data the dissociation constants for the nitrite and hydroxylamine enzyme complexes were calculated to be 6.3×10^{-5} M and 4.8×10^{-5} M respectively. These two enzymes have a relatively high affinity for substrates.

Reduced pyridine nucleotides as electron donors. The stimulatory effect of TPNH on the rates of nitrite and hydroxylamine reductions is shown in figure 4. A small amount of reduction occurred in the absence of added pyridine nucleotide but the rates of reduction were increased 2- to 7-fold in its presence.

The extremely active DPNH and TPNH oxidases of the Azotobacter preparations compete strongly for reduced pyridine nucleotide, and for this reason TPNH was continuously regenerated in the assay system by means of isocitric dehydrogenase or glucose-6-phosphate dehydrogenase. DPNH, regenerated by the glucose-6-phosphate dehydrogenase system, was also effective as an electron donor for both nitrite and hydroxylamine reductases. In view of this interfering oxidase-activity it was not possible to do stoichiometric or kinetic studies with reduced pyridine nucleotides, or to compare the relative effectiveness of DPNH and TPNH as electron donors.

Requirement for flavin. The enzymatic reduction of nitrite by Azotobacter extracts was stimulated 2- to 3-fold by the addition of a boiled extract of acetone powder of pig heart. This stimulatory effect could be replaced completely by FAD at 4×10^{-6} M final concentration. At optimum FAD concentration the addition of boiled pig heart extract gave no additional stimulation. Hydroxylamine reductase from Azotobacter was also stimulated 3- to 4-fold by added FAD at 8×10^{-6}

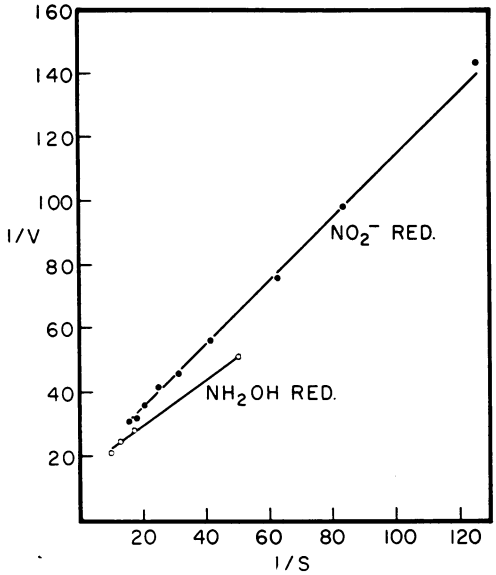

Figure 3. Effect of nitrite and hydroxylamine concentrations on the activities of nitrite reductase and hydroxylamine reductase, respectively. Standard assay conditions with 32 units of nitrite reductase and 36 units of hydroxylamine reductase. Lineweaver-Burk (1934) plot of data. S, substrate concentrations in millimoles per L. V, velocity of reaction (nitrite disappearance in millimoles per L, per 5 min; hydroxylamine disappearance millimoles per L per 7 min.)

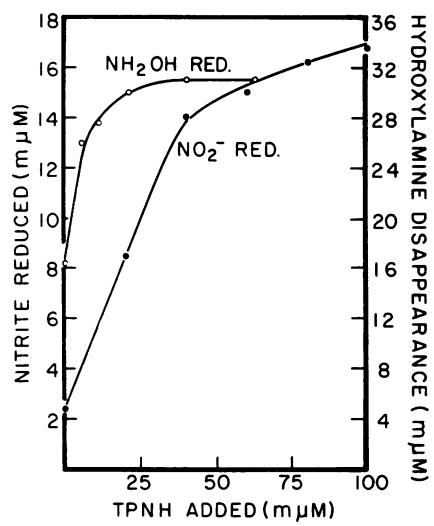

Figure 4. Effect of added reduced triphosphopyridine nucleotide (TPNH) on the activity of nitrite and hydroxylamine reductases. Conditions as in standard assay with 17 units of nitrite reductase and 32 units of hydroxylamine reductase except for varying TPNH concentrations.

M or by FMN at 4×10^{-6} M. The effect of a range of FAD and FMN concentrations on the nitrite and hydroxylamine reductases is shown in figure 5. FMN only slightly stimulated nitrite reductase, but resulted in a marked increase in hydroxylamine disappearance.

