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Nitrite Reductase of *Escherichia coli* Specific for Reduced Nicotinamide Adenine Dinucleotide

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ABSTRACT

KEMP, JOHN D. (University of California, Los Angeles), AND DANIEL E. ATKINSON. Nitrite reductase of *Escherichia coli* specific for reduced nicotinamide adenine dinucleotide. J. Bacteriol. 92:628–634. 1966.—A nitrite reductase specific for reduced nicotinamide adenine dinucleotide ($NADH_2$) appears to be responsible for in vivo nitrite reduction by *Escherichia coli* strain Bn. In extracts, the reduction product is ammonium, and the ratio of $NADH_2$ oxidized to nitrite reduced or to ammonium produced is 3. The Michaelis constant for nitrite is 10 μM. The enzyme is induced by nitrite, and the ability of intact cells to reduce nitrite parallels the level of $NADH_2$-specific nitrite reductase activity demonstrable in cell-free preparations. Crude extracts of strain Bn will also reduce hydroxylamine, but not nitrate or sulfite, at the expense of $NADH_2$. Kinetic observations indicate that hydroxylamine and nitrite may both be reduced at the same active site. The high apparent Michaelis constant for hydroxylamine (1.5 mM), however, seems to exclude hydroxylamine as an intermediate in nitrite reduction. In vitro activity is enhanced by preincubation with nitrite, and decreased by preincubation with $NADH_2$.

Cell-free extracts of nitrate-grown *Escherichia coli* strain Bn contain at least three systems that catalyze the reduction of nitrite at the expense of three different reducing agents. One is specific for reduced nicotinamide adenine dinucleotide ($NADH_2$), one for reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), and the third, which apparently involves a cytochrome, oxidizes flavin or viologens (4; Lazzarini, Ph.D. Thesis, Univ. California, Los Angeles, 1960). After the $NADPH_2$-specific enzyme had been partially purified and some features of its catalysis of nitrite reduction studied, it was found also to catalyze the reduction of sulfite (4). Kinetic evidence suggests that the same catalytic site is responsible for the reductions of both substrates (3). However, the presence of this enzyme was found to be correlated only with the ability to utilize sulfite as sulfur source, and not with the ability to grow with nitrate or nitrite as nitrogen source. Its repression by cysteine or sulfide also supports the conclusion that the $NADPH_2$-specific enzyme functions in the intact cell exclusively as sulfite reductase (3).

This paper reports results indicating that the $NADH_2$-specific enzyme is responsible for physiological nitrite reduction.

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MATERIALS AND METHODS

E. coli strain Bn (6) was grown on minimal medium previously described (7) containing 1% glucose and either nitrate or ammonium ion at 3 mM as the sole sources of carbon and nitrogen. The cultures were incubated in deep bottles at 35 C without aeration and were harvested after 14 hr at approximately maximal turbidity. Washed cells were resuspended at about four times the culture density for use in intact cell studies. Extracts were obtained by disrupting the cells for 2 min in a Branson sonifier, and by then centrifuging the resulting preparation at 144,000 $\times$ g for 60 to 90 min. Centrifugation removes the flavin-specific activity and also about 95% of the $NADH_2$ oxidase activity, which in the crude sonic-treated extract oxidizes $NADH_2$ much faster than does the nitrite reductase. Activity was assayed by following $NADH_2$ concentration spectrophotometrically at 340 mμ with a Beckman model B spectrophotometer attached to a Sargent SRL recorder. One unit of activity corresponds to the oxidation of 1 μmole of $NADH_2$ per min (corrected for the rate of $NADH_2$ oxidation in the absence of nitrite). The standard assay mixture contained, in 1 ml: pyrophosphate buffer (pH 8.0), 35 μmoles; $NADH_2$, 0.24 μmole; KNO_2, 2.0 μmoles; and enzyme preparation containing 0.5 to 1.0 mg of soluble protein. All results reported were obtained under conditions in which rate

had been shown to be proportional to enzyme concentration.

