STUDIES ON NITROGEN METABOLIZING ENZYMES FROM BACTERIAL SOURCES

(Nitrate and Nitrite Reductases from Achromobacter fischeri)

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This is to certify that the work incorporated in the thesis entitled: "Studies on nitrogen metabolizing enzymes from bacterial sources (nitrate and nitrite reductases from Achromobacter fischeri)" is the original work of Mr. Bashir M. Khan and was carried out under my supervision. The work is suitable for submission for the award of Ph.D. degree in Biochemistry.

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ABBREVIATIONS

BV, BVH	Benzyl viologen and its reduced form
DEPC	Diethylpyrocarbonate
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
ESR	Electron spin resonance
EPR	Electron paramagnetic resonance
FMN, $FMNH_2$	Flavin mononucleotide and its reduced form
FAD, $FADH_2$	Flavin adenine dinucleotide and its reduced form
Fd	Ferredoxin
Fld	Flavodoxin
GHz	Giga hertz
g	Tensor (characteristic of EPR spectra)
h	Hour or hours
L	Litre or litres
min	Minute or minutes
M_r	Relative molecular mass
MHz	Mega hertz
MV, MVH	Methyl viologen and its reduced form
NMR	Nuclear magnetic resonance
p-HMB	p-Hydroxymercuribenzoate
p-CMS	p-Chloromercuribenzene sulfonic acid
PMSF	Phenyl methyl sulfonyl fluoride
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
2-ME	β-Mercaptoethanol

CONTENTS

CHAPTER I

INTRODUCTION

GENERAL INTRODUCTION

Nitrogen is one of the major and essential constituent elements of life. It is available in various oxidized states in nature and can be utilized by plants and some of the microorganisms to the level of ammonia and amino groups which are ultimately responsible for providing organic nitrogen to many heterotrophic forms of life.

Nitrogen and carbon constitute approximately 2% and 40% of the dry weight of plant material, respectively. Based upon the approximate analysis and the estimated 200×10^9 tons of carbon fixed annually on a worldwide scale (Galston, 1961), 10×10^9 tons of nitrogen are incorporated into plant material. Burns and Hardy (1975) estimated that a total of 0.175×10^9 tons of dinitrogen is fixed in the biosphere annually. From these estimates it can be deduced that a large amount of nitrogen in plants is derived from nitrate or ammonium assimilation.

Nitrogen is mostly taken up as nitrate in plants and reduced to ammonia before being assimilated into amino acids. Although ammoniacal fertilizers are used almost exclusively, the ammonia derived from these fertilizers is oxidized to nitrate by soil microorganisms. The use of nitrogen fertilizers plays a predominant role in achieving the high yield of crops required to meet the world's food needs (Fowden, 1979; Hageman, 1979). However, the utilization of nitrogenous fertilizers, which are manufactured by high-cost technology,

is poor (Subbarao, 1977). This necessitates the development of an understanding of factors which regulate their uptake and assimilation.

Reduction of nitrate to ammonia is catalyzed by two enzymes, nitrate reductase and nitrite reductase. In green tissues assimilation of nitrate is intimately linked with photosynthetic reactions not only for the reduction of nitrate to ammonia but also for the generation of carbon compounds which are required for the incorporation of ammonia into amino acids. It has been suggested that nitrate reductase has a primary role in regulating the whole nitrogen metabolism (Vennesland and Guerrero, 1979; Hewitt et al., 1979; Losada et al., 1981), and the inflow of nitrate nitrogen into amino acids can be controlled by regulating the activity of nitrate reductase because it is (a) the first enzyme in the pathway, (b) substrate inducible, (c) relatively unstable both in vivo and in vitro especially when subjected to water stress or high temperature, and (d) its activity, relative to other enzymes in the pathway, is low and its K_m for nitrate is high (Beevers and Hageman, 1969).

General aspects of inorganic nitrogen metabolism

The essential features of inorganic nitrogen metabolism center about oxidation-reduction reactions. The nitrogen atom has a variety of oxidation states which range from + 7 to - 3 (Table 1). However, of all the oxidation states of nitrogen, nitrate, molecular nitrogen and ammonia are most widely distributed in nature.

Table 1: The oxidation-reduction states of some nitrogen compounds (Nicholas, 1963)

Oxidation-reduction state of nitrogen atom	Formula	Name
+ 7	N_2O_7	Nitrogen peroxide
	HNO_4	Pernitric acid
+ 6	NO_3	Nitrogen peroxide
	H_2NO_4	Pernitrous acid
+ 5	N_2O_5	Nitrogen peroxide
	HNO_3	Nitric acid
+ 4	N_2O_4	Nitrogen tetroxide
	NO_2	Nitrogen dioxide
+ 3	N_2O_3	Nitrogen sesquioxide
	HNO_2	Nitrous acid
+ 2	NO	Nitric oxide
	H_2NO_2	Hydronitrous acid
+ 1	NOH	Nitroxyl
	N_2O	Nitrous oxide
	$H_2N_2O_2$	Hyponitrous acid
	$NO_2:NH_2$	Nitramide; imido nitric acid
	$NH(OH)_2$	Dihydroxy ammonia
0	N_2	Nitrogen
	OH.NH.NH.OH	Dihydroxyl hydrazine
- 1	NH_2OH	Hydroxylamine
- 2	$H_2N.NH_2$	Hydrazine
- 3	NH_4OH	Ammonium hydroxide

Some autotrophic soil bacteria oxidize reduced forms of inorganic nitrogen to more oxidized forms: Nitrosomonas oxidizes ammonia to nitrite, and Nitrobacter oxidizes nitrite to nitrate. Such nitrification reactions serve as energy-yielding systems for these organisms. On the other hand certain species of bacteria reduce oxidized forms of inorganic nitrogen, such as nitrate or nitrite, to more reduced forms. Denitrification is a typical reaction in which the reduced products are gaseous compounds, such as NO, N_2O, and molecular nitrogen. Molecular nitrogen is utilized by certain species of bacteria including symbiotic and free-living cells by a process known as nitrogen fixation. The combined organic nitrogen compounds are decomposed by the cells in which they are synthesized or by other cells to simpler compounds ultimately to amino or other nitrogenous compounds. Therefore, nitrogen is utilized by living cells in a cyclic process.

The literature reviewed in this Chapter deals mainly with studies on nitrate- and nitrite reductases from bacteria, fungi and higher plants. Only a brief reference has been made to the significance of the nitrate metabolism. No attempt has been made to give an exhaustive review. The reviews on the subject by Nason and Takahashi (1958), Nason (1962), Takahashi et al.(1963), Hewitt and Nicholas (1964), Beevers and Hageman (1969), Payne (1973), Hewitt (1975), Hageman and Reed (1980), Vega et al. (1980), and Guerrero et al. (1981) have been of great help in writing this Chapter.

Metabolism of nitrate

The biological reduction of nitrate to nitrite occurs in bacteria, fungi, algae, higher plants and even in animal tissues. A number of classifioations have been proposed for various types of nitrate reduction (Nason and Takahashi, 1958; Verhoeven, 1956; Sato, 1956; Pichinoty, 1966; Pichinoty *et al.*, 1969), but none has been found satisfactory. Jensen (1904) suggested five categories according to the products of the reaction, whereas Verhoeven (1956) described three types of nitrate reduction as (a) "assimilation" in which nitrate is reduced only for the elaboration of the nitrogenous cell material, (b) "incidental dissimilation" in which nitrate acts as a non-essential hydrogen acceptor, (c) "true dissimilation" in which nitrate acts as the essential hydrogen acceptor which enables the organism to grow. An entirely different classification based on the function of the cytochrome system, was given by Sato (1956). He classified nitrate reducers into three categories: (a) whose cytochromes participate in nitrate reduction, the cytochromes involved, however, differ from species to species. Another important characteristic common to this group is the strong inhibitory action of oxygen on nitrate reduction, (b) those whose cytochromes do not participate in nitrate reduction, (c) those, that lack cytochrome. Takahashi *et al.* (1963) considered nitrate reduction as: (i) nitrate respiration, (ii) nitrate assimilation, and (iii) nitrate fermentation as illustrated in Table 2. However, it is now known that obligate anaerobes

Table 2: Classification of nitrate reducing systems

Category	Electron transport system	Pathway of reduction	Source
1. Nitrate respiration (facultative anaerobes)	Participation of cytochromes (particulate fraction)	1. Nitrate → Nitrite (Nitrite accumulating reaction).	Escherichia coli, Corynebacterium diphtheriae, Staphylococcus aureus, Serratia marcescense
		2. Nitrate → Nitrogen (denitrification)	Denitrifers
		3. Nitrate → Ammonia (Ammonia producing reaction)	Bacillus pumilus, Micrococcus, Bacillus licheniformis, Achromobacter fischeri
2. Nitrate assimilation (aerobes)	No participation of cytochromes (soluble molybdeflavo protein)	Nitrate → Ammonia → protein ← ←	Bacillus subtilis, Yeast, Azotobacter vinelandii, E. coli, Neurospora, Soybean leaves
3. Nitrate fermentation (obligate anaerobes)	No cytochrome	Nitrate → Nitrite	Clostridium welchii, Soybean cotyledon

can also synthesize cytochromes (Sadana and Jagannathan, 1954; Gibson and Larsen, 1955; Sadana and Jagannathan, 1956; Postgate, 1956). Garrett and Nason (1967) and Solomonson and Vennesland (1972) have concluded that the distinction between assimilatory reduction and respiratory nitrate reduction can not be made on the basis of the presence or absence of cytochromes.

