PYRIDINE NUCLEOTIDE–NITRATE REDUCTASE FROM EXTRACTS OF HIGHER PLANTS[1]

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Introduction

The major source of nitrogen for most higher plants and many microorganisms is nitrate, yet natural mechanisms by which it is reduced have remained obscure. Most of the experimental evidence concerning assimilation of nitrate by higher plants has been included in reviews by NIGHTINGALE (**24, 25**), STREET (**29**), and VIRTANEN and RAUTANEN (**30**). The early work of ECKERSON (**9**) showed that nitrite was formed when the expressed sap of plants was incubated with nitrate and glucose. Since long incubation periods were used in these experiments, the results do not show conclusively whether the nitrate reduction was catalyzed by enzymes from the sap or from contaminating microörganisms. Experiments by various investigators (**9, 12**) have demonstrated the formation of nitrite in tissues of nitrogen-deficient plants after nitrate had been added to nutrient solutions and had accumulated in various organs. Nitrite appearance was usually associated with the disappearance of carbohydrates and an increase in respiration. ANDERSON (**1**) reported the detection of nitrite in leaves and shoots of 25 plant species, and stated that the agent responsible for its formation was thermolabile and oxidizable. BÜRSTROM (**6**), ECKERSON (**10**), and others have shown that light is involved in nitrate assimilation by the aerial portions of plants. The mechanism of this effect, however, has not been apparent. In a recent study, BHAGVAT (**29**) reported that the aldehyde oxidase system, which catalyzes the oxidation of aldehyde by oxygen or by nitrate in absence of oxygen, is present in the potato. The physiological importance of this enzyme in nitrate assimilation has not been determined.

It is generally concluded (**24, 25, 29, 30**) that nitrite is a probable intermediate in nitrate metabolism and that the process of nitrate reduction is enzymatic. The characteristics and requirements of a widely distributed enzyme system in higher plants likely to play an important role in nitrate reduction have not been established. It is the purpose of this paper to describe the purification and properties of a pyridine nucleotide–nitrate reductase from soybean leaves already reported from this laboratory (**11**), and to present evidence for its occurrence in other higher plant species.

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Experimental methods and materials

Source of enzyme

The crude enzyme extract used in the purification procedure was obtained from the primary leaves of 8- to 10-day-old soybean seedlings (*Glycine max* Merr., variety Odgen) grown in flats (two feet by two feet) filled with sand or vermiculite. Each flat was supplied each day with approximately two liters of nutrient solution containing 0.005 M $Ca(NO_3)_2$, 0.0005 M KH_2PO_4, 0.002 M $MgSO_4$, 0.0025 M K_2SO_4, 0.5 p.p.m. Fe as $FeSO_4$, 0.02 p.p.m. Cu as $CuSO_4$, 0.015 p.p.m. Mo as Na_2MoO_4, and 0.25 p.p.m. B, Mn, and Zn as H_3BO_3, $MnSO_4$, and $ZnSO_4$, respectively. Solutions were prepared with demineralized water and adjusted to pH 5.8. Flats also were supplied daily with a volume of demineralized water equal to that of the nutrient solution.

The crude extract (fraction I) was made by grinding one weight of fresh primary leaves, three weights of cold 0.1 M K_2HPO_4 buffer (pH 9.0), and two weights of alumina powder (Alcoa A-301) in a Waring Blendor for two minutes at 4° C. The mixture was further ground for three minutes in a Ten Brock homogenizer at 0 to 4° C, then centrifuged in a Servall centrifuge at 20,000 times gravity for 10 minutes at 4° C. The supernatant fluid which was green in color but clear was used for purification. Measurement of activities in homogenates and crude extracts indicated that approximately 10% of the enzyme was dissolved by this procedure. Homogenates used in nutrition and survey experiments were prepared by grinding one weight of fresh tissue with three weights of cold 0.1 M phosphate buffer at pH 7.0, in a cold mortar, then in a Ten Brock homogenizer at 0 to 4° C.

The effect of nitrogen source on the enzyme activity was studied with soybean plants grown for five days (after a four-day germination period) in two-liter beakers containing nutrient solutions as follows: (a) nitrate solution, as indicated above for seedlings, (b) ammonium solution, containing nutrients indicated above, with the exception that 0.005 M $(NH_4)_2SO_4$ was substituted for $Ca(NO_3)_2$, (c) solution without nitrogen containing nutrients indicated above, with the exception that 0.005 M $CaCl_2$ was substituted for $Ca(NO_3)_2$. These solutions were diluted with an equal volume of water and adjusted to pH 5.8. The various plant species used in the survey were grown in six-inch ceramic pots, filled with vermiculite, and were supplied with nutrient solution and water as indicated for soybean seedlings grown in flats. Deficiencies of Fe, Mn, B, Zn, Mo, and N were obtained in 35-day-old soybean plants by a method previously described (**21**).

Co-factors, substrates and other materials

Triphosphopyridine nucleotide (TPN) of approximately 75% purity was prepared from sheep liver by a procedure utilizing ion exchange chromatography (unpublished method of Dr. A. Kornberg and Dr. B. L. Horecker). The reduced derivative of triphosphopyridine nucleotide (TPNH) was prepared enzymatically as follows: 40 mg. of TPN were dissolved in 15.4 ml.

of 0.1 M phosphate buffer, at pH 7.5, and 0.8 ml. of 0.1 M $MgCl_2$, and 3 ml. of 0.05 M D-isocitrate were added. The reaction was started by addition of 0.8 ml. of a phosphate buffer (pH 7.5) extract of washed, acetone-dried, pig heart, containing isocitric dehydrogenase (**26**). The reaction was allowed to proceed at room temperature for 30 to 45 minutes, and at intervals during this period 0.05 ml. aliquots were removed, diluted to 3.0 ml. with 0.1 M phosphate buffer at pH 7.5, and optical densities determined in a Beckman spectrophotometer at 340 mμ. The reaction was considered complete when a maximum increase in optical density was obtained. At the end of the reaction, the TPNH preparation was adjusted to pH 9.0 to 9.5, placed in a boiling water bath for three minutes, centrifuged for five minutes and the supernatant solution was used as a source of TPNH. This solution was shown to be active enzymatically by preliminary tests with partially purified glutathione reductase from peas (**20**). With this system the absorption decreased to the original starting value. The concentration of TPNH was determined spectrophotometrically by use of the extinction coefficient of 6.24×10^6 sq. cm. $\times$ mole^{-1} at 340 mμ (**14**).