Nitrite was measured by a modification (10) of the Griess method. Ammonia was separated by diffusion in a Conway dish and estimated by the phenol-hypochlorite reaction as described by Russell (8).

RESULTS

Nitrite reduction in intact cells. As previously reported (6), *E. coli* strain Bn can utilize either nitrate or nitrite as the sole source of nitrogen for growth. When nitrate is used, the concentration of nitrite in the medium typically increases to about 50 to 60% of the initial level of nitrate and then decreases to zero. Growth does not begin until a significant amount of nitrite has accumulated (6). When washed cells of strain Bn, grown on nitrate, were resuspended in a basal medium containing 1 mM nitrate, the concentration of nitrite varied with time, as shown in Fig. 1 (closed circles). The turbidity of the resuspended cells remained constant during the course of an experiment, suggesting that the cells did not multiply. When nitrite was substituted for nitrate in a similar experiment, the concentration of nitrite in the medium decreased with time (Fig. 1, open circles). These results are consistent with the assumption that free nitrite is an intermediate in nitrate reduction. Free ammonium ion does not accumulate in the medium during the reduction by intact cells of either nitrate or nitrite.

The linearity of the rate of nitrite reduction by nitrate-grown cells suggests that the enzyme system responsible for catalyzing this reduction is present at a constant level within the cells throughout the experiment. Cells grown in the presence of ammonium as the sole source of nitrogen, when resuspended under conditions identical to those in Fig. 1, reduce nitrite only after a significant lag period (Fig. 2A). An obvious explanation for this result is that ammonium-grown cells lack nitrite reductase, but can synthesize it when resuspended in the presence of nitrite; that is, nitrite reductase may be either repressed by ammonium or induced by nitrite. When cells of strain Bn were grown in the presence of 3 mM ammonium ion and 1 mM nitrite, they reduced nitrite with no lag (Fig. 2B), indicating that nitrite induces enzyme formation. In support of this conclusion, the time course of nitrite reduction by ammonium-grown cells was identical (and similar to that shown by the corresponding curve of Fig. 2A) whether or not ammonium was present in the resuspension medium along with nitrite (J. M. Schneider, M.S. Thesis, Univ. California, Los Angeles, 1964).

Induction of the enzyme. The NADH$_2$-specific nitrite reductase, observed earlier in cell-free homogenates of strain Bn (4; Lazzarini, Ph.D. Thesis, Univ. California, Los Angeles, 1960), was present in sonic-treated material of strain Bn grown in the presence of nitrate, but absent in similar extracts of cells grown in the presence of ammonium as sole nitrogen source. Cells grown in medium containing both ammonium and nitrite produced the enzyme. This demonstration, that the addition of nitrite results both in induction of the NADH$_2$-specific nitrite

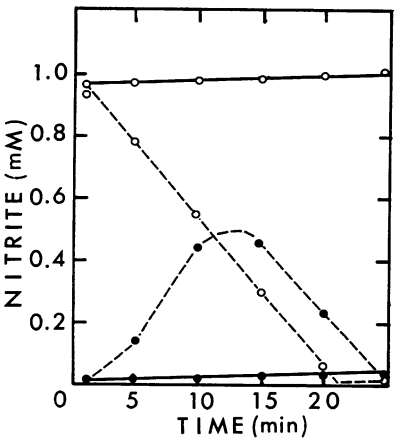

FIG. 1. *Reduction of nitrate and nitrite by intact cells. Escherichia coli, strain Bn, was grown in a medium containing 3 mM nitrate as the sole source of nitrogen. Washed cells were resuspended at 30 C in a solution containing the mineral components of the medium. Solid line, no glucose; broken line, 1% glucose. Closed circles, 1 mM nitrate; open circles, 1 mM nitrite.*