Fewson and Nicholas (1961a)and Nason (1962) proposed that nitrate reduction can best be distinguished into two major types: (a) Nitrate assimilation, in which nitrate and its reduction products are reduced to ammonia for the biosynthesis of nitrogen containing components, and (b) nitrate respiration or dissimilation, in which nitrate and/or its reduction products serve(s) as the terminal electron acceptors in place of oxygen, usually under anaerobic or partially anaerobic conditions. Depending upon the organism and its environment, nitrate may be reduced to nitrite, or in a series of steps, to more reduced forms of nitrogen. If molecular nitrogen, nitric oxide or nitrous oxide is the end product of the nitrate reduction, the process is called denitrification. The respiratory nitrate reduction is inhibited by oxygen. Because of the obvious physiological and enzymological similarities to oxygen respiration, it would be expected that nitrate respiration involves energy yielding reactions which under given conditions are necessary for the growth and well-being of the organism. One step reduction of nitrate is known to support the growth of various bacteria (Nason, 1962;

Matsubara, 1971). Nitrate reduction is reported to be linked to oxidative phosphorylation (Yamanaka et al., 1962; Ohnishi, 1963; Naik and Nicholas, 1966). Recently ATP production coupled to the denitrification of nitrate in Rhizobium japonicum and its bacteroids has been reported (Bhandari et al., 1984).

Nitrate metabolism in Achromobacter fischeri has been studied in this laboratory and is found unique in that the product of nitrate metabolism is ammonia but both the metabolism of nitrate and the synthesis of nitrate reductase and nitrite reductase are greatly inhibited by oxygen (Prakash and Sadana, 1973). Achromobacter nitrate reductase involves the participation of bacterial cytochrome c_{551} in the electron transport chain. Furthermore, the nitrite reductase of A. fischeri is itself a heme-containing protein (Prakash et al., 1966). In view of the cytochrome participation and the apparent competition by oxygen for the electrons in the conversion of nitrate to nitrite and further nitrite to ammonia, nitrate metabolism of A. fischeri apparently exhibited the characteristics of the respiratory-type (Sato, 1956). However, since the product of nitrate reduction is ammonia, it should belong to the assimilatory-type.

Nitrate reductase

Nitrate reductase catalyzes the reduction of nitrate to nitrite, the first step involved both in the assimilatory as well as dissimilatory nitrate reductions, according to the

following equation:

$$NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O$$

Many species of bacteria are able to reduce nitrate to nitrite. The actual agency of this reduction, however, remained obscure until Quastel et al. (1925) pointed out the existence of a specific enzyme acting on nitrate in resting bacteria. Yamagata (1938, 1939) obtained a cell-free preparation of this enzyme and proposed the name "Nitrate reductase" instead of "Nitratase" which has been previously used. Depending upon the type of nitrate reduction carried out by the organism, nitrate reductases can be divided into two major groups: (i) assimilatory nitrate reductase and (ii) dissimilatory or respiratory nitrate reductase.

Pichinoty (1964a, b; 1965) reported the existence of two types of nitrate reductase, A and B, in nitrate reducing bacteria which differ in some of their properties, particularly in their behaviour towards chlorate. Chlorate is the substrate of A and an inhibitor of B. He classified nitrate reducing bacteria into three groups in accordance with their possessing A, B and A and B. Nitrate reductase A, in general, belongs to the respiratory type and is membrane bound. Its formation is induced by nitrate and repressed by oxygen. The enzyme B (soluble), depending upon the species, has assimilatory (Pseudomonas putida, Micrococcus denitrificans) or respiratory (Providentia alcalifaciens, Aeromonas hydrophila) function (Pichinoty and Piechaud, 1968).

Two types of enzyme B are discernible in several species of bacteria. Enzyme B_{α} is activated by 1 M NaCl, KCl or CsCl whereas B_{β} is not (Pichinoty, 1971). A separate chlorate-reducing enzyme, designated enzyme C, which does not reduce nitrate, has been reported.

In Escherichia coli, nitrate reductase has a multifunctional character (Sato, 1956; Nicholas, 1963). It can perform simultaneously two different types of nitrate metabolism, nitrate respiration (anaerobiosis) and apparent nitrate assimilation (aerobiosis). The anaerobic nitrate reduction does not proceed beyond the nitrite stage and is profoundly retarded by oxygen, reflecting the character of nitrate respiration. However, it is not known whether the same nitrate reductase performs the two functions. In Aerobacter aerogenes, there is only one nitrate reductase which has a respiratory role under anaerobic condition and an assimilatory function under aerobic condition (Pichinoty, 1965; Stouthamer, 1967). On the basis of differences in factors regulating the synthesis of the enzyme that carried out two different functions, Van'T Riet et al. (1968) raised the possibility of two different nitrate reductases in A. aerogenes. In M. denitrificans, however, the existence of two nitrate reductases, one having respiratory and the other an assimilatory function has been reported (Pichinoty, 1964c).

In addition three different molybdenum-containing enzymes from animal tissues, namely xanthine oxidase, aldehyde oxidase, and xanthine dehydrogenase, are also known to catalyze the

reduction of nitrate to nitrite, although this is not considered to be their primary or physiological function (Bray, 1963; Nason, 1963). Ivanova and Pieve (1973) have reported that horseradish peroxidase can also catalyze nitrate reduction at a very high rate using diethyldithiocarbamate and sulfite mixture as electron donor. ESR studies in combination with redox titrations have shown that nitrite oxidase has some strong similarities, including the presence of molybdenum, with the respiratory nitrate reductase of a denitrifying bacterium (Ingledew and Halling, 1976). Indeed in cell-free membrane vesicle preparation from Nitrobacter, under anaerobic conditions, the nitrite oxidase can act as a nitrate reductase when a suitable reductant such as NADH is present (Aleem, 1970; 1977; Aleem and Sewell, 1981).

1. Assimilatory nitrate reductase

One of the earliest investigations reported by Kastle and Elvov (1904) consisted of a detailed and critical examination of potato tuber extract capable of catalyzing the reduction of nitrate to nitrite. The striking resemblance of this enzyme system to the aldehyde oxidase of potato, studied many years later by Bernheim (1928) and Bhagwat (1939), suggested that the two activities are catalyzed by the same or closely related proteins. It was subsequently shown that the aldehyde oxidase of animal tissues is a molybdo-flavoprotein (Mahler et al., 1954) which can catalyze the reduction of nitrate to nitrite, a property attributed to molybdoproteins in general (Mahler and Green, 1954).

Assimilatory nitrate reductases can be distinguished in two major groups based on the specificity of electron donors: (a) ferredoxin-dependent nitrate reductase, which is typically present in cyanobacteria (blue-green algae), and also presumably in chemoorganic and photosynthetic bacteria, and (b) pyridine nucleotide-dependent nitrate reductase, which is found in eukaryotic organisms. The differential specificity for the physiological reductant is correlated with differences in their prosthetic groups and physico-chemical properties. Also whereas ferredoxin-nitrate reductase of photosynthetic prokaryotes seems to be tightly bound to photosynthetic membrane, the pyridine nucleotide-dependent enzyme of eukaryotes is a soluble enzyme (Losada and Guerrero, 1979; Vennesland and Guerrero, 1979).

(a) Ferredoxin-nitrate reductase

Nitrate reductase from cyanobacteria Anabaena cylindrica (Hattori, 1970), Anacystis nidulans (Candau, 1979; Manzano et al., 1976), Nostoc muscorum (Ortega et al., 1976), Plectonema boryanum (Ida and Mikami, 1983; 1984) can not accept electrons directly from NAD(P)H but is rather dependent on reduced ferredoxin as the physiological electron donor. The reaction catalyzed by cyanobacterial nitrate reductase can thus be written as:

$$NO_3^- + 2Fd_{red} + 2H^+ \xrightarrow{2e^-} NO_2^- + 2Fd_{ox} + H_2O$$

$$\Delta G_o', \text{ pH } 7.0 = -38.6 \text{ Kcal.mol}^{-1}$$

Also reduced flavodoxin (a low molecular weight flavoprotein which physiologically substitutes for ferredoxin under conditions of iron starvation) can act as an electron source for the reduction of nitrate to nitrite catalyzed by Anacystis nitrate reductase (Manjano, 1977; Candau, 1979).

The nitrate reductases from both chemoorganic bacteria Azotobacter chroococcum (Guerrero et al., 1973), Acinetobacter calcoaceticus (Villalobo et al., 1977), R. japonicum (Daniel and Gray, 1976), Azotobacter vinelandii (Bothe and Haeger, 1981) and photosynthetic bacteria Rhodopseudomonas capsulata (Alef and Klemme, 1979), Ectothiorhodospira shaposhnikovii (Malofeeva et al., 1975), Rhodopseudomonas sphaeroides (Norma and Cardenas, 1982) can use reduced ferredoxin as the physiological electron donor. These enzymes are active with reduced viologens, but they can not use reduced pyridine nucleotides as reductant.

By using affinity chromatography with ferredoxin-Sepharose gel as the main step (Manjano et al., 1978) ferredoxin-nitrate reductase of Anacystis has been purified to homogeneity and partly characterized. The enzyme is a molybdo-protein having only one polypeptide chain with a relative molecular mass of 75,000. From the characteristics of the absorption spectra of homogeneous preparations of the enzyme the presence of either flavin or cytochrome in its molecule can be excluded (Candau, 1979). Whether this nitrate reductase contains non-heme iron remains to be determined as does the number of molybdenum atoms per enzyme molecule. It has been demonstrated

that Mo is an essential component for the catalytic activity of the enzyme. The Anacystis ferredoxin-nitrate reductase exhibits exceptionally high pH optimum (about 10.5) with dithionite-reduced methyl viologen as the electron donor (Manzano et al., 1976). Cyanide and p-HMB are powerful inhibitors of Anacystis nitrate reductase. The inhibition of this enzyme by iron-binding agents and the apparent lack of heme in the enzyme molecule suggest the participation of nonheme iron in its catalytic activity (Candau, 1979).

Ferredoxin-nitrate reductase from cyanobacterium P. boryanum has been purified recently using zinc form of chelating Sepharose 6B as the final step of purification (Ida and Mikami, 1984). Purified enzyme showed absorption spectrum characteristic of iron-sulfur protein. The relative molecular mass of the enzyme was 80,000. It showed a high pH optimum (pH 10.5) as in case of Anacystis enzyme (Ida and Mikami, 1983).