Inhibitors and activators. The effect of a range of inhibitors on nitrite and hydroxylamine reductase activities is shown in table 2. Sensitivity to metal binding reagents such as cyanide, versene, and 8-hydroxyquinoline suggests the essentiality of a metal component for both nitrite and hydroxylamine reductase activities. The identification of the essential metal component of nitrite reductase has not yet been made. The enzyme could be inactivated by dialysis against 10^{-4} M KCN in 0.1 M K_2HPO_4, pH 9.0, containing glutathione (10^{-3} M) in a pre-soaked dialysis sac. Removal of cyanide by subsequent dialysis gave an extract with greatly lowered activity compared to that of a non-cyanide treated dialyzed control. However, the activity of the enzyme was not restored by preincubation with salts of Fe^{++}, Fe^{+++}, Cu^{++}, Mn^{++}, Zn^{++}, $MoO_4^=$, $WO_4^=$, $BO_3^=$, Co^{++}, Ni^{++}, and Mg^{++} singly or in various combinations at 10^{-5} to 10^{-3} M final concentrations.

Cu^+ and Cu^{++} salts at approximately 10^{-4} M were found to cause a non-enzymatic loss of nitrite under the assay conditions. This reaction was found to require the presence of protein but active enzyme could be replaced by boiled enzyme or casein. At 10^{-7} to 10^{-6} M copper salts inhibited nitrite reductase activity.

The marked inhibition of nitrite reductase with 10^{-4} M *p*-chloromercuribenzoate and its partial reversal by glutathione (approximately 50 per cent) indicates the importance of sulfhydryl groups for enzyme.

The lack of inhibition of nitrite reductase by allylthiourea and hydrazine is of interest since these compounds were found by Hofman and Lees (1952) to be selective inhibitors of the stepwise oxidation of ammonia to nitrite via hydroxylamine in Nitrosomonas extracts.

Inhibition of hydroxylamine reductase activity by versene was overcome by the addition of excess Mn^{++}, and furthermore the addition of Mn^{++} to the preparation caused a marked stimulation of hydroxylamine reductase activity. The only effective metallic ion thus far tested was Mn^{++}, as shown in table 3. The dissociation constant (Km) of the enzyme-Mn complex, as estimated

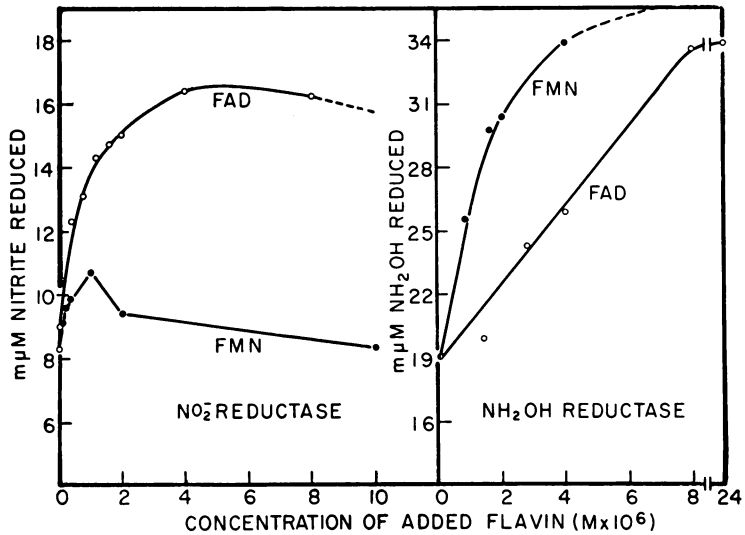

Figure 5. Effect of added flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN) on nitrite and hydroxylamine reductases. Conditions as standard assay with 16 units of nitrite reductase and 34 units of hydroxylamine reductases except for varying FAD and FMN concentrations.

from the saturation curve (figure 6) is approximately 4×10^{-5} M.

Since the glucose-6-phosphate dehydrogenase activity of Azotobacter extract was stimulated only 20 per cent by the addition of Mn^{++} as measured by TPNH formation, it is clear that Mn^{++} is involved in hydroxylamine reductase from Azotobacter. The nitrite reductase from Azotobacter was not influenced by Mn^{++}.

It was observed that the NH_2OH reductase system was stimulated 25 to 55 per cent when the reaction was conducted aerobically. There is no phosphate requirement for this system.

