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PROPERTIES OF A PARTICULATE NITRATE REDUCTASE FROM THE NODULES OF THE SOYBEAN PLANT*

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SUMMARY

A particulate fraction has been isolated from *Rhizobium japonicum* cells of soybean nodules which catalyzes the reduction of nitrate to nitrite with either DPNH or succinate. The properties of the complex have been studied with each of these electron donors.

When succinate is used as an electron donor for the nitrate reductase complex, the system exhibits many of the properties of succinic dehydrogenase and succinoxidase which have been reported. These include competitive inhibition by malonate and pyrophosphate, inhibition by fluoride and an activation by phosphate.

The nitrate reductase complex, with either succinate or DPNH as the electron donor was inhibited by Antimycin A, dicumarol, DNP and *p*-chloromercuribenzoate. Inhibition by the latter compound was prevented by either glutathione or cysteine. No evidence has been obtained for the involvement of a flavin in electron transport from either DPNH or succinate to nitrate; however, it is concluded that the failure to demonstrate a flavin requirement is associated with the difficulties in dealing with the particulate system.

From studies of the inhibition of the nodule nitrate reductase by metal chelating agents and from kinetic studies of the cyanide inactivation of the system it is concluded that metal ions are involved in electron transport at two cyanide-sensitive sites with either DPNH or succinate as the electron donor.

When succinate is used as the electron donor for the complex it is suggested that succinic dehydrogenase, a cytochrome, an Antimycin A-sensitive site, two cyanide sensitive sites, and nitrate reductase are involved in the electron transport to nitrate. When DPNH is used as a source of electrons for the system, the evidence indicates involvement of a vitamin K, or a related quinone, a cytochrome, an Antimycin A-sensitive site, two cyanide-sensitive sites, and nitrate reductase.

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References p. 153.

INTRODUCTION

It has been demonstrated that the nitrate reductase from both *Neurospora* and soybean leaves are molybdoflavoproteins with FAD as the prosthetic group[1-3]. The nitrate reductase from soybean leaves catalyzes the oxidation of either DPNH or TPNH by nitrate[2,4], but the *Neurospora* enzyme is relatively specific for TPNH as an electron donor[1,3]. NICHOLAS AND NASON have demonstrated with the *Neurospora* enzyme that both flavin and molybdenum function as electron carriers during the enzymic transfer of electrons from TPNH to nitrate[5,6]. Cytochromes apparently do not participate in electron transfer from substrate to nitrate in these systems.

In contrast to the molybdoflavoprotein nitrate reductases from *Neurospora* and soybean leaves, the nitrate reductases from *Escherichia coli*[8-11] and *Achromobacter fischeri*[14] contain cytochrome components which participate in electron transfer from substrate to nitrate. A soluble nitrate reductase from *Escherichia coli* has been described[7]; however, this system appears to be similar to the nitrate reductases from *Neurospora* and soybean leaf. SATO *et al.*[9,10] have presented evidence suggesting that a cytochrome *b* or b_1 functions in electron transfer in the particulate nitrate reductase from *E. coli*. The cytochrome, which is involved in electron transport from DPNH or TPNH to nitrate, in the nitrate reductase from *A. fischeri* appears to be a specific bacterial cytochrome with absorption bands similar to those of mammalian cytochrome *c*[14]. From *Micrococcus denitrificans* and *Pseudomonas denitrificans* VERNON[12] has purified a relatively low potential cytochrome *b* which is involved in the oxidation of DPNH but not of succinate by nitrate under anaerobic conditions. A very similar cytochrome also has been isolated from an unidentified nitrate-reducing *Pseudomonas*[13].

The properties of the nitrate reductase from soybean nodules[15] have not been established. It has been reported from this laboratory that the nodule nitrate reductase activity is correlated positively with nitrogen-fixing efficiencies of different strains of *Rhizobium japonicum* grown in symbiosis with the host plant[16]. The experiments reported in the present paper deal with some properties of the particulate nitrate reductase from *R. japonicum* cells of soybean nodules.

MATERIALS AND METHODS

Preparation of extracts

Nodules were obtained from 8 to 10 week old soybean plants (*Glycine max.* Merr., variety Lee) grown in sand cultures without combined nitrogen and also from soybean plants grown in the field. All seed were inoculated prior to planting with a commercial inoculum. The nodules were placed in crushed ice immediately after removal from the roots, and all subsequent preparatory operations were carried out at temperatures between 0 and 4°. The harvested nodules were washed thoroughly with cold distilled water and then quickly blotted dry. For each weight of nodules 3 weights of 0.1 *M* potassium phosphate buffer, 0.001 *M* with respect to EDTA, were used for homogenization with a cold mortar and pestle. The mass of the ground nodular tissue was separated from the bacterial cells and liquid by squeezing the homogenate through

Abbreviations: DPNH and TPNH, reduced forms of di- and triphosphopyridine nucleotide respectively; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; PCMB, *p*-chloromercuribenzoate; SN 5949, [2-hydroxy-3-(2-methyloctyl)-1-4-naphthoquinone], EDTA, ethylenediamine-tetraacetate; TRIS, tris(hydroxymethyl)aminomethane; GSH, glutathione.