Nitrate reductase from photosynthetic bacterium R. sphaeroides has been purified and characterized recently (Norma and Cardenas, 1982). The enzyme showed low specific activity (0.24 units per mg protein). A relative molecular mass of approximately 100,000 was estimated. The calculated frictional ratio of 1.33 indicated a slightly elongated shape of the enzyme. Studies with inhibitors and metal antagonists indicated that molybdenum and possibly iron participate in the enzymatic reduction of nitrate. The ferredoxin-nitrate reductase from R. capsulata has a relative

molecular mass of 185,000 and is composed of two subunits of 85,000 dalton. Molybdenum and heme iron has been detected in the enzyme molecule (Alef and Klemme, 1979).

Nitrate reductases from the aerobic chemoorganic bacteria A. chroococcum and A. calcoaceticus have been characterized partly and shown to be molybdoproteins of about 100,000 dalton whose activity is inhibited by cyanide and p-HMB and stimulated by cyanate (Guerrero and Vega, 1975; Guerrero et al., 1973; Villalobo et al., 1977).

(b) Pyridine nucleotide-dependent nitrate reductase

The first definite examination of the assimilatory nitrate reductase was reported from Neurospora (Nason and Evans, 1953) and soybean leaves (Evans and Nason, 1953), and characterized as a sulfhydryl molybdo-FAD-protein. Both FAD and Mo were shown to function as electron carriers in the following sequence (Nicholas and Nason, 1954a):

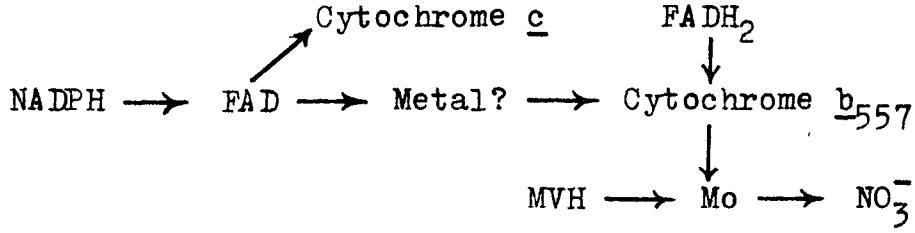
$$\text{NADPH} \longrightarrow \text{FAD} \longrightarrow \text{Mo} \longrightarrow NO_3^-$$

Oxidation states of Mo involved in the oxidation-reduction appears to be +5 and +6 (Nicholas and Nason, 1954b; Nicholas and Stevens, 1955).

The work of Nason and his colleagues has established several features of the enzymatic apparatus associated with nitrate reduction in Neurospora crassa. Assimilatory NAD(P)H-nitrate reductase (NAD(P)H: nitrate oxidoreductase, (EC 1.6.6.2)) of N. crassa is a soluble sulfhydryl protein, with FAD, cytochrome b_{557} (N. crassa), molybdenum, and an

unidentified second metal component as prosthetic groups (Garrett and Nason, 1967; 1969; Nason and Evans, 1953; Nicholas and Nason, 1954a; 1954b; Nicholas et al., 1954). It has a relative molecular mass of 230,000 (Garrett and Nason, 1969; Nason et al., 1970) and displays several other inducible enzymatic activities including FAD-dependent NADPH-cytochrome c reductase (Kinsky and McElroy, 1958), $FADH_2$-nitrate reductase, NADPH-nitrate reductase and reduced methyl viologen (MVH)-nitrate reductase (Garrett and Nason, 1969).

The following pathway of electron transfer in nitrate reduction in N. crassa has been suggested (Garrett and Nason, 1969):



both genetic and biochemical (Nason et al., 1970; Ketchum et al., 1970) evidences have shown that N. crassa nitrate reductase is composed of at least two subunits. The synthesis of one of these subunits is induced by nitrate. This component is characterized by its capacity to catalyze the reduction of cytochrome c by NADPH. The other subunit, which is a constitutive component of wild-type N. crassa and certain other mutants, is characterized by its capacity to catalyze the reduction of nitrate by $FADH_2$, reduced methyl viologen or NADPH, when this subunit is combined with the inducible subunit. Ketchum et al. (1970) and Nason et al. (1971) have

reported that molybdenum-containing constitutive subunit from N. crassa can be replaced by acid-treated molybdenum enzymes from diverse phylogenetic sources extending from prokaryotic through higher eukaryotic organisms. The molybdenum enzymes included bovine milk and intestinal xanthine oxidase, rabbit liver xanthine oxidase and chicken liver xanthine dehydrogenase. Acid-treated preparations of nitrogenase from Clostridium, Azotobacter and soybean bacteroids, liver aldehyde and sulfite oxidase from mammals, plant nitrate reductase and E. coli respiratory nitrate reductase can also replace the constitutive Mo-containing subunit of N. crassa (Nason et al., 1971). By contrast, inorganic molybdenum, and certain molybdenum-amino acid complexes, as possible catalytic models of nitrogenase, failed to yield NADPH-nitrate reductase activity after incubation with nit-1 extract grown in the presence of nitrate (Ketchum et al., 1970).

Certain mutant strains of N. crassa, designated as nit, are unable to grow on nitrate as a nitrogen source and produce aberrant enzymes which lack NADPH-nitrate reductase activity, and one or more of the other three activities (reduced FAD-nitrate reductase, reduced methyl viologen-nitrate reductase, and FAD-dependent cytochrome c reductase). nit-1 Mutant produces an enzyme which possesses a nitrate inducible FAD-dependent NADPH-cytochrome c reductase activity and lacks the other three activities. The combination of molybdenum-containing subunit from any of the various sources with the inducible subunit from N. crassa (nit-1) results in the

reconstitution of functional nitrate reductase having indistinguishable properties from those of wild-type N. crassa nitrate reductase (Nason et al., 1971). In vitro formation of nitrate reductase (EC 1.6.6.2) has also been attained by using extracts of N. crassa (nit-1) and extract of either photosynthetically- or heterotrophically-grown Rhodospirillum rubrum which contributes the constitutive component (Ketchum and Sevilla, 1973). Apparently, N. crassa nitrate reductase and the various molybdenum-containing enzymes share similar protein subunits. In explaining these results, Nason et al. (1971) postulated that the constitutive component is a molybdenum cofactor. Ketchum and Swarin (1973) have shown that loss of gene product in N. crassa (nit-1) can be replaced by a trypsin-and protease-insensitive dialysable component which is present in the extracts of bacteria that are capable of metabolizing dinitrogen and/or nitrate. The component is presumed to contain molybdenum and can probably be viewed as a cofactor as postulated by Nason et al. (1971). Evidence for possible existence of common genes affecting both nitrate reductase and nitrogenase has also been obtained in studies with mutants of Rhizobium (Ketchum and Swarin, 1973). In later papers Nason and his colleagues (Lee et al., 1974a; 1974b) have reported, using radioactive molybdenum, the partial reactivation effect specifically by salts and other derivatives of the metal showing that molybdenum (presumably as a component of a larger molecule of cofactor in the in vitro formation of the enzyme) is contributed by Neurospora extracts other than that of nit-1, and by acid-treated Mo-enzymes.

Nason et al. (1974) presented evidence, based on the in vitro assembly of an active Neurospora-like NADPH-nitrate reductase (EC 1.6.6.2), by incubating cell-free extract of N. crassa (nit-1) with (a) cell-free preparation of certain non-allelic mutants or uninduced wild-type or (b) acid-treated Mo-enzymes from diverse phylogenetic sources, that a Mo-cofactor is common to most of the Mo-enzymes. Mo-cofactor of approximately 1000 relative molecular mass was postulated to serve both as a link that binds the enzyme subunits of nit-1 to yield the active enzyme, and as an electron carrier. Amy and Rajagopalan (1979) purified Mo-cofactor from E. coli which diffused through a membrane of 2000 relative mass cut off and was insensitive to trypsin. It was associated with a carrier molecule ($\sim$40,000 dalton) but was easily removed by dialysis. Johnson et al. (1980) isolated a Mo-cofactor from sulfite oxidase, xanthine oxidase and nitrate reductase and showed this to be a novel pterin. The active factor was presumably composed of Mo and a reduced form of pterin. Much genetic and biochemical evidence indicates that the cofactor is a component of all molybdenum-containing enzymes, with the single exception of nitrogenase, which contains a cofactor containing iron, as well as molybdenum, designated as FeMoCo (Pienkos et al., 1977; Shah and Brill, 1977).

Recently, nitrate reductase has been purified from N. crassa wild strain, nit-1 and nit-3 and it has been shown that the native enzyme consists of two identical subunits, nit-1 enzyme being apoenzyme of wild-type nitrate reductase (Horner, 1983; Tachiki and Nason, 1983). The nit-1 mutant lacks not

only native nitrate reductase activity but also xanthine dehydrogenase activity. The nit-1 gene must, therefore, control the synthesis of a Mo-cofactor common to both nitrate reductase and xanthine dehydrogenase. Thus, the mutant nitrate reductase enzyme produced by nit-1 must be an apoenzyme, lacking the Mo-cofactor but complete in its polypeptide (Horner, 1983). The pleotrophy of N. crassa nit-1 is caused by a defect in the synthesis of molybdopterin. The reconstitution of the apoprotein of nitrate reductase of nit-1 is attained through specific incorporation of molybdopterin and there is no requirement of an exogenous peptide in the process (Kramer et al., 1984).

In vitro reconstitution of demolybdo nitrate reductase (also called cytochrome c reductase) of Chlorella vulgaris has been achieved by insertion of Mo from Na_2MoO_4. Measurements with ^{99}Mo showed that there was one Mo incorporated per subunit weight of 90,000 (Ramadoss et al., 1981). The experiments with Chlorella demolybdo nitrate reductase do not support the concept of the presence of a "molybdenum cofactor". This is unrelated, really, to the question whether a pteridine may be present in the enzyme in the domain that binds molybdenum (Gewitz et al., 1981; Johnson et al., 1980).