Products of nitrite and hydroxylamine reduction. The disappearance of nitrite from the assay system was accompanied by ammonia formation. The identification of the product as ammonia depends on its volatility at alkaline pH and the characteristic color formed with Nessler's reagent. Table 4 shows that for each molecule of nitrite reduced one molecule of ammonia was formed.

Thus far it has not been possible to consistently demonstrate the formation of significant amounts of ammonia concomitant with the enzymatic disappearance of hydroxylamine. The insignificantly small quantities of hydroxamic acid formed in the reaction mixture cannot account for the disappearance of the relatively large quantities of hydroxylamine. Also the disappearance of hydroxylamine cannot be ascribed to oxime formation, since the latter is determined by the same method as that for hydroxylamine. The lack of nitrite formation from hydroxylamine in this system (as well as the requirement for reduced pyridine nucleotides) also eliminates the oxidation of hydroxylamine as a possibility. Finally, the quantitative recovery of added ammonia in this mixture rules out the possibility that the failure to observe ammonia formation can be ascribed to its utilization in other enzyme systems. The enzymatic reduction product of hydroxylamine with the Azotobacter enzyme, therefore, has not yet been identified.

Adaptation experiments. In order to test whether nitrite and hydroxylamine reductases are adaptive enzymes, Azotobacter cells were grown in media in which sodium glutamate, ammonium sulfate, or N_2 replaced KNO_3 as the sole nitrogen source. The rates of growth in these different media were comparable (approximately 100 to 150 Klett units with a red filter after culture for 24 hr). Extracts were prepared from these cells in the usual manner. The results (table 5) showed that the formation of nitrite reductase was markedly stimulated in the presence of KNO_3. The crude cell free extracts, as well as the purified fraction from N_2 or glutamate grown

TABLE 2

Effect of inhibitors on nitrite and hydroxylamine reductases from Azotobacter agile

Compound	Concentration Final Molarity	Per Cent Inhibition	
		Nitrite reductase	Hydroxylamine reductase
KCN	10^{-2}		100
	2×10^{-3}		13
	10^{-3}	100	7
	10^{-4}	100	
	10^{-5}	100	
	10^{-6}	70	
Versene	10^{-3}	0	64
8-Hydroxyquinoline	10^{-3}		45
	10^{-4}	29	
Thiourea	10^{-3}	7	0
p-Chloromercuribenzoate	10^{-3}		15
	10^{-4}	100	
Hydrazine	10^{-3}	0	0
Allylthiourea	10^{-4}	0	

The above inhibitors were preincubated with the enzyme for approximately 2 to 3 min before the start of the reaction, except for *p*-chloromercuribenzoate which was preincubated for 10 min. 15 to 20 units of nitrite reductase and 20 to 35 units of hydroxylamine reductase were used in the standard assay.

TABLE 3

Effect of metals on hydroxylamine reductase

Metal Added (8×10^{-4} M)	NH_2OH Disappeared (mμmoles/15′)
Control	1.28
plus $MnCl_2$	28.6
$MgSO_4$	1.5
Na_2MoO_4	1.5
$ZnSO_4$	0
$CoSO_4$	11.1*
$CuCl_2$	31.2*
$FeSO_4$	0
Na_2WO_4	3.8

19 units of hydroxylamine reductase were used in the standard assay.

* Non-enzymatic.

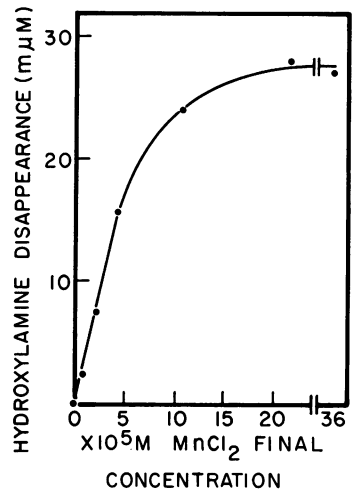

Figure 6. Effect of Mn^{++} on hydroxylamine reductase. Twenty-eight units of enzymes used in the standard assay.

TABLE 4

Relationship between nitrite disappearance and ammonia formation in the nitrite reductase reaction

Time of Incubation	Nitrite Lost	Ammonia Formed	NH_3/NO_2^-
min	*μmoles*	μmoles	
0	0	0	0
20	1.07	0.99	0.93
40	1.92	1.64	0.86
75	2.26	2.07	0.92

Ammonia was determined as described in Materials and Methods. Nitrite was determined in duplicate 0.05-ml aliquots of the reaction mixture. 535 units of enzyme were used in a 3-ml reaction mixture with components in the same proportion as in standard assay.

cells effected only a small disappearance of nitrite.