References p. 153.

four thicknesses of cheesecloth. The resulting filtrate was centrifuged at 12,500 × *g* for 20 min. The supernatant, containing the hemoglobin, was decanted from the sediment. The sediment, consisting almost entirely of bacilli, was washed with a solution (10 ml/g of wet cells) containing 0.1 *M* phosphate buffer at pH 7.0 and 0.001 *M* with respect to both GSH and EDTA. This solution is referred to hereafter as the GSH–EDTA-phosphate buffer. The cells were collected by centrifugation at 30,000 × *g* for 10 min and then were ground for 2 to 3 min with levigated alumina (2 g alumina per g of wet cells) in a cold mortar. After extracting the mixture for 10 min with GSH–EDTA-phosphate buffer at pH 8.3 (5 ml/g of wet cells), the alumina and cell debris were removed by centrifugation at 12,500 × *g* for 20 min. The supernatant was centrifuged at 30,000 × *g* for 5 min and the small amount of sediment obtained was discarded. The resulting supernatant, which contained 5 to 7 mg protein per ml, is designated as Fraction I. All nitrate reductase activity was removed from Fraction I by centrifugation at either 104,000 × *g* for 45 min or 30,000 × *g* for 2 h. This supernatant was discarded and, with the aid of a Ten Broeck homogenizer, the particulate matter was resuspended in GSH–EDTA-phosphate buffer at pH 7.5 (0.2 ml of buffer per ml of Fraction I). This fraction, designated as Fraction II, contained 0.8 to 1.4 mg protein per ml.

Source of chemicals

DPNH (90%), TPNH (90%), synthetic FMN, and FAD (60%) were obtained from the Sigma Chemical Company of St. Louis, Missouri. Benzyl- and methylviologens were obtained from Mann Research Laboratories of New York, N.Y. Methylene blue and the sodium salt of 2, 6-dichlorophenolindophenol were obtained from Eastman Kodak Company of Rochester, N.Y. The leuco form of methylene blue was prepared by bubbling H_2 through the aqueous solution in presence of a small amount of palladium-asbestos. Benzyl- and methylviologens were reduced by sodium hydrosulfite. Antimycin A was purchased from Wisconsin Alumni Research Foundation of Madison, Wisconsin. All other chemicals used were of C.P. grade.

Assays

The rate of nitrate reduction was measured by determining the amount of nitrite formed in the reaction mixture during a 10 min interval by the diazo-coupling procedure[2,17] utilizing N-(1-naphthyl)-ethylenediamine hydrochloride and sulfanilamide. When succinate was used as an electron donor, the reaction mixture consisted of the following: 0.1 ml of 0.01 *M* $NaNO_3$, 0.1 ml of 0.1 *M* phosphate buffer at pH 7.5, 0.1 ml of 0.1 *M* Na succinate, 0.1 ml, or quantities indicated, of Fraction II, and sufficient water to adjust the final volume to 0.5 ml. When DPNH was used as the electron donor, the reaction mixture was identical with that in which succinate was used, with the following exceptions: the phosphate buffer was at pH 7.0 instead of pH 7.5; Na succinate was omitted and, instead, 0.04 ml of $2.3 \cdot 10^{-3}$ *M* DPNH was added. When DPNH or TPNH was employed as the electron donor, the concentration of stock solutions was determined spectrophotometrically using $6.24 \cdot 10^6$ cm^2/mole as the extinction coefficient at 340 mμ[18]. Protein was determined on cell-free extracts by the Folin-phenol method of LOWRY *et al.*[19] using bovine serum albumin as the standard. All spectrophotometric measurements were made with a Beckman spectrophotometer, Model DU. All activities (v), unless otherwise stated, are in

References p. 153.

terms of millimicromoles of nitrite formed in 10 min per mg protein. All reactions were carried out under aerobic conditions and at a temperature of 30°. The activity of the nitrate reductase reaction with either DPNH or succinate as the electron donor was stimulated by anaerobic conditions. As indicated by the results to be presented, valid assays could be conducted under aerobic conditions.

RESULTS

Extent of purification

The specific activity of the nitrate reductase of Fraction II was 8 to 12-fold greater than that of Fraction I. Treatment of Fraction II with lysozyme or trypsin followed by collection of the particles by centrifugation did not result in any net increase in purification. Dispersion of the particles of Fraction II with cholate, deoxycholate or digitonin resulted in complete loss of nitrate reductase activity with either DPNH or succinate as electron donors.

Fraction II contained, in addition to the nitrate-activating system, cytochrome *c* reductase, cytochrome oxidase, and DPNH oxidase.

Effect of protein concentration on activity

The quantity of nitrite formed in 10 min was directly proportional to the concentration of Fraction II using either DPNH or succinate as the electron donor. Zero-order kinetics were obtained for nitrate reductase with either DPNH or succinate as the electron donor.