Information available on nitrate reductase from nitrate reducing yeasts is rather meagre. Preliminary experiments with Hansenula anomala (Silver, 1957; Pichinoty and Metenier, 1966), Candida utilis (Sims et al., 1968) and

Torulopsis nitratophila (Rivas *et al.*, 1973) have indicated that the enzyme resembles *Neurospora* nitrate reductase in that it is a metallo-flavoprotein, specific for NADPH as electron donor. Nitrate reductase of *C. utilis* was studied *in situ* for its regulatory properties (Choudary and Rao, 1976a) and characterized in detail for its electron donors (Choudary and Rao, 1976b). Nitrate reductase from *Rhodotorula glutinis* was found to be a soluble enzyme having a relative molecular mass of 230,000. This was considered to be composed of two subunits of 118,000 dalton (Guerrero and Gutierrez, 1977). Recently, nitrate reductase of *H. anomala* has been purified to homogeneity. The relative molecular mass of the enzyme was estimated to be 215,000 and it was composed of four subunits of relative molecular mass 52,000 (Zauner and Dellweg, 1983).

The presence of assimilatory nitrate reductase has been reported in algae and a variety of higher plants. The enzyme is a molybdo-flavoprotein specifically requiring NADH as electron donor for the reduction of nitrate to nitrite. It has been reported recently that both NADH and NADPH are utilized by a nitrate reductase isolated from a salt-tolerant alga (Heimer, 1976). That molybdenum is a component of nitrate reductase from algae and higher plants, has been convincingly demonstrated by the use of tungsten as a specific inhibitor (Paneque *et al.*, 1972; Notton and Hewitt, 1971a; 1971b), and by other ways (Aparicio *et al.*, 1971; Vega *et al.*, 1971; Cardenas *et al.*, 1971). By using ^{185}W,

it was possible to get *in vivo* a radioactive nitrate reductase-W complex from spinach (Notton and Hewitt, 1971a) and *Chlorella* (Paneque *et al.*, 1972) which maintained unaffected its NADH-diaphorase activity but was completely inactive as nitrate reductase. The association of ^{185}W with nitrate reductase was shown to be weaker than that of Mo. The pathway of electron transfer within the nitrate reductase complex, as suggested by Schrader *et al.* (1968), envisages a transfer of electrons from NADH to a flavin moiety and then to molybdenum which ultimately reduces nitrate attached at the active site of the enzyme.

Exception to pyridine nucleotide specificity are known in algae. Thus, nitrate reductase from *Cyanidium caldarium*, *Dunaliella tertiolecta*, and *Ankistrodesmus braunii* can accept electrons from both NADH and NADPH even at the high degree of purification (Rigano, 1971; LeClaire and Grant, 1972). The apparent ability of partially purified enzyme from leaves of soybean, maize, and foxtail to utilize NADPH was shown to be due to the presence of phosphatase which readily converted NADPH to NADH (Wells and Hageman, 1970). The stereospecificity of nitrate reductases for hydrogen removal from reduced pyridine nucleotides has been determined to be of the "A"-type regardless of the source of the enzyme (Guerrero *et al.*, 1977). Purified nitrate reductase from *C. utilis* exhibited "A" stereospecificity for NADH and NADPH while nitrite reductase from the same source exhibited "B" stereospecificity for NADH and NADPH. The "A" stereospecificity of nitrate reductase and "B" specificity of nitrite reductase in *C. utilis*

form an exception to the generalization that consecutive oxidoreductases have the same stereospecificity (Davies and Kenworthy, 1982).

Nitrate reductase from higher plants generally appears to be unstable and recoveries and enrichment during purification are usually quite low (Hageman and Hucklesby, 1971). Various causes have been suggested for the low yields and poor purification. It has been suggested that essential sulfhydryl groups may be oxidized during the purification. It is customary to incorporate sulfhydryl protectants, such as cysteine, mercaptoethanol or dithiothreitol in the extraction media. It has also been suggested that endogenous phenolics in crude plant extracts may inactivate essential sulfhydryl groups or cause protein precipitation. Addition of polyvinylpyrrolidone and ion exchange resins partially overcome this problem (Purvis et al., 1976). More recent purification techniques have involved affinity chromatography on Blue-Dextran-Sepharose or Blue Sepharose (Solomonson, 1975; Notton et al., 1977; Campbell and Smarrelli, 1978; Notton and Hewitt, 1979). Nakagawa et al. (1985) have purified a nitrate reductase from spinach leaves by chromatography on Bu Toyopearl 650-M, hydroxylapatite-brushite and Blue Sepharose CL-6B columns. The native enzyme (M_r 270,000) is composed of two identical subunits of 110,000-120,000 dalton.

In analogy with the enzymes from fungi, the partially purified nitrate reductase from several algae has associated cytochrome c reductase activity and contains cytochrome b_{557}

in addition to FAD and Mo (Payne, 1973). Working with highly purified nitrate reductase from spinach, small calabash and Chlorella, Losada et al. (1969) concluded that nitrate reductase molecule consists of two chemically separable moieties: a FAD-dependent NADH-diaphorase, which can use several oxidized compounds such as cytochromes as electron acceptor; and terminal nitrate reductase which can use reduced flavin nucleotides and viologen dyes as electron donor. The two activities participated sequentially in the transfer of electrons from NADH to nitrate.

Although there has been much controversy over the intracellular location of nitrate reductase in higher plants, the experimental evidence indicates the enzyme is soluble and located in the cytoplasm (Beevers and Hageman, 1969; Hewitt et al., 1976). However, Dalling et al. (1972a) found that nitrate reductase was bound indiscriminately to membrane (protein) surface.

Assimilatory nitrate reductases from various sources (squash cotyledons, spinach, corn, and soybean leaves, C. vulgaris and N. crassa) have been compared structurally using immunological approaches. It has been observed that all nitrate reductases studied have common antigenic determinants and are probably derived from a common ancestor. Although these assimilatory nitrate reductases have similar catalytic characteristics, they appeared to have diverged to a greater degree in structural features (Smarrelli and Campbell, 1981). In contrast to the generalization of Smarrelli and Campbell (1981) for common

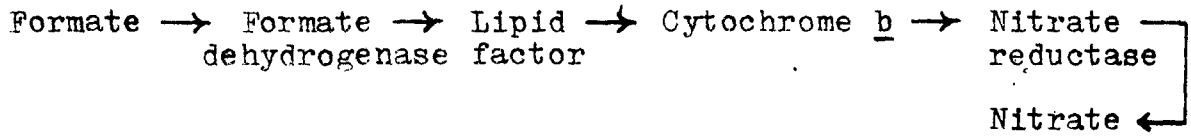
antigenic determinants in assimilatory nitrate reductases from various sources, Ferrario et al. (1983), using immunodiffusion and immunoprecipitation techniques, have recently reported that nitrate reductases from spinach leaves and roots have different antigenic determinants. Earlier work based on double immunodiffusion assays had shown that there are common antigenic determinants for nitrate reductase from E. coli and component I of nitrogenase from A. vinelandii. Further work using variety of immunoelectrophoretic techniques indicates that the cross-reaction between nitrate reductase and antiserum to component I of nitrogenase results from a contaminant antigen copurified with nitrate reductase (Byrne and Nicholas, 1982).

In contrast to nitrate reductases from the plant kingdom, there are few reports on nitrate reductases from the assimilatory-type nitrate reducing bacteria utilizing NAD(P)H as electron donor. Nicholas and Nason (1955) purified a soluble NADH-linked nitrate reductase from E. coli strain B; the enzyme was a metalloflavoprotein with FAD as the prosthetic group and molybdenum as a probable metal constituent. Taniguchi and Ohmachi (1960) isolated an inducible particulate NADH-specific nitrate reductase from A. vinelandii which was characterized as a sulfhydryl metalloenzyme, the activity of the enzyme was stimulated about 2-fold by added FAD or FMN.

2. Respiratory nitrate reductase

Taniguchi and Itagaki (1960) isolated a particulate nitrate reductase system from E. coli which included

cytochrome b as an intermediary electron carrier from formate or NADH to nitrate; this system possessed remarkably high activity of nitrate reductase. The best natural electron donors for nitrate reduction by the intact particle were $FMNH_2$, $FADH_2$, formate, and NADH, whereas reduced methyl-and benzyl viologen acted as best artificial electron donors. The enzyme was purified to a homogeneous state. It has a relative mass of one million and contained one atom of Mo and 40 atoms of Fe per molecule but no bound flavin or cytochrome. Itagaki et al. (1961) presented evidence indicating the involvement of cytochrome b as electron donor. The following pathway for the transfer of electrons was suggested:



the lipid factor could be replaced by vitamin K.

The anaerobic respiratory nitrate reductase of E. coli consists of subunits A, B, and C in a ratio of 2:2:4 (MacGregor, 1975). The functions of subunits A (active site, M_r 145,000) and C (cytochrome b, M_r 20,000) have been clearly defined. Subunit B has been implicated in the attachment of the enzyme complex to the cytoplasmic membrane. Subunit B has been referred to as B and B'. B' has a relative molecular mass of 58,000 and B, 60,000. An enzymatic activity with cytoplasmic membranes of E. coli has been reported which changes subunit B to a form B' with a slightly greater electrophoretic mobility on SDS-polyacrylamide gel (Choudhry et al., 1983).

The conversion of B to B' is a reversible process and is due to the removal of one or more small non-protein molecules.

Fewson and Nicholas (1961a) reported NADH-specific nitrate reductase from denitrifying cells of Pseudomonas aeruginosa which contained FAD, cytochrome c, and Mo as functional components. The following scheme for electron transport was suggested:

$$\text{NADH} \longrightarrow \text{FAD} \longrightarrow \text{Cytochrome } \underline{c} \longrightarrow \text{Mo}^{5+} \longrightarrow \text{NO}_3^-$$
$$\text{Cytochrome oxidase} \longrightarrow \text{O}_2$$

Recently, Carlson et al. (1982) have purified a dissimilatory nitrate reductase from anaerobic cultures of P. aeruginosa. The purified homogeneous enzyme had no associated cytochrome, but contained molybdenum and nonheme iron.