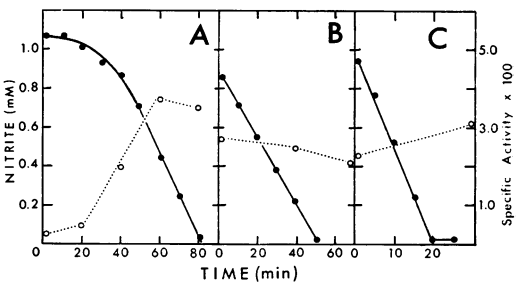

FIG. 2. *Comparison of the rate of reduction of nitrite by intact cells with the level of NADH$_2$-specific nitrite reductase present in extracts. Nitrogen sources for growth were: A, 3 mM NH_4^+; B, 3 mM NH_4^+ plus 1 mM NO_2^-; C, 3 mM NO_3^-. Washed cells were resuspended in medium containing 1 mM NO_2^- and 1% glucose. Solid lines indicate the concentration of nitrite in the medium (left-hand scale); broken lines indicate the level of NADH$_2$-specific nitrite reductase activity in cell extracts (right-hand scale).*

reductase and in the capacity of intact cells to reduce nitrite, strongly suggests that the $NADH_2$-specific enzyme is responsible for physiological nitrite reduction. Such a suggestion is strengthened by the observation that cells of strain Bn, grown in the presence of ammonium ions and resuspended in medium containing nitrite, produced the $NADH_2$-specific nitrite reductase activity (as indicated by assay in cell-free extracts) at a rate approximately parallel to the increase in ability of intact cells to reduce nitrite (Fig. 2A). When cells of strain Bn grown in the presence of nitrate or nitrite, with or without ammonium, were resuspended, the $NADH_2$-specific enzyme activity was present at high levels throughout the experiment (Fig. 2B and 2C). Washed and resuspended ammonia-grown cells of strain Bn synthesized nitrite reductase only when both nitrite and glucose were present (Fig. 3).

E. coli strain ATCC 10586 is unable to grow with nitrite or nitrate as sole nitrogen source. A suspension of cells of this strain, grown in a medium containing ammonium ions, did not reduce nitrite in the presence of glucose even after an incubation period of 80 min, about three times the induction period for strain Bn. No $NADH_2$-specific nitrite reductase activity could be detected when the incubated cells were disrupted.

Stability of the reductase. The $NADH_2$-specific nitrite reductase activity is unstable in centrifuged crude cell-free extracts. As much as 25% of the activity is typically lost in 6 hr when the preparation is kept at 2 C; 50% of the activity may be lost in 24 hr when frozen at −15 C. If a preparation is frozen in the presence of 1 mM nitrite, it retains most of its activity for at least 2 weeks.

Substrate and inhibitor effects. The $NADH_2$-specific nitrite reductase catalyzes the reduction of hydroxylamine, but not of nitrate or sulfite. The reduction of nitrite is not affected by 2 mM nitrate, but is totally inhibited by the same concentration of sulfite. The effects of sulfite are approximately those to be expected of a competitive inhibitor (Fig. 4) with a K_i of 30 μM. The reduction of nitrite and the oxidation of $NADH_2$ both appear to follow simple Michaelis-Menton kinetics with a K_m for nitrite of 10 μM (Fig. 4) and with a K_m for $NADH_2$ of 18 μM.

The apparent K_m for hydroxylamine at *p*H 8 was estimated at 1.5 mM, over 100 times the K_m for nitrite. The maximal velocity for hydroxylamine reduction is about 1.5 times the maximal velocity for nitrite reduction at *p*H 8.0. In the presence of a saturating level of hydroxylamine (10 mM), $NADH_2$ was oxidized in a standard assay at a rate of 0.63 μmole per min per ml of extract; at a saturating level of nitrite (0.1 mM), the rate of $NADH_2$ oxidation was 0.35 in the same units. When hydroxylamine at a concentration of 10 mM plus nitrite at a concentration of 0.1 mM were present together in a standard assay, the rate of oxidation of $NADH_2$ was 0.50 μmole per min per ml of extract, approximately midway between the rates observed with the individual substrates. This result suggests that hydroxylamine and nitrite may be reduced at the same catalytic site and that, as expected, they compete about equally when the ratio of their concentrations approximates the ratio of their Michaelis constants.