Effect of pH on activity

The reaction rate, using DPNH as a source of electrons, was about the same between pH 6.0 and pH 7.0, but it decreased fairly rapidly above or below this range of values. No difference in activity could be observed when acetate, phosphate, or TRIS was used as buffer at comparable pH values. The optimum pH, using succinate as the electron donor, was 7.8 and at pH values where phosphate and TRIS buffer could be compared, the activity with phosphate buffer was slightly greater than that with TRIS buffer.

Enzyme stability

Considerable unexplainable variation was found to exist in the stability of the enzyme when stored at — 15° in 0.1 *M* phosphate buffer at a pH of 7.5. Cysteine or GSH and EDTA at 0.001 *M* final concentration failed to eliminate the variation or to enhance the stability of the enzyme during storage. Storage of the enzyme under H_2 also failed to improve the stability. Storage of the Fraction II preparation for two weeks at — 15° generally caused a decrease in activity of 25 to 40% when succinate was used as the substrate and a loss in activity of 50 to 70% when DPNH was utilized in the assay. On some occasions complete loss of activity, with either succinate or DPNH as the electron donor, was observed after storage overnight at either — 15 or 4°. Incubation of the enzyme at 38° for 20 min resulted in complete loss of activity when assayed with DPNH, but incubation of the enzyme under these conditions resulted in a loss in activity of only 20 to 30% when assayed with succinate. Incubation at 38° for 30 min resulted in 90% loss in activity when assayed with succinate.

References p. 153.

Effect of concentrations of substrate and electron donors

The dependence of the velocity of the reaction on the concentrations of nitrate in the assay mixture is illustrated in Fig. 1. The data are presented in the form of a LINEWEAVER-BURK plot[20], and from this curve a Michaelis constant (K_m) of $7.8 \cdot 10^{-5}$ moles/l was calculated. The K_m value for nitrate obtained was essentially the same when either DPNH or succinate was used as the electron donor.

EVANS[15] has indicated that the nitrate reductase of *R. japonicum* from nodules appeared to be specific for DPNH as a source of electrons. The effect of reduced coenzyme concentration on nitrate reduction by the nodule system is shown in Fig. 7. In confirmation of the earlier observations, TPNH was completely inactive as an electron donor. The K_m value for DPNH as estimated from the curve in Fig. 2 is $7.4 \cdot 10^{-5}$ M. This value may serve as an indication of the relative DPNH requirement under the conditions described but in view of the complexity of the system it does not represent a property of the enzyme.

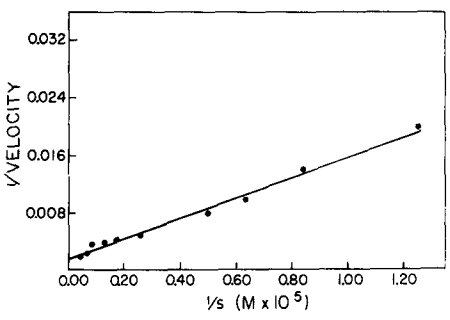

Fig. 1.

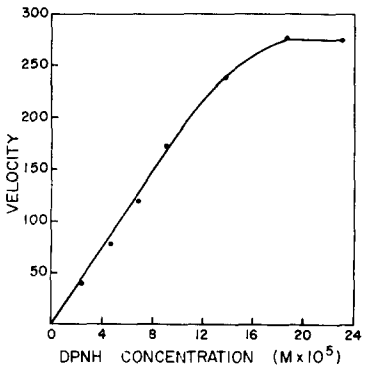

Fig. 2.

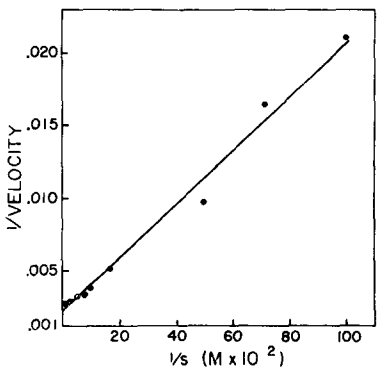

Fig. 3.

Fig. 1. Reciprocal plot of nitrate saturation for nitrate reductase using succinate as the electron donor. The standard assay procedure was used, with the exception that the nitrate concentration was varied as indicated in the double-reciprocal plot.

Fig. 2. Effect of increasing concentrations of DPNH on nitrate reductase activity. The standard assay procedure was used, with the exception that the DPNH concentration was varied as indicated.

Fig. 3. Reciprocal plot of succinate saturation for nitrate reductase. The standard assay procedure was used, with the exception that the succinate concentration was varied as indicated in the double-reciprocal plot.

TANIGUCHI *et al.*[10] have shown that either succinate or formate would serve as electron donors in the particulate nitrate reductase system from *E. coli*. The nodule nitrate reductase is inactive with formate as the electron donor but will effectively oxidize succinate. The influence of various succinate concentrations on the activity of the nodule nitrate reductase is shown in Fig. 3. The K_m calculated from the

References p. 153.

reciprocal plot is $8.4 \cdot 10^{-4}$ M. This value for succinate in the nodule nitrate reductase system compares reasonably well with the K_m values of succinic dehydrogenase from either yeast[21] or mammalian heart[22]. It also is comparable with the K_m values for succinoxidase from rat liver[23] and beef heart[24].