The respiratory nitrate reductase from M. denitrificans was purified and characterized by Lam and Nicholas (1969a). The enzyme was molybdoprotein but did not contain cytochrome or flavin. NADH, $FADH_2$, $FMNH_2$, succinate and reduced cytochrome could not donate electrons to the enzyme; only reduced benzyl viologen and methyl viologen were utilized as electron donors. The purified enzyme from the same source was shown by Forget (1971) to be able to accept electrons from reduced flavin nucleotides. The enzyme was characterized as a nonheme iron protein containing traces of Mo. The involvement of Fe and Mo, as functional components of nitrate reductase was later confirmed on the basis of EPR studies (Forget and Dervartanian, 1972). Chiba and Ishimoto (1973) reported a nitrate reductase from Clostridium perfringens having

ferredoxin as an intermediary electron carrier in nitrate reducing system with NADH as the electron donor. The following pathway for electron transport was suggested:

$$Na_2S_2O_4 \rightarrow NAD^+ \xrightarrow[\text{reductase}]{Fd\text{-}NAD^+} Fd \rightarrow NO_3^-R \rightarrow NO_3^-$$

Sadana and McElroy (1957) purified and characterized a nitrate-reducing system from A. fischeri and proposed the following pathway of electron transfer:

$$\begin{array}{l} \text{NADH} \\ \text{or} \\ \text{NADPH} \end{array} \rightarrow \begin{array}{l} \text{FMN} \\ \text{or} \\ \text{FAD} \end{array} \rightarrow Fe^{3+} \rightarrow \text{Bacterial cytochrome } c_{551} \rightarrow O_2$$

$$\text{Reduced benzyl viologen} \rightarrow \text{Nitrate reductase} \rightarrow \text{Nitrate}$$

The electron transport chain was separated into two soluble fractions, (a) the electron donor system, namely a NAD(P)H-cytochrome c - reductase with a requirement for FAD or FMN; and (b) the terminal nitrate reductase which mediated the transfer of electrons from reduced cytochrome to nitrate. When reduced benzyl viologen supplied the electrons, the bacterial cytochrome was not involved. On further purification, nitrate reductase free from cytochrome component was obtained (Sadana et al., 1963). Ultracentrifugal studies indicated that A. fischeri nitrate reductase was a much smallar molecule than the enzyme from E. coli reported by Taniguchi and Itagaki (1960).

Knook and Planta (1971) have shown that NADH-dependent nitrate reductase, involved in respiratory reduction of nitrate in A. aerogenes, requires ubiquinone-8 and cytochrome b, isolated from the same organism, as electron carriers. Cytochrome-linked nitrate reductase have also been purified

from Nitrobacter agilis (Street and Nason, 1965), R. japonicum (Evans, 1954; Lowe and Evans, 1964), Bacillus stearothermophilus (Downey, 1966), and Bacillus cereus (Hackenthal and Hackenthal, 1965). Respiratory nitrate reductase from Bacillus licheniformis (Van'T Riet et al., 1979) has been purified and characterized. The enzyme (M_r 193,000) consists of two subunits, having relative molecular mass of 150,000 and 57,000, present in equimolar ratio. Recently, Chikwem and Downey (1982) purified and characterized a respiratory nitrate reductase from B. stearothermophilus. The relative mass of the enzyme was estimated to be $\sim$210,000 having two subunits of 150,000 and 44,000 dalton in equimolar ratio. There are 6 atoms of nonheme iron and 12 moles of labile sulfide in one mole of the purified enzyme but no cytochrome. The 44,000 dalton B subunit is the smallest of all the characterized bacterial nitrate reductases and is very close to the size of B' subunit of E. coli. The various B components of other bacterial nitrate reductases are probably derived from this 44,000 dalton subunit.

Regulation of nitrate reductase activity

It is a widely accepted fact that nitrate reductase has a primary role in regulating the whole inorganic nitrogen metabolism (Vennesland and Guerrero, 1979; Hewitt et al., 1979; Losada et al., 1981). In green algae nitrate reductase has been found in two physiologically interconvertible forms (Aparicio and Maldonado, 1979). Although in higher plants the in vivo interconversion of the enzyme is not fully

characterized, *in vitro* terminal and total activities can be inactivated, as in green algae, by low potential reductant such as NAD(P)H or dithionite, especially if cyanide or acetylene are also present (Relimpio *et al.*, 1971; Maldonado *et al.*, 1981). All the activities of nitrate reductase complex from spinach are irreversibly inactivated by irradiation of the enzyme with blue light in the presence of flavin mononucleotide (Vargas *et al.*, 1982). Irreversible photoinactivation of *A. braunii* nitrate reductase has also been observed by De la Rosa and De la Rosa (1983). Recently, Cordoba *et al.* (1985) have reported that *in vitro* inactivation of *Chlamydomonas reinhardii* nitrate reductase by reduced pyridine nucleotides requires an active diaphorase moiety.

NADH-dependent inactivation of nitrate reductase seems to be mediated by superoxide anions, which is produced by interaction of the NADH-reduced flavin with molecular oxygen (Massey *et al.*, 1969; Aryan and Wallace, 1985). Superoxide would inactivate the enzyme by forming a stable complex with the reduced enzyme (De la Rosa *et al.*, 1981). Photooxidative inactivation of nitrate reductase has been shown to be due to the involvement of singlet oxygen generated by light absorption by FMN, rather than excited flavins or other oxygen species (Vargas *et al.*, 1982).

The cyanide-inactivated enzyme, whether from green algae and higher plants (Aparicio *et al.*, 1976; Roldan *et al.*, 1978) or from *N. crassa* (Roldan and Butler, 1980), can be reactivated chemically by ferricyanide or photochemically by

irradiation with blue light. Reactivation by these treatments presumably involves oxidation of the stable Mo^{IV}-CN complex to the unstable Mo^{VI}-CN complex, from which cyanide would be readily dissociated (Aparicio and Maldonado, 1979; Roldan and Butler, 1980). Jawali and Sane (1983) had shown that O_2^- is involved in the photoreactivation of spinach leaf CN^--inactivated nitrate reductase. Direct involvement of excited flavins in the photoreactivation of both CN^--and acetylene-inactivated spinach nitrate reductase have been demonstrated by Maurino et al. (1983).

Proposed mechanism for nitrate transport and reduction

It has been proposed (Butz and Jackson, 1977) that a tetrahedron-shaped transmembrane nitrate reductase tetramer functions as a carrier for nitrate transport. Both reduction and transport of nitrate are brought about by the same enzyme complex. An ATPase is visualized to be closely associated with the nitrate reductase tetramer. The tetramer is apparently oriented in such a manner that one monomer is exposed to the outside of the plasmalemma, while the other three are exposed to the cytoplasm. Orientation yields a reaction mechanism where the transport and reduction of one nitrate molecule is accompanied by the transport of two additional nitrate ions (i.e. a 3:1 transport reduction ratio). The proportion of transported nitrate that is reduced is apparently modulated by thiol-reversible ADP inhibition of nitrate reduction. This inhibition, however, is probably the result of

adenylate binding at sites on the proposed nitrate-activated ATPase to which nitrate reductase is tightly coupled. An analogous system consisting of nitrate reductase dimer that spans a unit membrane plus an ATPase has been proposed to be responsible for nitrate transport and reduction in algae and chloroplast (Butz and Jackson, 1977).

Mechanism of enzyme catalysis

The inhibition of nitrate reductase by p-HMB is generally prevented by reduced pyridine nucleotides (Garrett and Amy, 1978; Losada and Guerrero, 1979) suggesting that essential sulfhydryl groups of the protein participate in the binding of NAD(P)H to the enzyme. Studies with nitrate reductase from Neurospora reinforce this interpretation and suggest also the active participation of sulfhydryl groups in the electron flow from NADPH to the FAD prosthetic group (Garrett and Amy, 1978).

The participation of FAD in the activity of the diaphorase moiety of the enzyme and of molybdenum in that of the terminal moiety are now well established facts (Garrett and Amy, 1978; Hewitt and Notton, 1980; Losada and Guerrero, 1979; Vennesland and Guerrero, 1979). The molybdenum domain is thought to be the site where nitrate binds and is reduced. No general agreement, however, exists with respect to the oxidation state change of the molybdenum present in nitrate reductase during the reduction of nitrate to nitrite, but the Mo^{VI}/Mo^{IV} couple may well be the proximal reductant

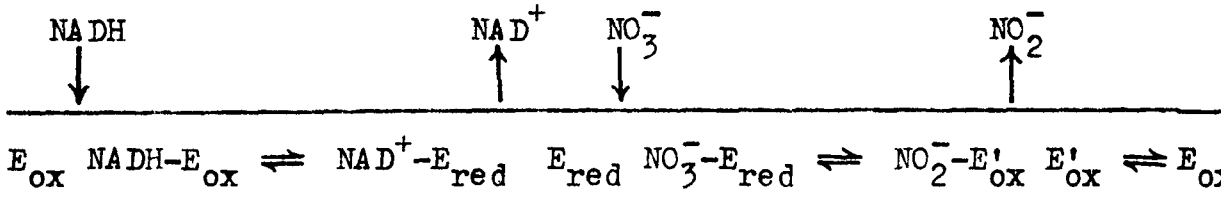
of nitrate (Hewitt and Notton, 1980; Jacob and Orme-Johnson, 1980; Losada and Guerrero, 1979; Vennesland and Guerrero, 1979). Electron spin resonance (ESR) spectra of the oxidized enzyme of Chlorella and Neurospora exhibit low absorption in the region near $g = 2$, but signals at g-values of 1.97 and 1.98, corresponding to an intermediate paramagnetic species of Mo(V), appear upon addition of NAD(P)H (Jacob and Orme-Johnson, 1980; Solomonson, 1979). The Mo(V) signals disappear upon reoxidation of the enzyme by nitrate. A loss of these signals is also observed upon cyanide addition to the NAD(P)H-reduced enzyme, probably because cyanide blocks the metal in a more highly reduced, nonparamagnetic oxidation state such as Mo(IV) (Solomonson, 1979). The lineshape and g value of these signals show pH dependence. The g_{av} for Mo(V) at pH 7.0 was 1.977 and at pH 9.0, 1.961. The signal observed at pH 7.0 exhibits interaction with a single exchangeable proton. The oxidized enzyme exhibits low spin ferric heme signals which are abolished upon reduction with NAD(P)H (Solomonson et al., 1984).