Diphosphopyridine nucleotide (DPN) of 65% purity was obtained from the Sigma Chemical Company, and the reduced derivative of diphosphopyridine nucleotide (DPNH) was prepared from this by a method described elsewhere (**27**). Flavin-adenine dinucleotide (FAD) of approximately 60% purity, prepared from yeast by the method of WARBURG and CHRISTIAN (**33**), was kindly furnished by Dr. L. A. Heppel. The FAD of 81% purity was obtained from the Sigma Chemical Company and the flavin mononucleotide (FMN) from Nutritional Biochemicals, Inc. In certain experiments as indicated, a boiled extract of acetone-dried pig heart was used as a source of FAD. The concentrations of FAD solutions were determined spectrophotometrically by the use of the extinction coefficient of 1.13×10^7 sq. cm. $\times$ moles^{-1} at 455 mμ (**33**).

Cytochrome c, used in the cytochrome c reductase assay (**15**), was obtained from the Sigma Chemical Company. The sodium salt of D-isocitric acid of 70% purity (30% citric acid) was prepared by ion exchange chromatography (**7**) from extracts of powdered Bryophyllum leaves kindly supplied by Dr. H. B. Vickery. The apoenzyme of D-amino acid oxidase was prepared from pig kidney, according to the method of NEGELEIN and BROMEL (**23**), modified by an unpublished procedure of Dr. Wilhelm Frisell. Purified phosphogluconic dehydrogenase and the barium salt of phosphogluconic acid were kindly furnished by Dr. B. L. Horecker. Snake venom, used as a source of nucleotide pyrophosphatase, was obtained from the Ross Allen Reptile Institute.

Grana were prepared from mature leaves of 18- to 21-day-old soybean plants by a modification of the method used by VISHNIAC and OCHOA (**31**). One weight of leaves and two weights of cold 0.05 M phosphate buffer (pH 7.0) containing 0.5% KCl were ground in a mortar at 0 to 4° C. The macerated material was squeezed through four layers of cheesecloth and

centrifuged at 2000 × gravity for 10 minutes at 0 to 4° C. The supernatant fluid, which was used as a source of grana, contained a small number of intact chloroplasts. Removal of soybean grana by centrifugation, followed by resuspension in the above buffer, resulted in almost complete loss of photochemical activity. The loss also was obtained when grana were prepared in 0.5 M sucrose, instead of phosphate buffer containing KCl. Chlorophyll content of grana was determined by the method of MacKinney (**19**).

Standard nitrate reductase assay

Enzyme activity was measured by colorimetric determination (**28**) of the nitrite formed during a 10-minute incubation period at 28° C. The procedure was as follows: 0.1 ml. of 0.1 M KNO_3 (10 μM), 0.11 ml. of 0.5 M pH 7.0 phosphate buffer (55 μM), 0.05 ml. of 2.6×10^{-5} M FAD (1.3×10^{-3} μM), and 0.04 ml. of 2×10^{-3} M TPNH (0.08 μM) were added to a small test tube, and the reaction was started by adding 0.2 ml., or quantities indicated, of enzyme extract. The final volume was 0.5 ml. The mixture was allowed to incubate for 10 minutes, then the reaction was stopped by adding 0.9 ml. of water and 0.5 ml. of sulfanilamide reagent, followed immediately with 0.5 ml. *N*-(1-naphthyl)-ethylenediamine hydrochloride solution. After 10 minutes, the optical density was determined with a Klett colorimeter using a 540 mμ filter. Optical densities of control reaction mixtures containing all constituents except TPNH were used to correct for turbidity. There was no chemical reduction of nitrate to nitrite by TPNH.

When homogenates or extracts high in protein were used as enzyme sources, 0.9 ml. of saturated $(NH_4)_2SO_4$ solution was added to each reaction mixture at the end of the incubation period, instead of 0.9 ml. of water. The sulfanilamide and *N*-(1-naphthyl)-ethylenediamine hydrochloride reagents were added, mixtures were centrifuged, and optical densities determined as above. The $(NH_4)_2SO_4$ precipitate adsorbed some of the diazo compound, and, therefore, the experimental error was increased when this procedure was used. Tests showed that no measurable quantity of nitrite disappeared in experiments utilizing enzyme extracts under the conditions described; however, there was some disappearance during long incubation periods using homogenates. This was reduced by including a final concentration of 10^{-3} M $NH_2OH \cdot HCl$.

One unit of the soybean nitrate reductase is defined as that amount of enzyme which will catalyze the formation of 10^{-3} micromoles of nitrite in five minutes when carried out under the conditions described, with the exception of time. In the experiments reported, units of enzyme were determined by incubating reaction mixtures for 10 minutes and dividing the $\mu M \times 10^{-3}$ formed by 2. This unit is equivalent to that of the Neurospora enzyme (**22**). Specific activity is defined as the units of activity per mg. of protein which was determined by the method of Lowry *et al.* (**17**). The enzyme activities of homogenates are expressed on a dry weight basis, as indicated.

Experimental results

Purification of enzyme

Crude extracts (fraction I) of the primary leaves of 8- to 10-day-old soybean seedlings were purified by a method involving calcium phosphate gel adsorption and ammonium sulphate precipitation. All steps of the purification were carried out at 0 to 4° C. The various fractions were centrifuged at a force of approximately 3000 times gravity. The pertinent data of a typical purification described in detail below are recorded in table I.

Ninety-five milliliters of calcium phosphate gel (11 mg. per ml.) were added to 190 ml. of crude extract obtained from 60 gm. of primary leaves. The suspension was allowed to stand 10 minutes in the cold, then was centrifuged for five minutes and the supernatant liquid was discarded. The gel, containing the adsorbed enzyme, was mixed with 95 ml. of 0.1 M phosphate buffer at pH 7.5, and allowed to stand for one minute. The gel suspension

TABLE I

PURIFICATION OF NITRATE REDUCTASE FROM NINE-DAY-OLD SOYBEAN LEAVES.

Fraction	Volume	Total activity	Recovery	Protein	Specific activity
	ml.	*units*	*%*	*mg./ml.*	*units/mg. protein*
I. Crude extract	190	2774	....	8.10	1.8
II. First calcium phosphate gel eluate	38	1281	46	0.80	42.2
III. 0–40% ammonium sulphate precipitate	15	645	23	1.10	39.1
IV. Second calcium phosphate gel adsorption eluate	3	199	7	0.55	120.6

again was centrifuged for five minutes and the supernatant fluid discarded. The enzyme was eluted by mixing 38 ml. of 0.1 M pyrophosphate buffer at pH 7.0 with the gel and allowing it to stand for 10 minutes. The suspension was centrifuged for 15 minutes and the supernatant fluid (fraction II) collected. This fraction contained 46% of the total activity and was purified 24-fold (table I).