Leucomethylene blue, reduced by palladium-asbestos and H_2, is an effective electron donor for nitrate reduction in this system. In this respect the nodule system is similar to the nitrate reductase from *E. coli*[10], but is different from the system in *A. fischeri*[14]. Benzyl- or methylviologen, reduced by hydrosulfite, will also donate electrons for the nodule nitrate reductase when assayed by the method of SADANA AND MCELROY[14]. Ascorbate, *p*-phenylenediamine, acetaldehyde, and hypoxanthine were inactive as electron donors.

Possible flavin requirement

A number of experiments were performed to determine whether or not there was a flavin requirement for the enzyme. Repeated precipitations of the enzyme with ammonium sulfate at an acid pH, essentially as described by HORECKER[25], caused 85% loss of activity, which could not be restored by preincubation with FAD, FMN, or boiled pig heart extracts. This loss of activity perhaps can be ascribed to adverse pH effects or to an irreversible resolution of flavin from the particulate enzyme complex. Irradiation of the enzyme with ultra-violet light (2535 Å or 3660 Å) for 2 h reduced the activity 50–75% with either DPNH or succinate as the electron donor. Restoration of activity was not accomplished by addition of FMN, or FAD. Addition of boiled Fraction II preparation to the irradiated enzyme, however, often stimulated the activity to extents of 30 and 50% in the succinate and DPNH assay systems respectively. Acriflavin at $2 \cdot 10^{-4}$ M final concentration (without preincubation) inhibited the nitrate reductase activity 46% in the succinate assay system and 40% in the DPNH assay system.

Inhibition by malonate and pyrophosphate

Since succinate was an effective electron donor for the nodule nitrate reductase system, it seemed probable that succinic dehydrogenase was involved in the over-all reaction. It was of interest, therefore, to study the effect of well known competitive inhibitors of succinic dehydrogenase on the nodule enzyme. As indicated by the curves of Fig. 4, the inhibition of nitrate reductase activity by malonate is competitive with respect to succinate. The K_i for malonate was calculated to be $1.7 \cdot 10^{-5} M$, which compares reasonably well with values reported and summarized for succinic dehydrogenase and succinic oxidase of various tissues[26]. Pyrophosphate, another competitive inhibitor of succinic dehydrogenase[27,28], inhibits nodule nitrate reductase when succinate is used as the electron donor (Fig. 5). No inhibition by pyrophosphate was observed when DPNH was used as an electron source.

Sulfhydryl inhibitors

The effect of sulfhydryl binding reagents on the nodule nitrate reductase is shown in Table I. Arsenite and iodoacetate were not inhibitory at the concentrations employed, but relatively low concentrations of PCMB strongly inhibited activity with either DPNH or succinate as the electron donor. Since the observed inhibition by PCMB is prevented by cysteine or GSH, it seems apparent that sulfhydryl groups

References p. 153.

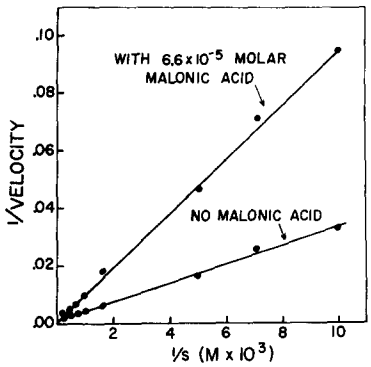

Fig. 4. Competitive inhibition of nitrate reductase by malonate using succinate as electron donor. Sodium malonate (pH 7.5) was included in the assay mixture at the concentration indicated; otherwise, conditions of assay were those of the standard procedure.

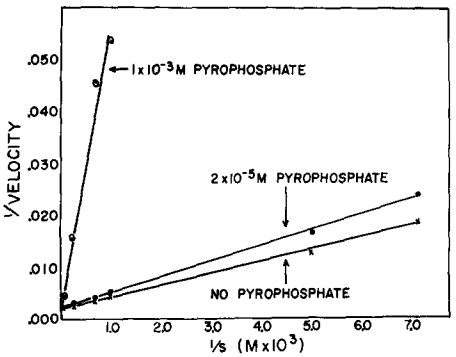

Fig. 5. Competitive inhibition of nitrate reductase activity by pyrophosphate using succinate as the electron donor. Sodium pyrophosphate (pH 7.5) was included in the assay mixture at the concentrations indicated; otherwise, conditions of assay were those of the standard procedure.

are essential for activity. When succinate is used as an electron donor for the nitrate reductase, it is presumed that at least a part of the observed inhibition by PCMB is associated with the succinic dehydrogenase component of the nitrate reductase complex, since sulfhydryl groups generally are required for succinic dehydrogenase activity. Since PCMB inhibition of the nodule nitrate reductase is observed when DPNH is used as an electron donor, it would appear that sulfhydryl groups also are involved at some site in the chain between DPNH and nitrate. In this regard NICHOLAS

TABLE I

INHIBITION OF NITRATE REDUCTASE FROM *Rhizobium japonicum* BY —SH REAGENTS AND ITS REVERSAL BY SULFHYDRYL COMPOUNDS

Reaction mixtures were prepared which contained the inhibitor indicated, Fraction II (GSH omitted in preparation), and all constituents of the standard assay mixture with the exception of electron donor. These were preincubated for 10 min at 0 to 4°, and then the standard assay was initiated by addition of electron donor.