Active participation of cytochrome b_{557} in the catalytic activity of the enzyme is sustained by spectrophotometric studies showing that this group is reduced by NAD(P)H and reoxidized by nitrate (Losada and Guerrero, 1979; Vennesland and Guerrero, 1979). Oxidized nitrate reductase of Neurospora exhibits ESR signals at $g = 2.98$ and 2.27 which have been ascribed to a low-spin ferric form of cytochrome b_{557}. These signals disappear after addition of NADPH. It seems that

reduction of the heme and production of the Mo(V) species occur at similar redox potentials (Jacob and Orme-Johnson, 1980). The localization of the heme group within the enzymatic electron transport chain remains undefined, although its site of action appears to be placed between FAD and molybdenum. The pathway of electrons from NAD(P)H to nitrate through nitrate reductase from eukaryotes may thus be depicted as:

$$NAD(P)H \rightarrow (FAD \rightarrow Cyt\ \underline{b}_{557} \rightarrow Mo) \rightarrow NO_3^-$$

Nitrate reduction by reduced pyridine nucleotide, catalyzed by Ankistrodesmus NAD(P)H-nitrate reductase, shows an iso ping pong bi bi steady state kinetic mechanism, with isomerization of the enzymatic form which binds NADH (Herrero et al., 1980).



For Aspergillus nitrate reductase, however, a random order rapid-equilibrium mechanism has been suggested (McDonald and Coddington, 1974). On the other hand, random order addition of nitrate or NADH, but ordered (ping pong) mechanism for release of products, has been proposed for the enzymes of squash and spinach (Hewitt and Notton, 1980). Purified respiratory nitrate reductase from E. coli is able to use either reduced viologen dyes or quinols as the electron donor and nitrate, chlorate or bromate as the electron acceptor. When

reduced viologen dyes act as the electron donor, the enzyme follows compulsory order, "Theorell Chance" mechanism, in which it is an enzyme-nitrate complex that is reduced rather than the free enzyme. In contrast if quinols are used as the electron donor the enzyme operates by a two-site enzyme substitution mechanism. It is concluded that the holoenzyme has two independent and spatially distinct active sites, one for quinol oxidation and other for nitrate reduction (Fraser and David, 1985).

Applied research with nitrate reductase

Because nitrate reductase is substrate-induced, relatively unstable, and the rate-limiting enzyme between nitrate and protein, the level of nitrate reductase should reflect the rate of supply of reduced nitrogen for plant growth. This has been confirmed by the work showing that nitrate reductase activity integrated over time is correlated with the accumulation of reduced nitrogen by a given genotype when nitrate is not limiting (Eilrich and Hageman, 1973; Brunetti and Hageman, 1976); however, attempts to correlate nitrate reductase activity as a criterion for the selection of cultivars with ability to produce more grain or grain protein have been variable (Hageman, 1979). In a study with six corn hybrids, levels of extractable nitrate reductase activity were correlated with grain and grain protein production; the enzyme activity, however, accounted for only 35% of the variability in grain and grain protein production. Similar correlation

between nitrate reductase activity and various yield components have been reported for wheat (Croy and Hageman, 1970; Reilly, 1976), sorghum (Eck and Hageman, 1974; Eck et al., 1975), soybean (Harper et al., 1972), alfalfa (Eskew et al., 1973), and rye grass (Bowerman and Goodman, 1971). In general, the correlations have not been sufficiently high to elicit interest of the plant breeders in the use of this procedure as a breeding tool.

The sensitivity of nitrate reductase activity to temperature treatment, both in vitro and in vivo, provides a possible means of selecting heat (draught) tolerant cultivars (Pal et al., 1976). The first instance of practical application of nitrate reductase methodology was with a legume, with Phaseolus vulgaris L.. Neyra et al. (1977) noticed seasonal rapid decline of nitrogenase activity after flowering concurrent with a resurgence of canopy nitrate reductase activity. Soil application of 40 Kg/N/ha at flowering caused a marked increase in nitrate reductase activity and bean yields were almost doubled.

Nitrite reductase

The term nitrite reductase is used for all enzymes which catalyze the reduction of nitrite regardless of the end product of reduction (Zarowny and Sanwal, 1963). However, nitrite can be metabolized by two pathways described as dissimilatory or assimilatory. In bacteria that possess the dissimilatory pathway, nitrite is reduced to gaseous nitrogen or other oxides of nitrogen by a series of respiratory processes (Payne, 1973; Thauer et al., 1977). Most organisms with the dissimilatory pathway are facultative bacteria which can grow anaerobically when supplied with an inorganic substitute for oxygen. An exception to this generalization is Propionibacteria pentosaceum (Gent-Ruijters et al., 1975), which is an obligate anaerobe.

For the assimilatory pathway it was proposed (Meyer and Schultze, 1894) that the reduction of nitrite to ammonia proceeded by three steps, each step involving the transfer of two electrons with the production of hyponitrite and hydroxylamine as intermediates. Although belief in this three step reductive pathway was maintained for almost six decades, current evidence shows that nitrite is reduced to ammonia by the enzyme nitrite reductase according to the following scheme:

$$NO_2^- + 6e^- + 8H^+ \longrightarrow NH_4^+ + 2H_2O$$

The reduction involves the transfer of six electrons and is catalyzed by a single protein.

Nitrite reductases are widely distributed in nature. The

enzyme has been reported from bacteria, fungi, algae and higher plants.

1. Bacteria: The dissimilatory nitrite reduction was first reported by Yamagata (1939) in a cell-free preparation of Bacillus pyocyaneus. Denitrifying-type nitrite reductase, which catalyzed the reduction of nitrite to nitric oxide, was subsequently reported from Thiobacillus denitrificans (Baalsrud and Baalsrud, 1954), Pseudomonas stutzeri (Chung and Najjar, 1956), Bacillus subtilis (Najjar and Allen, 1954), P. aeruginosa (Walker and Nicholas, 1961), and E. coli strain K 12 (Zarowny and Sanwal, 1963) grown anaerobically in the presence of nitrate. Yamanaka et al. (1960; 1961) obtained a cytochrome oxidase from P. aeruginosa which functioned as a nitrite reductase under anaerobic conditions. A nitrite reductase which reduced nitrite to nitrogen was reported by Asano (1959) in an aerobic denitrifier, a halotolerant Micrococcus strain 203. Newton (1969) isolated a cytochrome from M. denitrificans which functioned as a nitrite reductase and was shown to contain two hemes (heme c and heme d). Respiratory nitrite reductases have also been purified from Corynebacterium nephridii (Renner and Becker, 1970), Alcaligenes faecalis (Iwasaki and Matsubara, 1971), Achromobacter cycloclastes (Iwasaki and Matsubara, 1972), and Pseudomonas perfectomarinus (Payne et al., 1971) grown under anaerobic conditions. An ammonia-oxidizing bacteria, Nitrosomonas europaea, which ordinarily generate nitrite, was shown to contain a hydroxylamine-dependent nitrite reductase catalyzing reduction of

nitrite to nitric oxide (Hooper, 1968).

The partially purified preparation of hydroxylamine oxidase from N. europaea was shown by Ritchie and Nicholas (1972) to have retained nitrite reductase activity. This raised doubts as to the identity of nitrite reductase in the organism. The same authors, however, reported the separation of nitrite reductase from hydroxylamine oxidase (Ritchie and Nicholas, 1974). Evidence was presented for the probable identity of nitrite reductase as a copper protein.

A copper-containing assimilatory nitrite reductase was purified and crystallized from a denitrifying bacterium A. faecalis, strain S-6. The enzyme was composed of four subunits with a relative mass of $\sim$30,000, each containing one atom of Cu(II). NO was identified as the main reduction product from nitrite in the enzyme-catalyzed reaction (Tetsu et al., 1981). Recently, a dissimilatory nitrite reductase has been purified and characterized from a denitrifier Alcaligenes, species NCIB 11015 (Masuko et al., 1984). The EPR spectrum and Cu analysis clearly indicated that the enzyme contained 2 type I Cu atoms/mol but no other type of Cu. This is the first blue protein to be reported that exhibits catalytic activity despite possessing only I type Cu. Preuss and Klemme (1983) purified a Cu-containing dissimilatory enzyme from photosynthetically-grown phototrophic bacterium Rhodopseudomonas palustris. The enzyme (M_r 120,000) contained c-type cytochrome and Cu.

Two types of assimilatory nitrite reductases, marked by a well-defined electron donor specificity, have been described:

(a) Ferredoxin-nitrite reductase, characteristic of photosynthetic organisms and (b) NAD(P)H-nitrite reductase, found in non-photosynthetic organisms. The assimilatory nitrite reduction was first observed by Taniguchi et al. (1953) in Bacillus pumilus, grown aerobically in the presence of nitrite. Assimilatory nitrite reduction also occurs in Azotobacter species (Spencer et al., 1957; Vega et al., 1973), Clostridium pasteurianum (Valentine et al., 1963), Rhodospirillum (Taniguchi and Kamen, 1963) and in soil actinomycetes (Fedorov and Ilina, 1956). E. coli strain Bn, grown in deep standing cultures with nitrate as the sole source of nitrogen, has been shown to contain at least three nitrite reductases that reduced nitrite to ammonia (Lazzarini and Atkinson, 1961) but only the enzyme specific for NADH appeared to be responsible for physiological nitrite reduction (Kemp and Atkinson, 1966). Recently, nitrite reductase has been purified from Wolinella succinogenes and shown to contain 5 - 6 heme c groups per mol of enzyme (Liu et al., 1983). Schroeder et al. (1985) have shown that W. succinogenes contains two nitrite reductases, one in the cytoplasm and the other is integrated in the cytoplasmic membrane. The membrane enzyme consists of a single polypeptide chain (M_r 63,000) containing 4 heme c groups, and probably, an Fe-S cluster as prosthetic groups. A terminal nitrite reductase (M_r 45,000) has been purified from lupine root nodule bacteroids and shown to contain nonheme iron and FAD (Burikhanov et al., 1983).