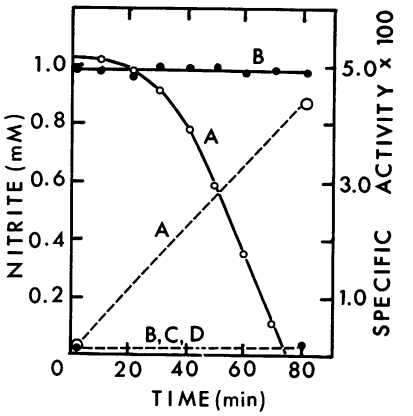

FIG. 3. *Effect of glucose on induction of $NADH_2$ nitrite reductase. Washed ammonium-grown cells of Escherichia coli, strain Bn, were suspended at 30 C in medium containing: A, 1 mM NO_2^-, 1% glucose; B, 1 mM NO_2^-, no glucose; C, no nitrite, 1% glucose; D, no nitrite or glucose. Solid lines indicate the concentration of nitrite in the medium (left-hand scale); broken lines indicate the level of $NADH_2$-specific nitrite reductase activity in cell extracts (right-hand scale).*

The rate of oxidation of $NADH_2$ by nitrite reductase typically increased by 50 to 70% during the 1st min of the assay. The initial increase in rate is unexplained; it was shown not to result from change in temperature and not to be affected by changes in the ratio of concentrations of nitrite and $NADH_2$ or by additions of the products, ammonium ion and NAD^+. After the first 60 to 80 sec, the reaction was linear with time for many minutes. Thus, the enzyme is neither inactivated nor further activated under assay conditions, when both $NADH_2$ and nitrite are necessarily present. (All rates reported were obtained by measuring slopes of the linear portions of the recorder plots.) However, preincubation with nitrite in the absence of $NADH_2$ caused a two- to fourfold increase in the rate of

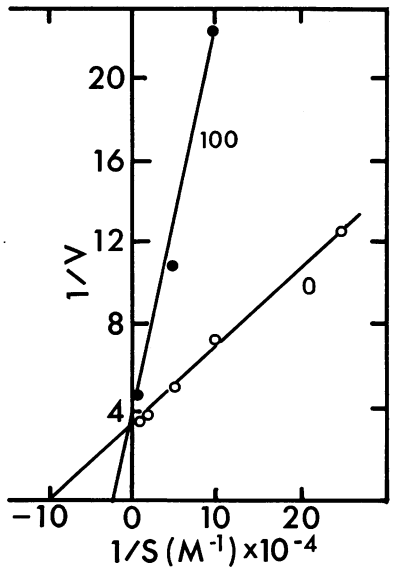

FIG. 4. *Effect of sulfite on nitrite reduction. Assay mixtures contained 35 mM pyrophosphate (pH 8.0); 0.24 mM NADH$_2$; nitrite as indicated on the abscissa; sulfite at 0 or 100 μM as indicated by figures on the lines; and 0.046 units of NADH$_2$ nitrite reductase activity from nitrate-grown cells. Assay volume, 1 ml. The reaction was started by addition of enzyme.*

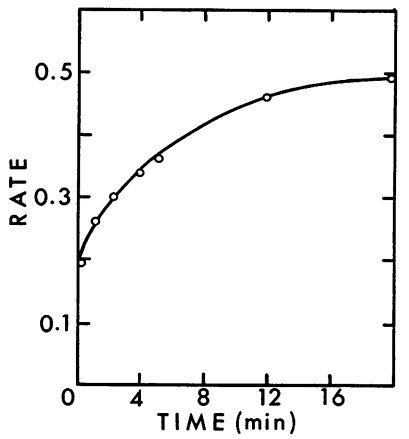

FIG. 5. *Effect of preincubation with nitrite on NADH$_2$ nitrite reductase activity. Assay mixtures containing 35 mM pyrophosphate (pH 8.0); 2 mM nitrite; and crude enzyme preparation containing 0.6 mg of protein were preincubated at room temperature for the times indicated on the abscissa before the addition of NADH$_2$ to a concentration of 0.24 mM to start the reaction. Assay volume, 1 ml. Rate is expressed as ΔA_{340}/min.*