Ammonium sulphate (9.2 gm.) was added to 38 ml. of the first eluate (fraction II), the suspension was allowed to stand for 10 minutes, then was centrifuged for 10 minutes. The precipitate was dissolved in 15 ml. of cold 0.1 M phosphate buffer at pH 7.0. This solution is referred to as fraction III. Seven and one half milliliters of calcium phosphate gel were added to 15 ml. of fraction III. After 10 minutes, the mixture was centrifuged and the supernatant liquid discarded. The gel was washed with 7.5 ml. of cold 0.1 M phosphate buffer at pH 7.5, centrifuged for five minutes and the gel collected. Three milliliters of 0.1 M pyrophosphate buffer at pH 7.0 were mixed with the gel, allowed to stand for 10 minutes, centrifuged for 15 minutes, and the eluate (fraction IV) collected.

The data in table I show that this procedure resulted in a 67-fold purification of the enzyme, with recovery of 7% of the total activity. The minimum purification obtained in repetitions of this procedure was 44-fold with 11% recovery. Since relatively large quantities of enzyme were necessary, the first eluate (fraction II) was used in many of the experiments. The range in purification of this fraction was 18.5- to 27-fold, and its protein content ranged between 0.8 and 1.0 mg. per ml. Fraction IV contained 0.5 to 0.9 mg. protein per ml. The turnover number for this fraction is 2.4 moles of nitrate reduced to nitrite per mole of protein per minute, assuming a molecular weight for the enzyme of 100,000. The quantity of purified enzyme (fraction II or IV) used in experiments with the standard assay procedure contained 0.05 to 0.2 mg. of protein.

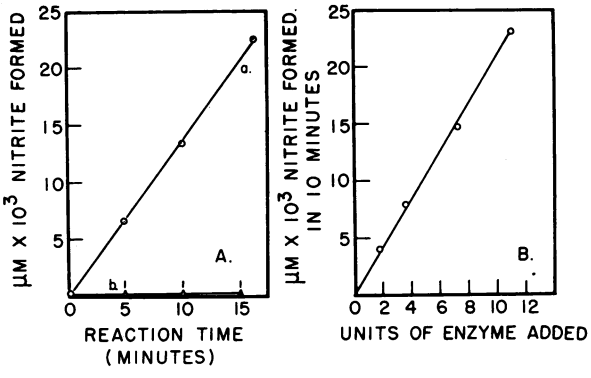

FIG. 1. A. Proportionality of enzyme activity with time. Procedure was that of the standard assay (see text) with exception of the time as indicated. The enzyme used was 6.7 units of fraction II (curve a) or this quantity boiled (curve b). B. Proportionality of activity with enzyme concentration. Procedure was that of the standard assay. Units of enzyme (fraction II) are indicated.

Tests of fractions II and IV for other enzymes indicated no diphosphopyridine nucleotidase activity (**16**), but very low glutathione reductase (**20**), and fairly strong cytochrome c reductase activities (**15**) were observed. The latter did not parallel nitrate reductase activity in the purification process, and it is concluded, therefore, that cytochrome c reductase and nitrate reductase are not the same enzymes. The inclusion of succinate in the reaction mixture with or without TPNH, under aerobic or anaerobic conditions, had no effect on the nitrate reductase activity. The purified enzyme (fraction IV) was very heat sensitive, as indicated by an experiment which showed 28, 46, 71, and 100% inactivation from five-minute exposures at 20, 30, 40, and 50° C, respectively. The activity was reduced 50 to 70% during storage periods of one week at – 15° C. As shown in figure 1, the activity was proportional to the quantity of enzyme added and also to the length of time reaction mixtures were incubated.

Requirements for optimum activity

pH optimum.—As indicated in figure 2, the pH optimum is at 6.0 with relatively high activity in the range 5.5 to 7.0. There was no marked difference in activity from use of phosphate as compared to acetate buffers on the acid side of the optimum or from phosphate and pyrophosphate buffers on the alkaline side of the optimum. When the soybean nitrate reductase was purified by ammonium sulphate fractionation only, the addition of pyrophosphate resulted in no stimulation of activity. In contrast, the Neurospora nitrate reductase (**22**), prepared by the same method, showed a striking response. Experiments reported in this paper were carried out at pH 7.0 for convenience.

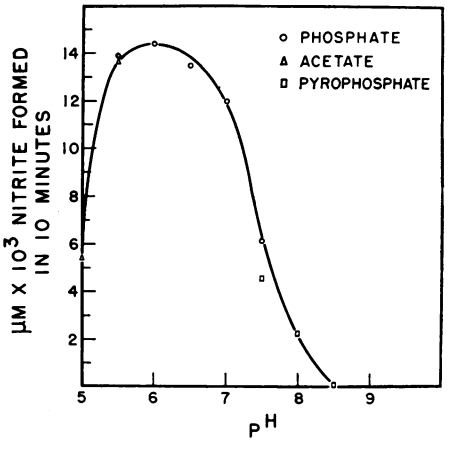

Fig. 2. Effect of pH on nitrate reductase activity. Procedure was that of the standard assay (see text) except that 42 μM of the buffers indicated were added. Six units of fraction IV were used as the enzyme.

Substrate concentration.—The final concentration of KNO_3 required to saturate the enzyme in presence of an optimum concentration of TPNH was 2×10^{-2} M (fig. 3). The concentration for one half maximal activity, which is a measure of the dissociation of the enzyme-nitrate complex (K_M) is 7.5×10^{-3} M, as determined from the saturation curve. The nitrate contamination usually found in TPNH was avoided by several reprecipitations of TPN in the course of its purification.

The curves in figure 4 show that TPNH or DPNH were almost equally effective as hydrogen donors for nitrate reduction. The maximum activity, in presence of an optimal concentration of nitrate and other known cofactors was obtained at a final concentration of 8×10^{-5} M for both reduced coenzymes. The concentration of TPNH required for one half maximal activity was 2.3×10^{-5} M and that for DPNH was 3.2×10^{-5} which would indicate a slightly greater affinity of the enzyme for TPNH than for DPNH. The

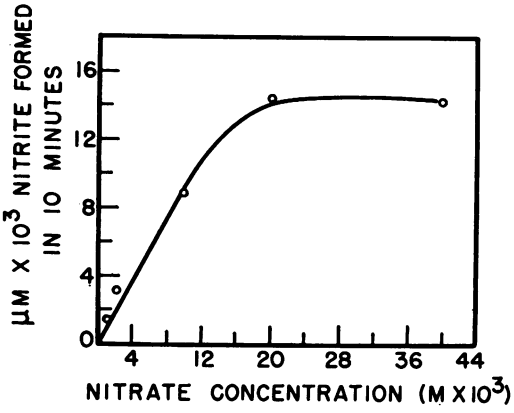

FIG. 3. Nitrate required for saturation of enzyme. Procedure was that of standard assay (see text) with exception of the final concentrations of KNO_3 used which are indicated. Seven units of fraction II were used as the enzyme.

differences, however, are of a magnitude where experimental error is probably an important consideration. The TPNH, prepared by chemical reduction with hydrosulphite (**32**), appeared to be as effective as enzymatically prepared TPNH, although it was less stable when stored at – 15° C.