The formation of hydroxylamine reductase was stimulated 2- or 3-fold by KNO_3, as shown in table 6.

Effect of aging and nitrogen source on nitrite and hydroxylamine reductases. Nitrite and hydroxylamine reductases in the crude extracts from nitrate grown cells could be stored for at least 2 days at 0 C without appreciable loss of activities. Hydroxylamine reductase activity in the crude extracts from the cells grown on other nitrogen sources was also stable under similar conditions,

TABLE 5

The effect of nitrogen source on the formation of nitrite reductase

Fraction	Nitrogen Source	Enzyme Units or Nitrite Reduced/ 0.05 ml Enzyme	Protein	Specific Activity
		(mμmoles /10′)	mg/ml	
Crude cell free extract	N_2	1.6	14.9	2.2
	Glutamate	2.9	15.6	3.7
	KNO_3	11.9	13.1	18.2
Gel eluate	N_2	2.9	9.4	6.2
	Glutamate	3.0	10.1	6.0
	KNO_3	15.1	10.2	29.6

Standard conditions of assay were used.

TABLE 6

The effect of nitrogen source on the formation of hydroxylamine reductase

Fraction	Nitrogen Source	Enzyme Units of Hydroxylamine Reduced/ 0.05 ml Enzyme	Protein	Specific Activity	
				Expt. 1	Expt. 2
		mμmoles/ 10′	mg/ ml		
Crude cell free extracts	N_2	21.3	23.6	18.1	16.8
	Glutamate	10.3	19.5	10.5	15.8
	$(NH_4)_2SO_4$	14.7	29.4	10.0	
	KNO_3	50.0	29.0	34.5	47.5

Standard conditions of assay were used.

TABLE 7

Effect of aging and nitrogen source on nitrite and hydroxylamine reductases from Azotobacter agile

Treatment	Units of mμmoles NO_2^- or NH_2OH Reduced per mg of Protein per 10′			
	KNO_3	N_2	$(NH_4)_2SO_4$	Glutamate
NO_2^- reductase				
Fresh	16.3	3.6	5.1	5.0
1 day old	19.8	0	1.7	7.9
2 day old	13.0		0.9	0
NH_2OH reductase				
Fresh	34.5	16.7	10.0	
1 day old	29.2		5.9	
2 day old	31.0	12.6	12.1	

Crude extracts stored for indicated time at 0 C. Standard conditions of assay were used.

whereas nitrite reductase activity in the crude extracts from N_2, ammonium sulfate, and glutamate grown cells was very labile (table 7). The addition of the crude extracts from nitrate grown cells (intact or boiled) did not stabilize the labile system. Nor did the extracts containing the labile nitrite reductase inhibit the activity of the extracts from nitrate grown *cells*.

DISCUSSION

Nitrite reductase from Azotobacter closely resembles that from Neurospora (Nason *et al.* 1954) with respect to electron donors, FAD requirements, and sensitivity to various metal binding agents. On the other hand, the hydroxylamine enzyme is more similar to that of soybean leaves (Nason *et al.* 1954), since manganese serves as an activator and either FAD or FMN meets the flavin requirement. The reduction product of the hydroxylamine reductase from Azotobacter has yet to be established, since ammonia is not formed.

Further evidence is needed in order to determine whether these systems constitute the main path of nitrate and nitrite assimilation on the intact Azotobacter cell. The quantitative recovery of an equivalent amount of ammonia as the product of nitrite reduction indicates that in these partially purified extracts no other major mechanisms for nitrite metabolism were functioning. The simultaneous adaptive formation of nitrite reductase, and additional hydroxylamine reductase in the presence of nitrate, also suggest their potential physiological importance in nitrate assimilation.

If one assumes that the reduction of nitrite proceeds by a series of two electron transfers it will be seen that two intermediate compounds must occur between nitrite (+3 oxidation state of N) and ammonia (−3 oxidation state of N). One of these would be on the oxidation level of hyponitrous acid (+1) and the other on that of hydroxylamine (−1). It is clear that in Azotobacter the hydroxylamine reductase is a distinct enzyme from nitrite reductase, as shown by the Mn^{++} requirement, inhibition experiments, stability in crude extracts, and the pH curves. The adaptive formation of hydroxylamine reductase in the presence of nitrate suggests that it may play some role in nitrate assimilation. The nature of the compound at the oxidation level of hyponitrous acid is unknown.