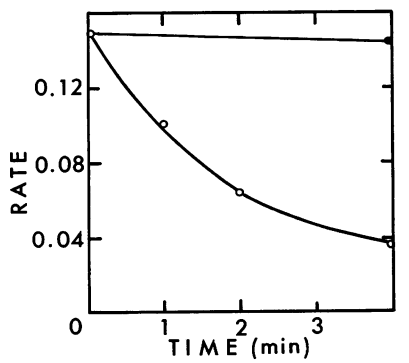

FIG. 6. *Effect of preincubation with NADH$_2$ on NADH$_2$ nitrite reductase activity. Assay mixtures containing 35 mM pyrophosphate (pH 8.0) and crude enzyme preparation containing 0.6 mg of protein were incubated at room temperature with: open circles, 0.24 mM NADH$_2$; closed circle, 0.24 mM NADPH$_2$. At the times indicated on the abscissa, the reaction was started by the addition of nitrite to a concentration of 2 mM. The mixture preincubated with NADPH$_2$ received NADH$_2$ to a concentration of 0.24 mM simultaneously with the addition of nitrite. Assay volume, 1 ml. Rate is expressed as ΔA_{340}/min.*

the reaction, and preincubation with NADH$_2$ in the absence of nitrite inactivated the enzyme. The activation curve resembles the course of a first-order reaction, with a half-time of about 3 to 4 min (Fig. 5). Preincubation of the enzyme (diluted to assay concentration) with buffer alone sometimes causes some degree of activation, but never as great as with nitrite. Preincubation with NADH$_2$ in the absence of nitrite inactivates the enzyme (Fig. 6). Inactivation seems to be specific for NADH$_2$; NADPH$_2$, for example, is ineffective.

The ratio of activities of the control preparation and one preincubated with nitrite remains constant over the *p*H range used (Fig. 7) with the optimum for both preparations at about 7.5. Preincubation with nitrite affects only the V_{max} of the reaction; the K_m values for both nitrite and NADH$_2$ are unchanged.

Stoichiometry of the enzyme reaction. Measurements of the quantity of nitrite necessary to oxidize given amounts of NADH$_2$ are complicated by the fact that NADH$_2$ interferes with the nitrite assay. Therefore, limiting amounts of NADH$_2$ were added and nitrite was measured only after the NADH$_2$ was completely oxidized. The ratio of NADH$_2$ oxidized to nitrite reduced was 3 (Table 1), which indicates reduction to ammonia with no accumulation of intermediates, even when nitrite reduction is not complete. Ammonia accumulates as the product of the reduction and the ratio of NADH$_2$ oxidized to ammonia produced was also 3 (Table 2), regard-

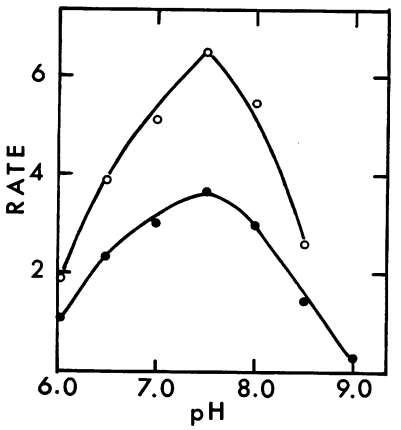

FIG. 7. *Effect of pH on the activity of $NADH_2$ nitrite reductase activity. Assay mixtures contained: 35 mM pyrophosphate at the indicated pH; 1 mM nitrite; 0.24 mM $NADH_2$; and crude enzyme preparation containing 0.7 mg of protein. Closed circles, control; open circles, other components preincubated 15 min before addition of $NADH_2$. Assay volume, 1 ml. Rate is expressed as $(\Delta A_{340}/min) \times 10$.*