INORGANIC IONS.—No consistent increase in activity could be obtained by preliminary incubation at 10^{-4} M final concentrations of $ZnSO_4$, $MnSO_4$, Na_2MoO_4, $Na_2B_4O_7$, $MgSO_4$, $FeSO_4$, $FeCl_3$, or $CuSO_4$. Attempts to dialyze the enzyme prior to incubation with these compounds resulted in complete

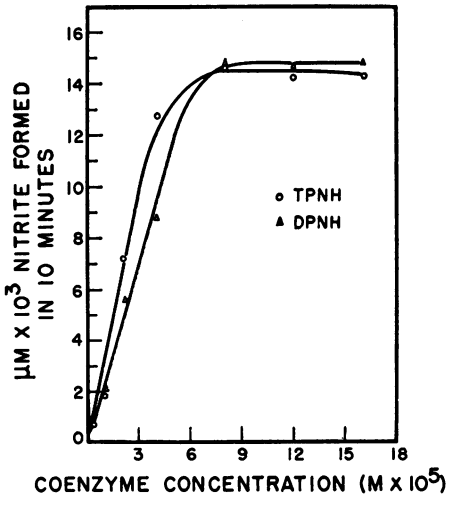

FIG. 4. Comparison of TPNH and DPNH as hydrogen donors in soybean nitrate reductase system. Procedure was that of the standard assay (see text) with the exception that TPNH and DPNH concentrations, on a final volume basis, were varied as indicated. The enzyme used was 7.3 units of fraction IV.

TABLE II

DISSOCIATION OF FLAVIN NUCLEOTIDE FROM ENZYME BY AMMONIUM SULPHATE PRECIPITATION.

Samples of enzyme fraction II	Units of enzyme		Nitrite formed in 10 minutes			
			Before precipitation		After two precipitations	
	Before precipitation	After precipitations	With FAD	Without FAD	With FAD	Without FAD
			$\mu M \times 10^3$			
A	10.0	9.5	20.2	19.6	19.1	14.1
B	16.0	13.0	32.2	30.3	25.9	15.8
C	7.8	6.0	15.6	14.1	12.1	5.8

inactivation which could not be reversed. There was no difference in nitrate reduction by this enzyme system under anaerobic, as compared to aerobic conditions.

Dissociation of flavin nucleotide prosthetic group

The enzyme activity of the first eluate (fraction II) was not appreciably affected by additions of FAD or FMN. A very marked response to FAD, however, was demonstrated after twice precipitating the enzyme with $(NH_4)_2SO_4$ (table II). Dissociation of the prosthetic group by this method was accomplished as follows: solid $(NH_4)_2SO_4$ was added to the enzyme solution (45% saturation), then after 10 minutes at 4° C, the suspension was centrifuged and the precipitate dissolved in the original volume of cold 0.1 M phosphate buffer at pH 7.0. The process was then repeated. Three different extracts obtained by this method from three preparations of fraction II

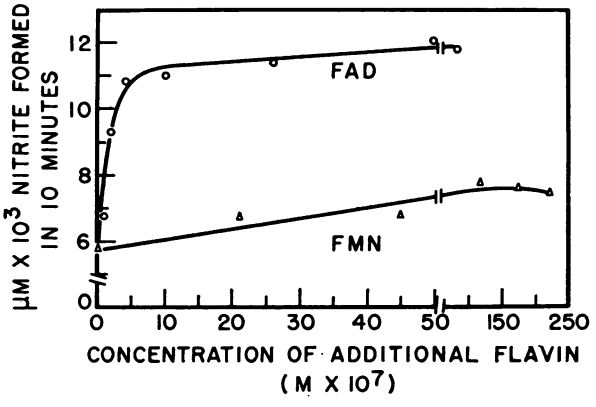

FIG. 5. Flavin nucleotide requirement of nitrate reductase. Procedure was that of the standard assay (see text) with the exception of the final concentrations of FAD or FMN indicated. All constituents except TPNH were incubated five minutes at 4° C prior to the nitrate reductase assay. Six units of preparation C (see text) were used as the enzyme.

contained 0.3 to 0.6 mg. protein per ml. They will be referred to as preparations A, B, and C.

The activity of the twice-precipitated enzyme (preparation C) as a function of FAD and FMN concentrations is illustrated in figure 5. A final concentration of 1×10^{-6} M additional FAD was sufficient for almost maximum activity. One half maximal reactivation of the enzyme was obtained with FAD at approximately 1×10^{-7} M final concentration and with FMN at about 3.7×10^{-6} M. The affinity of the enzyme, therefore, is much greater for FAD than FMN. These results show that the activity of the enzyme which was decreased approximately 50% by two $(NH_4)_2SO_4$ precipitations was completely restored by FAD and that FMN additions caused a very slight activation. The greater effectiveness of FAD in reactivating the nitrate reductase does identify the natural prosthetic group, however, the following experiments show that FAD is the nucleotide associated with the enzyme.

The flavin nucleotide associated with the twice-precipitated nitrate reductase (preparations A and B) was further identified as FAD by the method

TABLE III

FLUORIMETRIC ANALYSIS OF SOYBEAN NITRATE REDUCTASE FLAVIN NUCLEOTIDE.

Analyzed	Galvanometer deflections due to flavins		
	Flavin from boiled enzyme		FAD
	Preparation A	Preparation B	
Before hydrolysis (f_1)	2	3	11
After hydrolysis (f_2)	13	13	36
Per cent. flavin as FAD	98	89	81

of BURCH *et al.* (**4**), which is based on the increase in fluorescence obtained by hydrolyzing FAD to FMN and adenylic acid. Flavin nucleotides were liberated from the enzyme by heating 3.5 ml. aliquots at 100° C for five minutes in the dark. The supernatant liquids (collected by centrifugation) and a known sample containing 3.8×10^{-3} μM of FAD were hydrolyzed by incubating them in the dark with 100 μM phosphate buffer at pH 7.5 and 1.5 mg. of snake venom (5 mg. protein per ml.) containing pyrophosphatase, in a final volume of 5 ml. for 30 minutes at 37° C. The snake venom showed no fluorescence due to flavins. Control solutions (before hydrolysis, in table III) of the boiled enzyme and FAD were incubated with all constituents used with those hydrolyzed, except pyrophosphatase.