Electron donor	*Addition*	*Final concentration* *M*	*Inhibition* *No sulfhydryl reagent* %	*Glutathione* (10^{-4} *M*) %	*Cysteine* (10^{-4} *M*) %
Succinate	PCMB*	$2 \cdot 10^{-5}$	95	0	0
	PCMB	$5 \cdot 10^{-5}$	100	0	2–3
	PCMB	$1 \cdot 10^{-4}$	100	12	10
	Sodium arsenite	$4 \cdot 10^{-3}$	0	0	0
	Sodium arsenite	$2 \cdot 10^{-2}$	0	0	0
	Iodoacetate	$2 \cdot 10^{-2}$	0	0	0
DPNH	PCMB	$2 \cdot 10^{-5}$	76	0	0
	PCMB	$5 \cdot 10^{-5}$	84	0	0
	PCMB	$1 \cdot 10^{-4}$	87	0	2
	Sodium arsenite	$4 \cdot 10^{-3}$	0	0	0
	Sodium arsenite	$2 \cdot 10^{-2}$	0	0	0
	Iodoacetate	$2 \cdot 10^{-2}$	0	0	0

* *p*-Chloromercuribenzoate.

References p. 153.

AND NASON[3] have concluded that the major role of sulfhydryl groups in the TPNH-nitrate reductase from *Neurospora* is associated with the enzymic reduction of FMN or 2,3', 6-trichlorophenolindophenol by TPNH; however, SADANA AND MCELROY have shown that sulfhydryl groups are essential for the activity of a DPNH-nitrate reductase from *A. fischeri* when reduced benzylviologen was used as a source of electrons. The experiments reported here do not permit a decision on the site of PCMB inhibition when DPNH is used as a source of electrons.

Inhibition by metal-chelating agents

The sensitivity of the nodule nitrate reductase to various metal-binding agents suggests the presence of a metal constituent in the system (Table II). In these experiments, reaction mixtures, containing all reactants except electron donors, were preincubated for 10 min at 0 to 4° in presence of the various inhibitor compounds; then the electron donors were added to initiate the usual procedure. Essentially no differences were obtained in the magnitude of the inhibitions given in Table II when the electron donor was included and the substrate omitted during the preincubation period. The degree of inhibition by various metal-binding agents such as *o*-phenan-

TABLE II

THE EFFECT OF VARIOUS METAL-CHELATING AGENTS ON THE ACTIVITY OF NITRATE REDUCTASE FROM *Rhizobium japonicum*

Reaction mixtures were prepared which contained the inhibitors indicated, Fraction II, and all constituents of the standard assay mixture with the exception of electron donor. These were preincubated for 10 min at 0 to 4°; then the standard assays were initiated by addition of electron donor.

Addition	*Final concentration* *M*	*Inhibition*	
		With succinate %	*With DPNH* %
o-Phenanthroline	$1 \cdot 10^{-3}$	48	50
	$2 \cdot 10^{-3}$	75	70
Sodium azide	$2 \cdot 10^{-5}$	72	—
	$1 \cdot 10^{-4}$	84	96
	$2 \cdot 10^{-4}$	99	100
Versene	$2 \cdot 10^{-2}$	0	0
	$4 \cdot 10^{-2}$	0	0
Thiourea	$1 \cdot 10^{-2}$	0	0
	$2 \cdot 10^{-2}$	0	0
Potassium ethyl xanthate	$1 \cdot 10^{-2}$	16	14
	$2 \cdot 10^{-2}$	51	31
2,2-Bipyridyl	$1 \cdot 10^{-3}$	17	0
	$2 \cdot 19^{-3}$	38	0
Sodium citrate	$1 \cdot 10^{-2}$	0	0
	$2 \cdot 10^{-2}$	0	0
Sodium diethyl dithiocarbamate	$1 \cdot 10^{-2}$	6	0
	$2 \cdot 10^{-2}$	21	0

References p. 153.

throline, sodium azide, and potassium ethyl xanthate was essentially of the same magnitude whether succinate or DPNH was used as a source of electrons.

Dialyses of extracts at 0 to 4° for periods ranging from 4 to 16 h against metal-free phosphate buffer[6], 0.001 *M* with respect to both KCN and GSH, (followed by a 4 h dialysis against metal-free phosphate buffer) completely inactivated the enzyme whether succinate or DPNH was used as the electron donor. This loss of activity could not be restored by the addition of metals such as iron, molybdenum, manganese, cobalt, zinc and copper. Little loss of activity was observed when the extracts were dialyzed for 4 to 16 h against metal-free phosphate buffer[6] in the absence of cyanide.