TABLE 1. *Stoichiometry of nitrite reduction catalyzed by Escherichia coli $NADH_2$-specific nitrite reductase*[a]

$NADH_2$ added and used	Nitrite used		$NADH_2$/nitrite used (ratio)
	Amt	Per cent of total	
μmoles	*μmoles*		
0.743	0.271	78	2.7
0.240	0.078	22	3.0
0.557	0.199	52	2.8
0.522	0.188	48	2.8

[a] Assay mixtures contained, in 3 ml: 110 μmoles of pyrophosphate (*p*H 8.0); 0.10 units of crude enzyme preparation; 0.35 μmoles of nitrite; and $NADH_2$ in limiting amounts, as indicated. $NADH_2$ disappearance was measured spectrophotometrically, and remaining nitrite was determined after the added $NADH_2$ had been completely oxidized.

less of whether nitrite or $NADH_2$ was present in limiting amounts.

DISCUSSION

In cell-free preparations, the $NADH_2$-specific nitrite reductase of *E. coli* strain Bn catalyzes the reduction of hydroxylamine as well as of nitrite. Any interpretation of this observation must take into account, however, the approximately 150-fold difference between the apparent Michaelis constants for the two substrates. Thus, nitrite at

TABLE 2. *Stoichiometry of ammonia production catalyzed by Escherichia coli $NADH_2$-specific nitrite reductase*[a]

Nitrite added	$NADH_2$		Ammonia produced	$NADH_2$ used/NH_3 produced
	Added	Oxidized		
μmoles	*μmoles*	*μmoles*		
3	1.26	1.26	0.42	3.0
3	0.84	0.84	0.27	3.1
0.15	0.84	0.45	0.17	2.5

[a] Assay mixture contained, in 3 ml: 100 μmoles of pyrophosphate (*p*H 7.5); 0.14 units of crude enzyme preparation; and nitrite and $NADH_2$ as indicated. $NADH_2$ disappearance was measured spectrophotometrically, and ammonia production was measured after the reaction had reached completion.

50 μM (five times its K_m) is reduced to ammonium at nearly the maximal rate, but hydroxylamine at the same concentration (about 3% of its K_m) would be reduced very slowly. This comparison seems virtually to eliminate free hydroxylamine as an intermediate in the reduction of nitrite to ammonium. Both the observation that V_{max} for hydroxylamine exceeds that for nitrite, and the suggestion (from kinetic competition) that both substrates may be reduced at the same site are consistent with the hypothesis that no free intermediates are released from the enzyme during the 6-electron reduction of nitrite to ammonium, but that the enzyme is capable of reacting with free hydroxylamine to form a complex that is similar to, or identical with, a normal intermediate in nitrite reduction. Such a hypothesis has been previously proposed, with somewhat stronger experimental support, for both nitrite and sulfite reduction by the $NADPH_2$ specific sulfite reductase of *E. coli* (3).

The $NADH_2$ and $NADPH_2$ enzymes are similar in several ways: (i) both seem to accept either sulfite or nitrite at the active site, although sulfite is reduced only by the $NADPH_2$ enzyme; (ii) both catalyze the reduction of hydroxylamine; (iii) it seems likely that both catalyze a 6-electron reduction (nitrite to ammonium or sulfite to sulfide) at a single site. These resemblances suggest that the two enzymes might have evolved from a relatively recent common molecular "ancestor." Nothing is known of the physical properties or amino acid composition of either enzyme, so this suggestion cannot be pursued further at present.