A marked increase in fluorescence due to flavin nucleotides was obtained by hydrolyzing all samples (table III). Since the fluorescence of FAD is 0.14 times that of FMN, the per cent. of FAD in a mixture is given by the following equation (**4**):

$$\text{Per cent. FAD} = \frac{f_2 - f_1}{0.86\ f_2} \times 100 \qquad (1)$$

where f_1 and f_2 are measures of flavin nucleotide fluorescence before and after hydrolysis, respectively. From the increase in fluorescence obtained by hydrolysis with nucleotide pyrophosphatase, it is calculated that 98, 89, and 81% of the flavins in preparation A, B, and the FAD solution, respectively, is accounted for as FAD. By use of the fluorescence data and that of a known riboflavin standard, it was calculated that preparation A contained 2.8×10^{-4} μM FAD per ml. and preparation B contained 2.6×10^{-4} μM per ml. (table IV).

Additional proof that the soybean nitrate reductase contained FAD was provided by its activity with the protein of D-amino acid oxidase specific for FAD (**33**). In this experiment, 2.6 ml. of the boiled preparation A and B adjusted at pH 8.5, and 0.2 ml. of the apoenzyme of D-amino acid oxidase were placed in the main compartment of a Warburg vessel. Two tenths milliliter of 0.33 M DL-alanine was placed in the side arm, and KOH in the center well. The mixture was preincubated for 30 minutes at 4° C, and oxygen uptake was determined at 30° C. The oxygen uptake of other reaction mixtures containing from zero to 2.5×10^{-3} μM of FAD, instead of the

TABLE IV

FLAVIN-ADENINE DINUCLEOTIDE CONTENT OF SOYBEAN NITRATE REDUCTASE.

Preparation	Method of analysis	
	Fluorimetric	d-Amino acid oxidase
	μM per ml.	*μM per ml.*
A	2.8×10^{-4}	2.3×10^{-4}
B	2.6×10^{-4}	1.6×10^{-4}

boiled enzyme preparations, was determined at the same time, in order to estimate the FAD content of the unknown samples. Preparations A and B contained 2.3×10^{-4} and 1.6×10^{-4} μM FAD per ml., respectively, as determined by this method. This compares favorably with the values for the corresponding samples determined by the previously described fluorimetric procedure (table IV).

Stoichiometry of reaction

In order to determine the stoichiometry of the nitrate reductase reaction, an experiment designed to measure concomitant disappearance of TPNH and formation of nitrite was carried out in spectrophotometric cuvettes with the approximate proportion of constituents used in the standard assay. Methylene blue was added to the solvent cuvette to compensate for the absorption due to the enzyme. At various intervals during the reaction period, aliquots of the reaction mixtures were removed from cuvettes and assayed for nitrite. As shown by the spectrophotometric measurements (fig. 6), there was a very rapid oxidation of TPNH in presence of the complete nitrate reductase system, none when the enzyme was omitted, and a fairly rapid endogenous rate when all constituents, except KNO_3 were present.

Since nitrite was formed in the complete system only, the endogenous rate (without nitrate) was caused by some factor other than nitrate. The observed values of nitrite formation and TPNH oxidation (difference between curve A and curve B of fig. 6) are reported in table V. These results are in agreement with the conclusion that nitrate reduction by this system proceeds according to the following equation:

$$NO_3^- + TPNH + H^+ \rightarrow NO_2^- + TPN + H_2O \qquad (2)$$

The appreciable endogenous disappearance of TPNH in the absence of nitrate suggests that the enzyme (fraction II) possibly contained a TPNH oxidase system, as described by Conn *et al.* (**8**). It is interesting that the optical density of the complete reaction mixture (curve A, fig. 6) increased

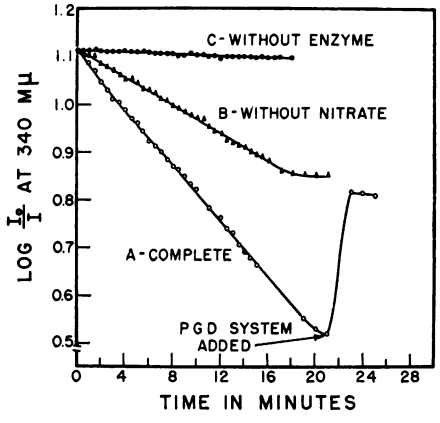

Fig. 6. Disappearance of TPNH in presence of soybean nitrate reductase system. Reaction mixtures contained the following, in a final volume of 3.5 ml.: curve A, 45 units of enzyme (fraction II), 625 μM phosphate buffer at pH 7.0, 0.01 μM FAD, 100 μM KNO_3, and 0.63 μM TPNH; curve B, the constituents added were the same as indicated for curve A except that KNO_3 was omitted; curve C, quantities of constituents were the same as indicated for curve A with the exception that the enzyme was omitted. After 21 minutes 0.02 ml. of purified phosphogluconic dehydrogenase and 47. μM of sodium phosphogluconate were added to the reaction (curve A). The phosphogluconic dehydrogenase contained 0.32 units of enzyme and 0.5 mg. protein.

to a level equal to that of the endogenous curve (without nitrate) when the phosphogluconic acid dehydrogenase system was added (after 21 minutes), indicating that some of the TPN had been destroyed. Otherwise, the absorption should have increased to the original starting value.

Effect of inhibitors

The sensitivity of the enzyme to various inhibitors suggests the presence of a metal constituent in the nitrate reductase system (table VI). The procedure in table VI was that of standard assay with the exception that inhibitors as indicated and all reactants except TPNH were incubated five minutes at 4° C prior to starting the reaction with TPNH. The enzyme

TABLE V

CONCOMITANT DISAPPEARANCE OF THE REDUCED DERIVATIVE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE CALCULATED FROM DIFFERENCES BETWEEN CURVES A AND B OF FIGURE 6 AND PRODUCTION OF NITRITE DETERMINED FROM ALIQUOTS OF REACTION MIXTURE (CURVE A, FIG. 6)

Time	TPNH disappeared	Nitrite formed
min.	$\mu M \times 10^3$ *per ml.*	$\mu M \times 10^3$ *per ml.*
0.0	0.0	0.0
2.0	5.4	6.0
4.0	10.1	13.5
6.0	18.0	15.5
8.5	22.3	25.5
10.0	23.5	28.4
13.0	30.6	32.0
15.0	37.7	40.0
21.0	53.3	56.0

consisted of five units of fraction II. KCN at final concentrations of 10^{-3} and 10^{-4} M inhibited the activity to an extent of 83 and 27%, respectively, and NaN_3 at these concentrations inhibited 98 and 39% respectively. A concentration of 2×10^{-4} M potassium ethyl xanthate inhibited the activity 49% and 10^{-4} M $CuSO_4$ inhibited 32%. There was no inhibition by 100% CO or 10^{-3} M NaF.