The time course of the inactivation of nodule nitrate reductase by cyanide where succinate is used as the electron donor is illustrated in Fig. 6. Similar inactivation kinetics were obtained in experiments where DPNH was used in the assay system. Although the inactivation kinetics by cyanide appear to be of first order, they do not follow the first order expression reported by TSOU[28] for succinic dehydrogenase (Fig. 7). The fact that the two curves representing 0.001 *M* and 0.005 *M* concentrations of KCN fail to intercept the ordinate at a common point at zero time suggests an instantaneous inactivation at one or more sites in addition to a slow first order inactivation at another site. The inactivation by cyanide is irreversible and, in contrast with the observations of TSOU on succinic dehydrogenase of beef heart[28], was accelerated rather than prevented by including succinate (0.01 *M*) in the incubation mixture. These experimental conditions, however, were not comparable to those used by TSOU.

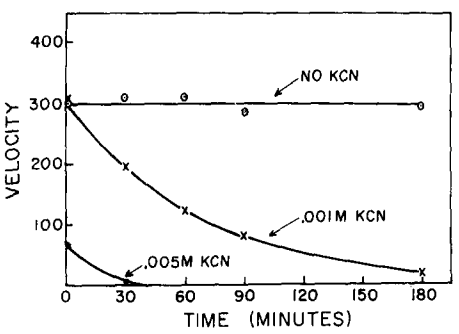

Fig. 6. Time-course of inactivation of nitrate reductase by cyanide. The Fraction II preparation was incubated at 4° with the indicated final concentrations of KCN. At the indicated time intervals aliquots were withdrawn and the standard assay, using succinate as the electron donor, was initiated by addition of an aliquot of the Fraction II–KCN mixture.

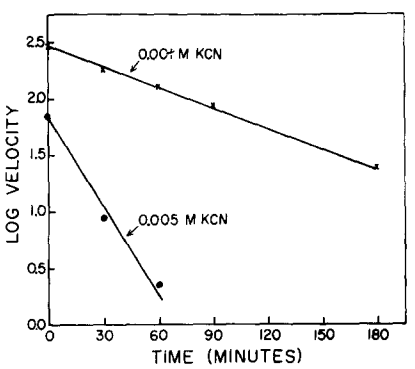

Fig. 7. The logarithm of velocity shown in Fig. 6 plotted against time.

Other inhibitors

A specific requirement for 2-methyl-1,4-naphthoquinone (menadione) for *E. coli* pyridine nucleotide nitrate reduction has been shown by WAINWRIGHT[11]. A requirement for this compound has been demonstrated for the nitrate reductase in acetone powders of *R. japonicum* collected from nodules[29], and the details of these experiments will be reported at a later date. The menadione requirement is exhibited in the assay system containing DPNH as an electron donor but not in the system containing

References p. 153.

succinate. Dicumarol is a competitive inhibitor with respect to menadione in the assay systems containing acetone powder suspensions of *R. japonicum*. WAINWRIGHT[11] has suggested that the inhibition by dicumarol of the *E. coli* nitrate reductase also is competitive with respect to menadione. It was of interest, therefore, to examine the effect of dicumarol on the activity of particulate nitrate reductase (Fraction II) from *R. japonicum* of nodules. A summary of these inhibition experiments is presented in Table III. The observed inhibitions by dicumarol (Table III) are in contrast with observations on the nitrate reductase from *A. fischeri*[14] where dicumarol failed to affect activity in assays using DPNH as a source of electrons.

TABLE III

EFFECT OF NONSPECIFIC INHIBITORS ON THE ACTIVITY OF NITRATE REDUCTASE FROM *Rhizobium japonicum*

In these experiments standard conditions of assay were modified to include the additions indicated. With the Antimycin A experiments an ethanol control was necessary since the ethanol solvent for Antimycin A was inhibitory. The values for the inhibition by Antimycin A were corrected for the inhibition by ethanol.

Addition	*Final concentration*	*Inhibition*	
		With succinate %	*With DPNH* %
Dicumarol	$4 \cdot 10^{-5}$ *M*	38	51
	$1 \cdot 10^{-4}$ *M*	64	70
Antimycin A	10 μg	30	24
	50 μg	74	90
2,4-Dinitrophenol	$5.4 \cdot 10^{-4}$ *M*	23	36
	$2.7 \cdot 10^{-3}$ *M*	50	60
Potassium fluoride	$2 \cdot 10^{-3}$ *M*	27	0
	$1 \cdot 10^{-2}$ *M*	33	0
	$2 \cdot 10^{-2}$ *M*	67	0

The effect of Antimycin A on the *R. japonicum* nitrate reductase complex is shown in Table III. Essentially no difference in the magnitude of inhibition resulted from the substitution of DPNH for succinate in the assay system. No inhibition was obtained by the addition of the naphthoquinone, SN 5949, to assays containing either DPNH or succinate. Since it is believed[30,31] that Antimycin A blocks electron transfer from cytochrome *b*, the data presented in Table III suggests the participation of a cytochrome *b* component in the nitrate reductase complex when either DPNH or succinate is used as the electron donor. In this respect the *R. japonicum* nitrate reductase complex is similar in some respects to the *E. coli* nitrate reductase[10] but not to the system in *A. fischeri*[14].