2. Fungi: Nitrite reductase, first characterized in extract from N. crassa by Nason et al. (1954), catalyzed the reduction of nitrite to ammonia via hydroxylamine using reduced pyridine nucleotides as electron donor. It has a relative mass of 290,000. The reduction of nitrite is stimulated by the presence of FAD when reduced pyridine nucleotides serve as electron donor. The enzyme possessed NAD(P)H-diaphorase activity with cytochrome c or 2,6-dichlorophenolindophenol as electron acceptor. Nicholas et al. (1960) further purified the N. crassa nitrite reductase and concluded that it was NADH-dependent and contained FAD, Fe, Cu and -SH groups. The purified enzyme characteristically showed absorption maxima at 390 and 578 nm with the formation of additional absorption bands at 588, 556 nm when treated with NADPH and FAD. When nitrite is added to the reduced enzyme, absorption maxima are at 585 and 560 nm indicating the formation of a complex between the enzyme and nitrite or a reduction product thereof. The spectral properties of the enzyme are characteristic of a siroheme, a tetrahydroporphyrin of the isobacteriochlorin-type, first isolated from E. coli (Murphy et al., 1974a) and demonstrated in the purified nitrite reductase for N. crassa (Vega et al., 1975).

3. Algae: Photochemical reduction of nitrite was first observed by Kessler (1953) in cultures of Ankistrodesmus. He later demonstrated that nitrite served as an efficient Hill reagent for oxygen evolution of Scenedesmus braunii and that light immediately stimulated nitrite reduction (Kessler, 1955).

Huzisige and Satoh (1960) observed a similar light dependence of nitrite reduction by Euglena gracilis. In the blue-green algae A. cylindrica, the photochemical nitrite reduction was first demonstrated by Hattori (1962) who also showed nitrite reduction with molecular hydrogen as electron donor (Hattori, 1963). A soluble nitrite reductase was isolated and purified from cell-free extracts of D. tertiolecta (Grant, 1970). The enzyme resembled nitrite reductase of higher plants in that it was a ferredoxin-dependent enzyme. Zumft (1972) obtained an electrophoretically homogeneous enzyme from Chlorella fusca which required ferredoxin, reduced chemically or photosynthetically, or by reconstituted enzymatic system, as its natural electron donor. The relative molecular mass of the enzyme was estimated to be 63,000. Recently, an assimilatory soluble nitrite reductase from Anabaena sp. 7119 has been reported by Mendez et al. (1981), which catalyzes the reduction of nitrite to ammonia. Ferredoxin was shown to be an efficient electron donor, when reduced either photosynthetically with subcellular particles, enzymatically with a NADPH-generating system or chemically with dithionite.

4. Higher plants: The enzyme nitrite reductase, which catalyzes the reduction of nitrite to ammonia (Hewitt, 1975; Guerrero et al., 1981; Vega et al., 1980), is widespread in higher plants. Enzymatic reduction of nitrite was first described briefly by Nason et al. (1954); soybean leaf extracts catalyzed ammonia formation from nitrite in the presence of either NADH or NADPH and manganese ions.

Similarly, in a brief report, Vaidyanathan and Street (1959) reported NADH-dependent disappearance of nitrite as ammonia in tomato extracts. Stoichiometry of the disappearance of nitrite and formation of ammonia was established for the first time by Hageman et al. (1962). Photosynthetic nitrite reduction was reported in wheat leaves by Vanecko and Varner (1955) but the reaction product was not identified. The participation of photosynthetically reduced electron donor in nitrite reduction was reported in a similar grana system of tomato (Sanderson and Cocking, 1964). Subsequently several groups demonstrated that ferredoxin was the physiological electron donor (Losada et al., 1963; Paneque et al., 1964; Betts and Hewitt, 1965). This nonheme iron protein replaced viologen dyes as the electron carrier in the dark (Joy and Hageman, 1966; Hattori and Vesugi, 1968). Since then a number of groups have attempted to isolate and purify nitrite reductases from higher plants (Beevers and Hageman, 1969; Ramirez et al., 1966; Hewitt et al., 1968; Shin and Oda, 1966).

Nitrite reductase from higher plants Cucurbita pepo (Hucklesby et al., 1976), calabash (Cardenas et al., 1972a), and spinach (Cardenas et al., 1972b; Vega and Kamin, 1977) have been purified to electrophoretic homogeneity.

Two forms of nitrite reductase have been isolated from scutella, roots and etiolated leaves of maize (Hucklesby et al., 1972; Dalling et al., 1973). The physical and biochemical characteristics of one form of the enzyme are nearly

identical with that of the enzyme from the green leaf. Only one form of the enzyme was found in the green leaf. Except for the differences in the thermal stability and ion charge, the properties of the second form are nearly identical with that of the enzyme from green leaves. The enzymes from the nonchlorophyllous tissue, like the enzyme from green leaves, can utilize reduced dyes or ferredoxin but not NAD(P)H or FMN or FAD as electron donor.

Recently, nitrite reductases have been purified from various sources using affinity chromatography as the final step of purification. Nitrite reductase from spinach leaves has been purified using affinity chromatography on ferredoxin-Sepharose (Ida, 1977). Nitrite reductase purified from barley leaves, using affinity chromatography, did not dissociate on treatment with SDS. It was composed of only one polypeptide chain having relative molecular mass 61,000. The spectral studies indicated that the enzyme contained a heme group.

Gupta et al. (1984) purified and characterized an assimilatory nitrite reductase from cell suspension cultures of Paul's Scarlet rose. The enzyme was purified to electrophoretic homogeneity using ferredoxin-Sepharose affinity chromatography. The enzyme activity from rose cells was precipitated by antiserum prepared against pea leaves nitrite reductase and formed immunoprecipitin bands during immunodiffusion and rocket immunoelectrophoresis.

The structure of nitrite reductase can be altered during

purification and the modified enzymes display differences in relative activities with different electron donors (Hirasawa et al., 1984). Native nitrite reductase (ferredoxin-linked nitrite reductase) from spinach leaves has a relative molecular mass of 86,000. The native enzyme can be separated into two protein components by treatment with DEAE Sephadex A-50. The two components have M_r 61,000 (modified nitrite reductase) and M_r 24,000 (coupling protein). The modified enzyme shows considerably less ferredoxin-linked nitrite reduction than native enzyme but retains its methyl viologen activity. The immunochemical characterization of nitrite reductases from spinach leaves, spinach roots and other higher plants has been carried out by Hirasawa et al. (1984). They have reported that native and modified nitrite reductases appear to be immunologically very similar. They based their conclusion on the immunodiffusion and immunoprecipitation behaviour. Nitrite reductase from spinach leaves and roots appeared to be identical proteins on the basis of their antigenic behaviour. A high degree of similarity between Spinacia oleracea and Chenopodium album nitrite reductase was also observed. No immunological cross reaction could, however, be detected between nitrite reductase from S. oleracia and the enzymes from C. pepo, Zea mays, Hordeum vulgare and Brassica rape.

Gupta and Beevers (1984) have reported that modulation of nitrite reductase activity in response to environmental perturbations appears to be due to de novo synthesis and

degradation and not due to reversible activation-inactivation of the enzyme protein.

Most of the workers seem to agree that intracellular location of nitrite reductase occurs in chloroplast fraction after aqueous or non-aqueous isolation (Harel et al., 1977; Rathnam and Das, 1974; Rathnam and Edwards, 1976; Wallsgrove et al., 1979). In non-green tissues nitrite reductase was found to be localized in the stroma of the chloroplast (Dalling et al., 1972b).

Prosthetic groups

Purified ferredoxin-nitrite reductases of higher plants and algae are redish-brown in colour and show similar absorption spectra characteristic of a heme-containing protein (Ho et al., 1976; Hucklesby et al., 1976; Vega and Kamin, 1977; Zumft, 1972). These spectra show peaks in the region 380 - 390 nm and 572 - 580 nm. The heme prosthetic group of spinach nitrite reductase was identified as "siroheme", previously shown to be a component of sulfite reductase (Hewitt, 1975; Vega et al., 1980). ESR spectroscopy has confirmed the presence of "siroheme" and provided evidence for the additional presence of an iron-sulfur centre in higher plants nitrite reductase (Aparicio et al., 1975; Cammack et al., 1978; Stoller et al., 1977; Vega and Kamin, 1977). Oxidized spinach nitrite reductase shows an ESR spectrum with resonance absorption at g-values of 6.72, 5.21, and 2.03, characteristic of high-spin ferric-heme with rhombically distorted tetragonal symmetry. Under strongly reducing conditions (reduced

ferredoxin + CO), ESR signals with g-values of 2.04 and 1.94 (reduced iron-sulfur centre) appear (Lancaster et al., 1979). The ESR spectrum of marrow nitrite reductase exhibits resonance absorption at g-values of 6.86, 4.98 and 1.95. Upon addition of dithionite plus methyl viologen, signals at g = 2.04, 1.94 and 1.92 are observed (Cammack et al., 1978). The iron-sulfur centre prosthetic group in spinach nitrite reductase has been identified as a tetranuclear cluster (4Fe - 4S) (Lancaster et al., 1979), also found as a typical cluster of sulfite reductase.

Iron appears to be the sole metal component of ferredoxin-nitrite reductase. Analytical and ESR data (Lancaster et al., 1979) indicated that the spinach enzyme contains a minimum of five iron atoms. This is compatible with a composition of one siroheme and one tetranuclear iron-sulfur centre (4Fe - 4S) per enzyme molecule (Lancaster et al., 1979; Vega et al., 1980).

The midpoint redox potential of the siroheme prosthetic group of plant nitrite reductase has been determined by EPR spectroscopy by reductive titration with reduced methyl viologen, reduced with metallic zinc. The disappearance of the high-spin heme EPR signal at g-values of 6.7 and 5.2 was followed. The data give a good fit for an n = 1 titration with a midpoint potential of -50 mV, at pH 7.8 for the spinach enzyme (Stoller et al., 1977), and -120 mV at pH 8.5 for the marrow enzyme (Cammack et al., 1978).

The midpoint potential of the iron-sulfur centre of

plant nitrite reductase has also been determined by reductive titration with dithionite but following the increase of the EPR signal at g = 1.94. By this method a midpoint potential of -550 mV (assuming n = 1), at pH 9.0 has been reported for spinach nitrite reductase (Stoller et al., 1977). Similarly, values of -570 mV (pH 8.1), -615 mV (pH 8.7), and -660 mV (pH 9.8) have been found for the marrow enzyme (Cammack et al., 1978).