Results of an experiment showing reversal of *p*-chloromercuribenzoate inhibition of the enzyme activity by cysteine hydrochloride are recorded in

TABLE VI

EFFECT OF INHIBITORS ON SOYBEAN NITRATE REDUCTASE ACTIVITY.*

Inhibitor	Final concentration	Inhibition
	M	%
o-Phenanthroline	2×10^{-3}	10
aa-Dipyridyl	2×10^{-3}	10
Diethyldithiocarbamate	2×10^{-3}	23
Potassium ethyl xanthate	2×10^{-3}	56
Potassium ethyl xanthate	2×10^{-4}	49
Potassium ethyl xanthate	2×10^{-5}	29
Thiourea	2×10^{-3}	56
Thiourea	2×10^{-4}	29
Sodium fluoride	10^{-3}	0
Potassium cyanide	10^{-3}	83
Potassium cyanide	10^{-4}	27
Sodium azide	10^{-3}	98
Sodium azide	10^{-4}	39
Cupric sulphate	10^{-4}	32
Carbon monoxide 100% (in dark)	....	0
Hydroxylamine hydrochloride	10^{-3}	12
8-Hydroxyquinoline	2×10^{-3}	11

*No preliminary incubation.

table VII. The procedure used was as follows: reaction mixtures (final volume of 0.7 ml.) containing 50 μM phosphate buffer at pH 7.0, 10 μM KNO_3, four units of enzyme (fraction II) and concentrations of *p*-chloromercuribenzoate indicated in table VII were incubated five minutes at 4° C. Cysteine hydrochloride as indicated and an equivalent amount of Na_2CO_3 for neutralization were added to reaction 5 through 8 and water to maintain equal volumes in reactions 1 through 4. Reactions again were incubated five minutes at 4° C. Afterward 0.001 μM of FAD (as boiled pig heart extract), and 0.08 μM TPNH were added to all mixtures and the nitrate reductase assay was carried out in the usual manner.

Preincubating the enzyme with concentrations of 10^{-3} M and 10^{-6} M *p*-chloromercuribenzoate resulted in 94 and 51% inhibition, respectively. If the reaction mixtures containing *p*-chloromercuribenzoate were incubated a second time with cysteine hydrochloride (8.3×10^{-4} M) before assaying for nitrate reductase, the inhibition was reduced to 56% with concentrations

TABLE VII

INHIBITION OF NITRATE REDUCTASE BY *p*-CHLOROMERCURIBENZOATE AND REVERSAL BY CYSTEINE HYDROCHLORIDE.

Reaction	*p*-Chloromercuribenzoate concentration	Cysteine hydrochloride concentration	Inhibition
	M	*M*	%
1	10^{-3}	....	94
2	10^{-4}	....	90
3	10^{-5}	....	81
4	10^{-6}	....	51
5	10^{-3}	8.3×10^{-4}	56
6	10^{-4}	8.3×10^{-4}	15
7	10^{-5}	8.3×10^{-4}	2
8	10^{-6}	8.3×10^{-4}	0

of 10^{-3} M *p*-chloromercuribenzoate and no inhibition was observed where 10^{-6} M *p*-chloromercuribenzoate was used. These results strongly suggest the presence of a sulfhydryl group on the enzyme surface (**13**).

Photochemical reduction of nitrate

Many investigators have reported a pronounced effect of light on nitrate assimilation by plants. During the course of this investigation, it was observed that homogenates of soybean leaves harvested early in the morning showed very little capacity to reduce nitrate without the addition of TPNH or DPNH. However, homogenates of leaves that had been exposed to bright sunlight for periods of a few hours in most cases were very active in reducing nitrate in the absence of reduced coenzymes. This might be ascribed to the photochemical reduction of pyridine nucleotides by grana (**2, 31**), which was coupled with the nitrate reductase system.

The design and results of an experiment showing the photochemical reduction of nitrate by use of grana and nitrate reductase obtained from

soybean leaves are given in table VIII. Complete reaction mixtures 1 and 6 (final volume of 1.5 ml.) contained: 150 μM phosphate buffer at pH 7.0, 6 μM KNO_3, 0.2 μM TPN or TPNH as indicated, grana containing 0.11 mg. chlorophyll, and 15.6 units of fraction II (no FAD requirement) as the enzyme. Constituents omitted in other reaction mixtures are indicated. Quantities of KCl in the grana preparation or pyrophosphate in the enzyme extract were added to reaction mixtures not including grana or enzyme. Light reactions were carried out under an intensity of 1400 foot-candles and dark reactions were shielded with aluminum foil. All reactions were incubated in a water bath at 28° C.

As indicated, 6 μM of KNO_3 were added to each reaction mixture of 1.5 ml. final volume. This was only one fifth of the concentration used in the

TABLE VIII

PHOTOCHEMICAL REDUCTION OF NITRATE WITH NITRATE REDUCTASE AND GRANA FROM SOYBEAN LEAVES.

Reaction time	1 Nitrate reductase KNO_3 TPN Grana	2 KNO_3 TPN Grana	3 Nitrate reductase TPN Grana	4 Nitrate reductase KNO_3 Grana	5 Nitrate reductase KNO_3 TPN	6 Nitrate reductase KNO_3 TPNH Grana
min.			$\mu M \times 10^3$ *nitrite**			
			Light			
0	0.0	0.0	0.0	0.0	0.0	0.0
10	12.3	1.5	6.6	1.8	0.9	19.5
20	25.7	4.8	18.0	2.4	0.0	38.1
30	34.5	7.2	22.5	2.7	0.0	65.2
			Dark			
0	0.0	0.0	0.0	0.0	0.0	0.0
10	3.6	0.3	1.8	1.5	0.3	16.2
20	4.5	5.4	3.6	3.6	0.0	27.6
30	5.1	7.2	2.7	6.6	0.0	28.2

*Quantity of nitrite in entire reaction volume of 1.5 ml., determined from aliquots.

standard nitrate reductase assay. The smaller quantity was necessary because the concentration of nitrate required for optimum nitrate reductase activity resulted in a striking decrease in photochemical reduction of TPN. Washing soybean grana preparations resulted in a serious decrease of their photochemical activity, and therefore, unwashed preparations were used that contained small quantities of nitrate from the leaves.