At relatively high concentrations, dinitrophenol inhibits the activity of the nitrate reductase complex (Table III). Fluoride also inhibits activity in the succinate assay system but fails to inhibit when DPNH is used as a source of electrons. It seems probable that fluoride inhibition is associated with the succinic dehydrogenase portion of the complex.

References p. 153.

Activation and electrolyte requirement

During the experimentation with the particulate nitrate reductase (Fraction II) prepared from nodules from the field, it was observed that the addition of potassium phosphate buffer to the enzyme preparations resulted in a striking decrease in the turbidity of the suspensions. Similar effects of phosphate on particulate preparations have been observed by others[32]. The effect of various phosphate and chloride concentrations on the absorbancy at 620 mμ of a suspension of nodule nitrate reductase (Fraction II) is shown in Fig. 8. It is apparent from these curves that the maximum clarification was obtained at a concentration of approximately 0.06 *M* phosphate and that various chloride concentrations had little influence on the absorbancy.

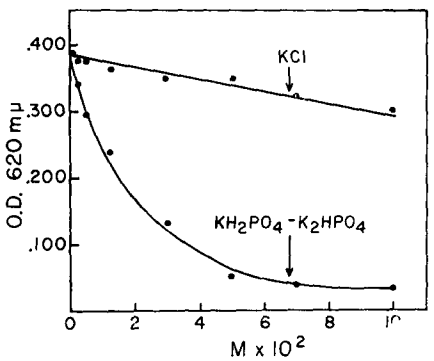

Fig. 8. The effect of increasing KCl and phosphate concentration on absorbancy of a Fraction II preparation. A Fraction II preparation (0.1 ml), resuspended in 0.001 *M* phosphate buffer pH 7.5, was incubated at room temperature (25°) for 5 min with the indicated final concentrations of either KCl (pH 7.5) or KH_2PO_4–K_2HPO_4 (pH 7.5). The final volume was adjusted to 1.0 ml with distilled water. After the prescribed incubation period the absorbancies of the mixtures were determined.

Arsenate also caused a decrease in the absorbancy of the enzyme suspension, the magnitude of which was comparable to that caused by phosphate. Although these effects on the absorbancy of Fraction II were routinely observed with extracts prepared from nodules from the field, the effects on absorbancy were less pronounced with extracts prepared from nodules of plants grown in sand cultures in the greenhouse.

When the Fraction II preparation was resuspended in 0.001 *M* phosphate buffer, pH 7.5, and assayed for nitrate reductase activity with either DPNH or succinate as the electron donor, virtually no activity was obtained unless certain salts were included in the assay. From the data in Table IV it is apparent that there is a requirement for either phosphate or arsenate for nitrate reductase activity when succinate is used as the electron donor. On the other hand when DPNH is used as the source of electrons, a non-specific requirement for salt is exhibited. The influence of various concentrations of phosphate on the activity of the succinate–nitrate reductase complex was determined and the results are illustrated in Fig. 9. Typical Michaelis-Menton kinetics were exhibited. The activation constant (K_A) was calculated from the reciprocal plot of Fig. 9 and a value of $5.8 \cdot 10^{-2}$ *M* was obtained.

The specific effect of phosphate or arsenate on the succinate–nitrate reductase complex and the non-specific effect of salt on the DPNH–nitrate reductase complex was shown repeatedly with extracts (Fraction II) prepared from nodules from the field. When Fraction II preparations were prepared from nodules of plants cultured in the greenhouse on a N-free nutrient solution, an absolute requirement for phosphate or arsenate for the activity of the succinate nitrate reductase could not be demonstrated, however, stimulation of activity by phosphate was consistently observed.

References p. 153.

The failure to observe the absolute requirement for phosphate or arsenate on the succinate–nitrate reductase complex preparation from nodules of soybeans from sand culture perhaps may be ascribed to differences in the endogenous level of phosphate ion concentration in nodules from the two different sources.

TABLE IV

THE EFFECT OF ELECTROLYTES AND MANNITOL ON THE RELATIVE ACTIVITY OF NITRATE REDUCTASE FROM *Rhizobium japonicum*

Addition	*Final molarity*	*Relative activity*	
		With succinate	*With DPNH*
Potassium phosphate, pH 7.5	$2 \cdot 10^{-1}$	100	100
Sodium arsenate, pH 7.5	$2 \cdot 10^{-1}$	125	100
Potassium chloride	$2 \cdot 10^{-1}$	0	100
Sodium chloride	$2 \cdot 10^{-1}$	0	95
Sodium sulfate	$2 \cdot 10^{-1}$	2	100
Potassium acetate	$2 \cdot 10^{-1}$	2	88
Ferrous sulfate	$2 \cdot 10^{-4}$	0	0
Sodium molybdate	$2 \cdot 10^{-4}$	0	3
Mannitol	$2 \cdot 10^{-2}$	0	4

When succinate was used as the electron donor in these experiments, the reaction mixture consisted of the following: 0.1 ml of 0.01 *M* $NaNO_3$, 0.1 ml of 0.1 *M* Na succinate, pH 7.5, 0.1 ml of a Fraction II preparation resuspended in 0.001 *M* phosphate buffer at pH 7.5, additions as indicated, and sufficient water to adjust the final volume to 0.5 ml. When DPNH was used, the reaction mixture was the same as that used for succinate except for the following: 0.1 ml of 0.001 *M* phosphate buffer, pH 7.0, was included, Na succinate was replaced with 0.04 ml of $2.2 \cdot 10^{-3}$ *M* DPNH, and the additions were at pH 7.0 unless otherwise indicated.