The inability of N_2, when acting as nitrogen

source for growth of Azotobacter to induce the formation of large amounts of nitrite and hydroxylamine reductases is of interest with respect to the mechanism of nitrogen fixation. Moreover, nitrite was never detected in the cells or in the growth medium of these cells. The results suggest that nitrite and hydroxylamine are not intermediate products of the nitrogen fixation reaction. This would constitute evidence against the possibility that N_2 is first oxidized to nitrite or nitrate and then reduced to ammonia via the pathway of nitrate assimilation. Suzuki and Suzuki (1954) observed that the hydroxylamine reduction activity of the whole cells of *Azotobacter agile* could be induced by nitrate but not by N_2, and came to the conclusion that the hydroxylamine reducing system is not involved in nitrogen fixation in Azotobacter.

Higgins *et al.* (1956) have reported that tungstate is a dietary antagonist of molybdate in animal nutrition, and a competitive inhibitor of molybdate in *Aspergillus niger* when nitrate is the sole nitrogen source. It has already been shown that molybdenum is required in the nutrient medium for the enzymatic reduction of nitrate, and that it is the metal component of nitrate reductase from Neurospora (Nicholas *et al.*, 1954; Nicholas and Nason, 1954*a*, 1954*b*), from soybean leaves (Nicholas and Nason, 1955a, Evans and Hall, 1955), and probably from *Escherichia coli* (Nicholas and Nason, 1955*b*). It has recently been possible to demonstrate that tungstate also acts as a competitive inhibitor of molybdate in the growth of Azotobacter when nitrate or N_2 is the sole nitrogen source. (Takahashi and Nason, 1957). These results suggest that a molybdenum enzyme, probably similar to that characterized in Neurospora and soybean leaves, is involved in nitrate reduction of Azotobacter. These data also point to a molybdenum system for N_2 fixation in Azotobacter, in support of the reported stimulatory action of molybdenum first indicated by Bortels (1930) and since observed by a number of other investigators.

SUMMARY

The properties of a soluble nitrite and hydroxylamine reductase system found in extracts of *Azotobacter agile* (*A. vinelandii*) are described. The enzymes utilize reduced pyridine nucleotides as electron donors and require added flavin for maximal activity, flavin adenine dinucleotide specifically in the case of nitrite reductase, and flavin adenine dinucleotide or the mononucleotide with the hydroxylamine enzyme. Inhibitor studies indicate that the systems have an essential metal component. Mn^{++} was demonstrated to be a specific activator of hydroxylamine reductase. The dissociation constants of enzyme nitrite and enzyme hydroxylamine complexes were calculated to be 6.3×10^{-4} M and 4.8×10^{-4} M respectively. The product of the reduction of nitrite by these extracts was identified as ammonia. Approximately one molecule of ammonia is formed for each mole of nitrite utilized. The enzyme has a pH optimum at 7.1 in tris buffer. The product of hydroxylamine reduction by these extracts was not identified. The pH optimum for the enzyme ranges from 6.5 to 8.0. Nitrite and hydroxylamine reductases are adaptive enzymes whose formation is stimulated by nitrate but not N_2, ammonium sulfate, and glutamate. The results indicate that these enzymes play a role in nitrate and nitrite assimilation but not in nitrogen fixation.

REFERENCES

BORTELS, H. 1930 Molybdän als Katalysator bei der biologischen Stickstoffbindung. Arch. Mikrobiol., **1**, 333–342.

BURRIS, R. H. 1956 Studies on the mechanism of biological nitrogen fixation. In *Symposium on Inorganic Nitrogen Metabolism*, pp. 316–343, edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.

CSÁKY, T. Z. 1948 On the estimation of bound hydroxylamine in biological materials. Acta chem. scand., **2**, 450–454.

EVANS, H. J. AND HALL, N. S. 1955 Association of molybdenum with nitrate reductase from soy bean leaves. Science, **122**, 922–923.

GEST, H., JUDIS, J., AND PECK, H. D., JR. 1956 Reduction of molecular nitrogen and relationships with photosynthesis and hydrogen metabolism. In *Symposium on Inorganic Nitrogen Metabolism*, pp. 298–315, edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.