A very definite photochemical reduction of nitrate was demonstrated (table VIII, reaction 1) in the light. The low rate of nitrate reduction observed in the dark indicates that the reaction mixture contained a weak system for reducing TPN that was not directly dependent upon light. The minute quantity of nitrite formed in reaction 2, containing no purified nitrate reductase, implies that the grana preparation contained a low con-

centration of nitrate reductase. The omission of nitrate from the system (reaction 3) resulted in a fairly high nitrite formation in the light, but very little in the dark. The grana preparation undoubtedly provided a supply of nitrate in this reaction. A small quantity of nitrite was formed in reaction mixtures without TPN (reaction 4) in both light and dark, indicating that the grana contained coenzymes or other substances effective in nitrate reduction. There was almost no endogenous nitrite formation in reaction 5 (without grana). It is interesting to observe that reaction 6, containing TPNH proceeded more rapidly in the light than the dark. It seems logical that in presence of active grana a greater proportion of TPNH would remain reduced in the light than in the dark, thus preventing the depletion of the TPNH supply by oxidation in the nitrate reductase system and other systems undoubtedly present in the grana. Light had no effect on the nitrate reductase system without grana. This experiment was repeated, using 0.45 μM of $NaNO_2$ in reaction mixtures, instead of KNO_3. Determination of nitrite in all reaction mixtures, after incubating for 30 minutes, showed no nitrite disappearance. These results, however, are not quantitative, since nitrite was formed in certain reactions from the nitrate associated with the unwashed grana. Since the enzyme activity (reactions 1 and 6 in the light) was proportional to the incubation time, it seems improbable that sufficient nitrite disappeared to affect the results appreciably. Repetitions of the experiment (table VIII) with different enzyme and grana preparations indicated the same trends of results as those reported.

Effect of nutrition on nitrate reductase activity

Soybean seedlings were grown for five days in triplicate cultures containing nutrient solutions with nitrogen sources as follows: (a) $Ca(NO_3)_2$, (b) $(NH_4)_2SO_4$, (c) without nitrogen. The enzyme activity of homogenates from the primary leaves of these plants was determined by the standard assay procedure, using hydroxylamine hydrochloride to inhibit partially the nitrite disappearance. Homogenates of all leaves, regardless of source of nitrogen provided, showed high nitrate reductase activity ranging between 8×10^{-3} and 1.1×10^{-2} μM nitrite formed in 10 minutes per mg. of dry leaf weight. Diphenylamine tests indicated the absence of nitrates in nutrient solutions without N or with $(NH_4)_2SO_4$, but a positive nitrate test was obtained with leaves of plants grown in these solutions. Therefore, the question of whether or not the enzyme is adaptive remains unanswered.

The nitrate reductase activities of homogenates of young soybean leaves from 30-day-old plants showing symptoms of Fe, Mn, Zn, B, Mo, and N deficiencies were compared with those of control plants. The variation of results from day to day was so great that no conclusions could be drawn. No such extreme variation in the enzyme activity of seedlings was observed.

Distribution of the enzyme

Results from a survey of the distribution of the nitrate reductase in a limited number of higher plant species is reported in table IX. The stand-

ard assay was used with the following exceptions: incubation times were as indicated; 0.5 μM $NH_2OH \cdot HCl$ was added to each reaction (0.5 ml. final volume) to partially inhibit nitrite disappearance; 0.2 ml. of homogenates was used as the enzyme. Equal concentrations of DPNH and TPNH were used. It was necessary to incubate reaction mixtures containing homogen-

TABLE IX

THE OCCURRENCE OF NITRATE REDUCTASE IN CERTAIN PLANT SPECIES.

Plant species		Nitrite formed in 1 hr.*		
		No coenzyme	TPNH	DPNH
	days	$\mu M \times 10^3$ *mg. dry wt.*		
Potato, variety Irish Cobbler, *Solanum tuberosum* L.				
Roots	30	0.0	1.7	1.2
Leaves	30	1.8	2.4	2.5
Barley, variety Alpha, *Hordeum vulgare* L.				
Roots	30	0.3	3.7	2.2
Roots	17	0.5	1.0	....
Leaves	17	1.2	1.3	....
Muskmelon, variety Honey Rock, *Cumcumis melo* L.				
Roots	30	0.0	2.2	1.3
Roots	17	1.8	4.0	....
Leaves	17	0.0	0.2	....
Wheat, variety Nittany, *Triticum vulgare* Vill.				
Roots	30	0.7	6.8	4.6
Tomato, variety Rutgers, *Lycopersicum esculentum* Mill.				
Roots	38	0.1	4.5	11.4
Roots	31	5.1	16.0	23.5
Corn, variety Lancaster Surecrop, *Zea Mays* L.				
Roots	28	0.2	1.2	1.5
Soybean, variety Odgen, *Glycine max.* Merr.				
Roots	17	0.0	1.5	....
Leaves (primary)	17	0.0**	0.6**	....
Leaves (young trifoliate)	17	6.2**	12.5**	....
Leaves (meristematic)	17	5.1**	12.0**	....
Leaves (young trifoliate)	30	1.0	1.3	....
Leaves (primary)	8	33.3**	63.7**	....

*Single determinations except soybean samples which are means of duplicate determinations.

**Incubation time was 10 minutes, results calculated on an hour basis.

ates of most plants for an hour, in order to obtain a quantity of nitrite that could be measured accurately. The results show that the addition of reduced coenzymes consistently increased quantities of nitrite accumulated. This is considered as strong evidence that the nitrate reductase purified from soybean leaves or a similar enzyme is present in at least six other plant species.

The enzyme appeared to be concentrated in the meristematic and young trifoliate leaves of 17-day-old soybean plants. The primary leaves of 17-day-old plants contained little enzyme, in contrast to a very high concentration in primary leaves of eight-day-old seedlings which suggests that the enzyme is concentrated in young, metabolically active tissues.

Nitrite disappearance

In studying the enzymatic reduction of nitrate to nitrite, it was necessary to determine whether or not nitrite disappeared in reaction mixtures. This could be determined with enzyme preparations free of nitrate, by substituting nitrite (1.5×10^{-2} μM) for nitrate in the standard assay procedure and determining the quantity that disappeared. By use of this method, no nitrite disappearance could be detected with the various enzyme fractions obtained in the purification. There was a small non-enzymatic disappearance of nitrite, however, if TPNH and nitrite were allowed to remain in contact with the acid sulfanilamide reagent for a few minutes before adding the solution of *N*-(1-naphthyl)-ethylenediamine hydrochloride reagent. This possibly was caused by deamination of the amino group of the adenine in TPNH.