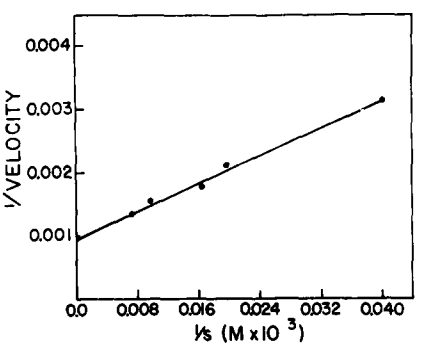

Fig. 9. Reciprocal plot of phosphate saturation for nitrate reductase using succinate as the electron donor. A Fraction II preparation, resuspended in 0.001 *M* phosphate buffer at pH 7.5, was assayed with succinate as the electron donor. The standard assay procedure was used, with the exception that the concentration of phosphate buffer in the assay mixture was varied as indicated in the double-reciprocal plot. The term 1/S represents merely the reciprocal of phosphate concentration and does not imply that phosphate is a substrate.

Phosphate, but not arsenate, activation of succinic oxidase[34–38] and succinic dehydrogenase of beef heart[33,37], but not of yeast[38], has been observed. In addition a phosphate requirement, replaceable by arsenate, tellurate, or selenate, has been observed in the pyridine–nucleotide nitrate reductase from *Neurospora*[39,40]. From the results presented here on the nodule nitrate reductase we cannot specify the site of phosphate or arsenate action. It would appear, however, that a portion of the phosphate or arsenate stimulation may be associated with the succinate-activating portion of the system. Additional work on the site(s) of phosphate stimulation is in progress. It is of interest, however, that maximum activation by phosphate or arsenate of succinate–nitrate reductase was observed only when a pronounced decrease of absorbancy of Fraction II was observed.

References p. 153.

DISCUSSION

From the reports in the literature it seems apparent that there are diversified pathways for the transport of electrons from donors to nitrate in the organisms that have been investigated. The nitrate reductases from *Neurospora*[5,6] and soybean leaves[2,4] comprise systems whereby electrons are transferred from reduced pyridine nucleotides to nitrate via a molybdoflavoprotein. There is no evidence for the existence of a cytochrome constituent in these systems. On the other hand, there seems to be conclusive evidence that *E. coli*[10], *A. fischeri*[14], *M. denitrificans*, *P. denitrificans*[12] and a *Pseudomonas*[13] contain nitrate reductase with a cytochrome component which functions in the transfer of electrons from donor to nitrate. These nitrate reductase complexes with cytochrome components, undoubtedly involve oxidation and reduction of other cofactors such as flavin and metal ions; however, involvement of these has not been demonstrated in all cases. SADANA AND MCELROY[14] have indicated with the *A. fischeri* nitrate reductase that the cytochrome component may be bypassed provided reduced benzylviologen is used as an electron donor.

From the evidence presented here it would appear that the nitrate reductase complex from *R. japonicum* collected from nodules also may contain a functional cytochrome constituent. This presumption is based primarily on the fact that with either DPNH or succinate as the electron donor, the activity of the enzyme is inhibited by Antimycin A. This observation suggests that the Antimycin-sensitive site is common in the routes of electron transport from either of these donors.

A direct demonstration of the involvement of a cytochrome in nitrate reduction by spectrophotometric means has been complicated by the pronounced light-scattering of the Fraction II preparation. It has been shown by difference spectra, using a Beckman DK spectrophotometer, however, that the addition of succinate to the Fraction II preparation in an anaerobic cuvette causes the appearance of a broad band in the region from 415 to 425 mμ. The addition of nitrate from the side arm of the cuvette resulted in a decrease in the Soret band to an extent of 0.008 OD units indicating a change in the steady-state of what is considered to be a cytochrome component.

In general the nitrate reductase complex from *R. japonicum* of nodules compares most favorably with the particulate nitrate reductase complex from *E. coli*. In this connection the most obvious physiological role of nitrate reductase of *R. japonicum* is that it is involved in "nitrate respiration" rather than in nitrate assimilation. No nitrite reduction was observed using either Fraction I or Fraction II.

It has been reported that the nitrate reductase of various *R. japonicum* strains is correlated positively with nitrogen-fixing efficiencies of the various strains in symbiosis with the host plant[16]. The relation of the nitrate reductase complex from *R. japonicum* of nodules to symbiotic nitrogen fixation is not clear. Further experimentation on the possible role of nodule nitrate reductase in symbiotic nitrogen fixation as well as other properties, including specificity of electron acceptor, of the particulate and soluble enzyme will be presented elsewhere.

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