The physiological functions of the two enzymes appear to be quite different. The $NADPH_2$ enzyme, which is repressed by cysteine (3, 5)

seems to be a typical biosynthetic enzyme that catalyzes the reduction of sulfite as a step in sulfur amino acid formation. The $NADH_2$ enzyme can play a similar role in permitting growth on nitrate or nitrite in the absence of ammonium or organic nitrogen compounds. It can thus participate in assimilatory nitrite reduction, and this may be its main metabolic function. The observation that it is induced by nitrite rather than repressed by ammonium makes it appear more likely, however, that the $NADH_2$ enzyme evolved as a nitrite detoxifying system, or perhaps as an electron sink in nitrate respiration, and that any biosynthetic function is fortuitous.

The peculiar pattern of activation by one substrate (nitrite) and inactivation by the other ($NADH_2$) is unexplained as to both mechanism and physiological function, if indeed it occurs in the intact cell. Inactivation, like nitrite reduction, is specific for $NADH_2$; thus inactivation might involve a reduction of the enzyme that is related in some way to its normal catalytic function, especially since inactivation is prevented by the presence of nitrite, the normal electron acceptor.

Activation by nitrite could easily be rationalized in terms of any physiological function proposed for the reductase, but we see no apparent advantage for the cell that would result from in vivo inactivation by $NADH_2$. In particular, this response to the level of $NADH_2$ would not seem appropriate for an enzyme participating in nitrate respiration.

Zarowny and Sanwal (11) observed a $NADH_2$-specific nitrite reductase in extracts of *E. coli* K-12 grown with nitrate as nitrogen source. The reported properties differ from those of the enzyme from strain Bn described here. The comparison follows:

Optimal *p*H: K-12, 7.8 to 8.0; Bn, 7.5
K_m for nitrite: K-12, 40 μM; Bn, 10 μM
K_m for $NADH_2$: K-12, 330 μM; Bn, 18 μM

The *p*H optima are essentially identical, and the fourfold discrepancy in K_m value for nitrite is probably not significant, especially in view of the difference in assay methods (Zarowny and Sanwal measured nitrite disappearance during a 15-min incubation). The difference in reported values of the K_m for $NADH_2$ would certainly seem large enough to be real, although, in the absence of initial rates for the K-12 enzyme, this conclusion may not be entirely definite. The most clear-cut difference between the observations on the two preparations relates to the accumulation of product. Reduction of nitrite by the Bn extracts was accompanied by accumulation of a stoichiometric amount of ammonium. In contrast, neither ammonium nor any other reduced product was detected in the K-12 extracts. Thus, the relation between the two enzymes is not clear.

Green plants reduce nitrate to ammonium on a massive scale, but identification of the physiological electron donor has been difficult. Hageman, Cresswell, and Hewitt (2) and Cresswell et al. (1) prepared extracts from *Cucurbita pepo* that reduced either nitrite or hydroxylamine quantitatively to ammonium. Reduced benzyl viologen could serve as the reducing agent; alternatively, in the presence of a catalytic level of benzyl viologen, $NADPH_2$, but not $NADH_2$, was an effective electron donor. These workers proposed that $NADPH_2$ is the physiological ultimate donor in the plant, with benzyl viologen being required in extracts to replace an unknown natural electron transport cofactor. The *Cucurbita* system seems to be similar to the nitrite and sulfite reductases of *E. coli*, except that no evidence has been obtained for any cofactor other than reduced pyridine nucleotides in the case of the bacterial enzymes. The ratio of the Michaelis constant for hydroxylamine to that for nitrite is even larger for the *Cucurbita* system than for the *E. coli* enzyme, so that in the plant system also, as Cresswell et al. (1) point out, participation of free hydroxylamine as an intermediate is very unlikely. Sanderson and Cocking (9), working with tomato preparations, also observed viologen-mediated electron transfer from reduced pyridine nucleotides to nitrite with the production of ammonium. Their results differed from those of Hageman, Cresswell, and Hewitt (2) mainly in that $NADH_2$, as well as $NADPH_2$, could supply electrons to the tomato system.

ACKNOWLEDGMENT

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