In preliminary experiments it was found that a protein precipitate collected by 35% $(NH_4)_2SO_4$ saturation of the supernatant liquid from the first calcium phosphate gel adsorption would catalyze a slow disappearance of nitrite. In one experiment 6.8×10^{-3} μM nitrite disappeared in 20 minutes in the presence of the enzyme and TPNH, whereas none disappeared when TPNH was omitted in the reaction or when boiled enzyme was used. The nitrite reductase activity could be inhibited approximately 80% by adding a final concentration of 10^{-3} M $NH_2OH \cdot HCl$ to the reactive mixture. This concentration resulted in 12% inhibition of the nitrate reductase activity. Tests indicated no nitrite formation from incubations of $NH_2OH \cdot HCl$, TPN, and enzyme.

The nitrite reductase system indicated in soybean leaves also has been observed in washed homogenates of tomato roots. This system requires further investigation.

Discussion

The nitrate reductase purified from soybean leaves is similar to that found in Neurospora (**11, 22**). They are not identical, however, as indicated by a variety of evidence. The two enzymes have different pH optima, show marked differences in specificity for reduced coenzymes I and II, behave differently in the purification procedure, show different dissociation properties of the flavin nucleotide prosthetic group, and have different turnover numbers.

It was shown that the activity of the twice-precipitated soybean nitrate reductase was markedly stimulated by FAD, but responded very slightly to FMN. Tests with D-amino acid oxidase and fluorimetric analyses indicated

that enzyme solutions contained FAD and that 89 to 98% of the total flavin present in the enzyme extract was FAD. From this information and the data in table IV, it was calculated that the enzyme extract contained a maximum of 3.4×10^{-5} μM FMN per ml. This or greater quantities of FMN were not sufficient to produce an appreciable stimulation of the activity of the twice-precipitated enzyme in the standard assay procedure. In view of these facts, it seems conclusive that the natural prosthetic group of the enzyme is FAD and not FMN.

The strong inhibition of the activity by *p*-chloromercuribenzoate and reversal by cysteine hydrochloride suggest that a sulfhydryl group is involved in the active surface. This seems a probable explanation for the sensitivity of the enzyme to cupric ions. The pronounced inhibition by potassium cyanide, sodium azide, thiourea and potassium ethyl xanthate indicates the necessity of a metal for enzymatic activity. Since it is well known that deficiencies of Mo and Mn result in nitrate accumulation (**3, 5**), it has been suggested that one of these metals is involved. The answer to this problem, however, remains for future investigation.

The possible physiological significance of this enzyme is obvious. Reduced pyridine nucleotides necessary for nitrate reduction could be readily supplied by the large number of enzyme systems that reduce these coenzymes by oxidation of various substrates. Some of these enzymes that are known to be present in higher plants (**3, 30**) are hexose phosphate dehydrogenase, malic dehydrogenase, glutamic dehydrogenase, isocitric dehydrogenase, formic dehydrogenase, triosephosphate dehydrogenase, and alcohol dehydrogenase. The energy required for the production of TPNH or DPNH by these systems would be derived from the intermediates of carbohydrate metabolism, and, therefore, would offer a logical explanation for the disappearance of carbohydrates that has been observed (**9, 12**) to accompany nitrate reduction *in vivo*.

The energy for nitrate reduction, via reduced coenzymes I and II, is not necessarily derived from the oxidation of carbohydrate intermediates as was demonstrated by the photochemical reduction of nitrate by the following reactions:

$$H_2O + TPN \xrightarrow[\text{and grana}]{\text{light}} 1/2O_2 + TPNH + H^+ \qquad (3)$$

$$TPNH + H^+ + NO_3^- \xrightarrow[\text{reductase}]{\text{nitrate}} NO_2^- + TPN + H_2O \qquad (4)$$

VISHNIAC and OCHOA (**31**) used anaerobic conditions when reaction 3 was utilized in a coupled system to fix carbon dioxide. In the experiments reported in this paper, reactions 3 and 4 catalyzed by grana and nitrate reductase from soybean leaves, proceeded at a relatively rapid rate under aerobic conditions.

Results of these experiments offer a plausible explanation of the direct effect of light on nitrate reduction which has been reported by BÜRSTROM

(**6**). It is interesting to consider that a concentration of 0.02 M nitrate, that was optimum for nitrate reductase activity, resulted in approximately 75% inhibition of the photochemical reduction of TPN. This inhibitory effect may explain the observation of LUNDEGÅRDH (**18**) that leaves of nitrogen-deficient oat plants were much more active photosynthetically than leaves of comparable plants provided with adequate nitrate.

Summary

The purification and properties of an enzyme from soybean leaves is described which catalyzes the reduction of nitrate to nitrite by the oxidation of TPNH or DPNH. Evidence is presented that this or a similar enzyme is present in the six other higher plant species examined.

Experiments show that the enzyme is a flavoprotein with FAD as the prosthetic group. It is sensitive to heavy metal inhibitors, including potassium cyanide, sodium azide, thiourea and potassium ethyl xanthate. Inhibition by *p*-chloromercuribenzoate was reversed by cysteine hydrochloride, which suggests that a sulfhydryl group is present on the active enzyme surface. No inhibition was obtained by use of CO or NaF.

The pH optimum activity is at 6.0. No stimulation of activity is obtained by additions of $ZnSO_4$, $MnSO_4$, $Na_2B_4O_7$, Na_2MoO_4, $MgSO_4$, $FeSO_4$, $FeCl_3$, or $CuSO_4$ at final concentrations of 10^{-4} M.

Determinations of the TPNH that disappeared in the reaction and the quantities of nitrite that appeared concomitantly, indicate that one mole of nitrate is reduced by one mole of the reduced coenzyme.

By use of grana and purified enzyme from soybean leaves, the photochemical reduction of nitrate has been demonstrated. The significance of these results are discussed in relation to previous reports of the effect of light on nitrate assimilation.

No measurable quantity of nitrite disappeared in reaction containing purified nitrate reductase fractions. Disappearance, however, was observed in certain homogenates and other preparations. Nitrite disappearance could be partially inhibited by the addition of 10^{-3} M final concentration of $NH_2OH \cdot HCl$.

Preliminary evidence indicates that TPNH at least is involved in nitrite as well as nitrate reduction.

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