HIGGINS, E. S., RICHERT, D. A., AND WESTERFELD, W. W. 1956 Tungstate antagonism of molybdate in *Aspergillus niger*. Proc. Soc. Exptl. Biol. Med., **92**, 509–511.

HOFMAN, T. AND LEES, H. 1952 The intermediary metabolism of *Nitrosomonas*. Biochem. J. (London), **52**, No. 4, xx.

KAPLAN, N. O., COLOWICK, S. P., AND NEUFELD, E. F. 1952 Pyridine nucleotide transhydrogenase. II. Direct evidence for and mechanism of the transhydrogenase reaction. J. Biol. Chem., **195,** 107–119.

LINEWEAVER, H. AND BURK, D. 1934 The determination of enzyme dissociation constants. J. Am. Chem. Soc., **56,** 658–667.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193,** 265–275.

MCELROY, W. D. AND SPENCER, D. 1956 Normal pathways of assimilation of nitrate and nitrite. In *Symposium on Inorganic Nitrogen Metabolism*, pp. 137–152, edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.

NASON, A. 1956 Enzymatic steps on the assimilation of nitrate and nitrite in fungi and green plants. In *Symposium on Inorganic Nitrogen Metabolism*, pp. 109–136, edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.

NASON, A., ABRAHAM, R. A., AND AVERBACH, B. C. 1954 The enzymatic reduction of nitrite to ammonia by reduced pyridine nucleotides. Biochim. et Biophys. Acta., **15,** 160–161.

NASON, A. AND EVANS, H. J. 1953 Triphosphopyridine nucleotide nitrate reductase in Neurospora. J. Biol. Chem., **202,** 655–674.

NEWTON, J. W., WILSON, P. W., AND BURRIS, R. H. 1953 Direct demonstration of ammonia as an intermediate in nitrogen fixation by Azotobacter. J. Biol. Chem., **204,** 445–451.

NICHOLAS, D. J. D. AND NASON, A. 1954*a* Molybdenum and nitrate reductase. II. Molybdenum as a constituent of nitrate reductase. J. Biol. Chem., **207,** 353–360.

NICHOLAS, D. J. D. AND NASON, A. 1954*b* Mechanism of action of nitrate reductase from Neurospora. J. Biol. Chem., **211,** 183–197.

NICHOLAS, D. J. D. AND NASON, A. 1955*a* Role of molybdenum as a constituent of nitrate reductase from soy-bean leaves. Plant Physiol., **30,** 135–138.

NICHOLAS, D. J. D. AND NASON, A. 1955*b* Diphosphopyridine nucleotide-nitrate reductase from *Escherichia coli*. J. Bacteriol., **69,** 580–583.

NICHOLAS, D. J. D., NASON, A., AND MCELROY, W. D. 1954 Molybdenum and nitrate reductase. I. Effect of molybdenum deficiency on the Neurospora enzyme. J. Biol. Chem., **207,** 341–351.

SHUG, A. L., HAMILTON, P. B., AND WILSON, P. W. 1956 Hydrogenase and nitrogen fixation. In *Symposium on Inorganic Nitrogen Metabolism*, pp. 344–360, edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.

SUZUKI, N. AND SUZUKI, S. 1954 Hydroxylamine reduction and hydrazine oxidation by *Azotobacter vinelandii*. Science Repts. Tohoku Univ. Fourth Ser., **20,** 195–201.

TAKAHASHI, H. AND NASON, A. 1957 Tungstate as a competitive inhibitor of molybdate in nitrate assimilation and in N_2 fixation by Azotobacter. Biochim. et Biophys. Acta, **23,** 433–435.

TANIGUCHI, S., SATO, R., AND EGAMI, F. 1956 The enzymatic mechanisms of nitrate and nitrite metabolism in bacteria. In *Symposium on Inorganic Metabolism*, pp. 87–108, edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.

VANSELOW, A. P. 1940 Preparation of Nessler's reagent. Ind. Eng. Chem. Anal. Ed., **12,** 516–517.

VERHOEVEN, W. 1956 Some remarks on nitrate metabolism in microorganisms. In *Symposium on Inorganic Nitrogen Metabolism*, pp. 61–86, edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.

WILSON, P. W. 1952 The comparative biochemistry of nitrogen fixation. Advances in Enzymol., **13,** 345–375.

ZUCKER, M. AND NASON, A. 1955 A pyridine nucleotide-hydroxylamine reductase from Neurospora. J. Biol. Chem., **213,** 463–478.