Iron has a role in the formation of active nitrite reductase both in Azotobacter (Guerrero and Vega, 1975) and in Neurospora (Vega et al., 1975). The absorption spectrum of purified nitrite reductase of Neurospora shows absorption maxima at 280, 390 (soret) and 580 (α)nm and a shoulder at 450 nm. These are indicative of a hemoprotein containing a flavin (Greenbaum et al., 1978; Vega et al., 1975). The flavin component of the enzyme has been identified as FAD, and the heme chromophore as siroheme (Garrett and Amy, 1978; Vega, 1976; Vega et al., 1975). Greenbaum et al. (1978) suggested that this enzyme might contain nonheme iron also. Recently, Prodouz and Garrett (1981) have concluded that N. crassa nitrite reductase is a homodimer of large molecular weight subunit housing an electron transfer complex of FAD, iron-sulfur centre and siroheme to mediate the NAD(P)H-dependent reduction of nitrite to ammonia.

The assimilatory NADH-nitrite reductase from E. coli K 12 was reported by Coleman et al. (1978) to have no absorbance maximum in the range 380 - 600 nm, but their

analytical data show a flavin content of 0.07 mole FMN and 0.4 mole FAD per mole nitrite reductase. Jackson et al. (1981) further characterized the enzyme from E. coli K 12. They have shown that the enzyme contains one non-covalently bound FAD, probably 5 Fe atoms and 4 acid-labile sulfur atoms per subunit. The absorption spectrum of the enzyme showed maxima at 386, 455, 530, ∿575 nm and a shoulder at 480 - 490 nm. The heme chromophore of the enzyme is also shown to be siroheme. The nitrite reductase from the same source was examined by Cammack et al. (1982) by low temperature EPR spectroscopy. The enzyme, stored in the presence of NO_2^- and ascorbate, gave the spectrum of a nitrosyl derivative with hyperfine splitting due to nitrosyl N. On removal of these reagents, a series of signals centered around g = 6 were observed typical of high-spin ferric heme. Cyanide converted these into a low-spin form. On reduction of the enzyme with NADH, an axial spectrum at g = 1.92, 2.01 was observed. The temperature dependence of the signal was indicative of a (2Fe - 2S) Fe-S cluster. The midpoint potential of this cluster was estimated to be -230 mV. Reduction of the enzyme with dithionite yielded further signals, which are at present unidentified at g = 2.1 - 2.8. No signal could be observed that would assign to a (4Fe - 4S), cluster such as those found in sulfite reductases and other nitrite reductases containing siroheme.

In denitrifying prokaryotes, at least two types of dissimilatory nitrite reductases can be distinguished. A cytochrome cd-containing enzyme with a four heme moiety (EC 1.9.3.2) has

been isolated from various Pseudomonas species (Horio et al., 1961; Kodama, 1970), Paracoccus denitrificans (Lam and Nicholas, 1969b), T. denitrificans (Sawhney and Nicholas, 1978), whereas heme-free Cu-containing enzymes (EC 1.7.99.3) have been reported to occur in A. cycloclastes (Iwasaki and Matsubara, 1971), A. faecalis (Kakutani et al.,1981) and denitrifying strain of the phototrophic bacterium R. sphaeroides (Sawada et al., 1978). Nitrite reductase (cyt cd) of T. denitrificans was studied with Mossbauer, EPR and optical spectroscopy and biochemical techniques by Boi et al. (1982). They have shown that the enzyme contains 2 heme c and 2 heme d per mole of the enzyme. The d heme exhibited EPR resonance at g = 2.5, 2.43 and 1.7 similar to those reported for chlorine diimidazole complexes. The c heme exhibited EPR resonance for g = 3.6, suggesting a nitrogenous 6th ligand. Upon reduction, c heme remains low-spin (S = 0) and d heme becomes high-spin (S = 2). Heme d_1 is the trivial name given to the prosthetic group of dissimilatory nitrite reductase found in many chemoautotrophic denitrifying bacteria. Recently, Timkovich et al. (1984) have identified the ten substituents of the macrocycle core of heme d_1 and termed acrylochlorin: four methyls, two hydroxymethyls, two formate, a propionate and an acrylate. The macrocycle is a chlorin, i.e. one of the four pyrrole rings has a saturated β-pyrrolic bond, and thus bears four substituents. The meso positions are unsubstituted and bear methine protons.

A Cu-containing dissimilatory nitrite reductase has been

purified from phototrophic bacterium R. palustris (Preuss and Klemme, 1983). Trace metal analysis showed Cu as the metal component of the enzyme. Very low amount of c-type cytochrome has also been detected in the highly purified enzyme. The contamination of the cytochrome c in the enzyme can not be excluded. Masuko et al. (1984) purified a Cu-containing dissimilatory enzyme from Alcaligenes sp. NCIB 11015. The EPR spectrum and Cu analysis clearly indicated that the enzyme contained 2 type I Cu atoms per mole but no other type of Cu. This is the first blue protein to be reported that exhibits catalytic activity despite possessing only I type Cu.

A respiratory nitrite reductase (ammonia-forming) has been purified from A. fischeri (M_r 80,000) which contains only two c-type heme groups, and utilizes either reduced flavin or viologen dyes as electron donor (Prakash and Sadana, 1972). The enzyme catalyzes the six-electron reduction of nitrite to ammonia. Liu and Harry (1981) have isolated a new type nitrite reductase from a strain of Desulfovibrio desulfuricans. The purified enzyme has a minimal M_r 66,000 and contains six c-type heme groups per molecule. The enzyme catalyzes the six-electron reduction of nitrite to ammonia. The multiheme nature of this nitrite reductase has recently been confirmed by EPR spectroscopy (Liu et al., 1980) which revealed 4 - 6 distinct ferric heme resonances, including high and low spin, from the purified enzyme.

Mechanism of enzyme catalysis

Multielectron reduction reactions involve some of the most important and yet least understood enzymatic processes known. Two of these reactions, catalyzed by cytochrome c oxidase ($O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$) and nitrogenase ($N_2 + 8H^+ + 6e^- \longrightarrow 2NH_4^+$) respectively, involve enzymes with multiple subunits and multiple prosthetic groups. In contrast, two other multielectron reduction reactions, sulfite reduction to sulphide ($SO_3^{2-} + 8H^+ + 6e^- \longrightarrow H_2S + 3H_2O$) and nitrite reduction to ammonia ($NO_2^- + 8H^+ + 6e^- \longrightarrow NH_4^+ + 2H_2O$), are catalyzed by monomeric enzymes which contain only two prosthetic groups, one iron-sulfur centre (4Fe - 4S) and a novel heme, termed as "siroheme" on a single polypeptide chain of M_r 60,000 (Lancaster et al., 1979; Siegel et al., 1982; Krueger and Siegel, 1982). Although sulfite- and nitrite reductases are physiologically distinct proteins within a given organism (Krueger and Siegel, 1982), each enzyme is capable of catalyzing both types of multielectron reduction reactions under appropriate conditions. The A. fischeri nitrite reductase, however, did not show sulfite reductase activity (Prakash and Sadana, 1972).

The six-electron enzymatic reduction of nitrite to ammonia was earlier postulated to proceed by a series of two electron transfers, each catalyzed by a different enzyme (Nason, 1962; Hewitt and Nicholas, 1964; Takahashi et al., 1963). However, working with E. coli nitrite reductase, Lazzarini and Atkinson (1961) concluded that the enzyme catalyzed the complete six-

electron reduction of nitrite to ammonia with no obligate free intermediates. The relevant findings were: (a) there was no indication of separation of enzymes catalyzing the different steps in the course of moderate degree of purification of the overall system, (b) possible intermediates at the +1 oxidation state of nitrogen (hyponitrite and nitrous oxide) were not reduced, (c) although hydroxylamine was reduced, free hydroxylamine was not an obligate intermediate in the reduction of nitrite, (d) there was no evidence for the participation of a dissociable organic cofactor, so that the movement of hydroxylamine from one site to the other in the form of an organic compound seems unlikely. This conclusion was supported by studies of Kemp and Atkinson (1966) who showed that E. coli nitrite reductase catalyzes the reduction of nitrite as well as hydroxylamine to ammonia and that the Michaelis constant for hydroxylamine was 150 times greater than that for nitrite. The high K_m for hydroxylamine seems to exclude hydroxylamine as a free intermediate in nitrite reduction. Nitrite reductase from A. fischeri has also been shown to catalyze complete six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). They have also shown that both the substrates, nitrite and hydroxylamine, are reduced at the same active site and no free intermediate could be determined during the six-electron reduction of nitrite. The K_m for hydroxylamine was approximately two order of magnitude greater than that for nitrite. The high K_m value for hydroxylamine seems to preclude it as a free intermediate in the reaction. Recently Sadana

et al. (1981) used GC/MS method for detection of $^{15}NH_2OH$ oxime and indicated that free hydroxylamine does not accumulate. Similar conclusions were reached with ferredoxin-nitrite reductase from Chlorella, spinach and squash leaves (Beevers and Hageman, 1969; Losada, 1972). Results reported by Vega et al. (1973) corroborate this view and show that in A. chroococcum, nitrite is stoichiometrically reduced to ammonia without the formation of hydroxylamine as a free intermediate.

Since ferredoxin is a single electron donor, the reduction of nitrite to ammonia might proceed by a series of one electron stages; such a scheme was proposed by Fewson and Nicholas (1961b), however, if such intermediates do occur, it appears unlikely that they are released in the free form. Hewitt et al.(1968) and Cresswell et al. (1965) suggested a tentative hypothetical scheme, based on a hemiacetal structure, for the reduction of nitrite and hydroxylamine by a single protein (Fig. 1). The scheme is compatible with no free intermediates. However, as the scheme is based on a sequence of two electron steps, it would need further modification to accommodate the specific requirement for a single electron donor (either ferredoxin or benzyl viologen), as suggested by the authors (Hewitt et al., 1968).

Like nitrite reductase, assimilatory sulfite reductases have been obtained in a homogeneous state which catalyze the six-electron reduction of sulfite to sulfide without formation of free inorganic sulfur-containing intermediates

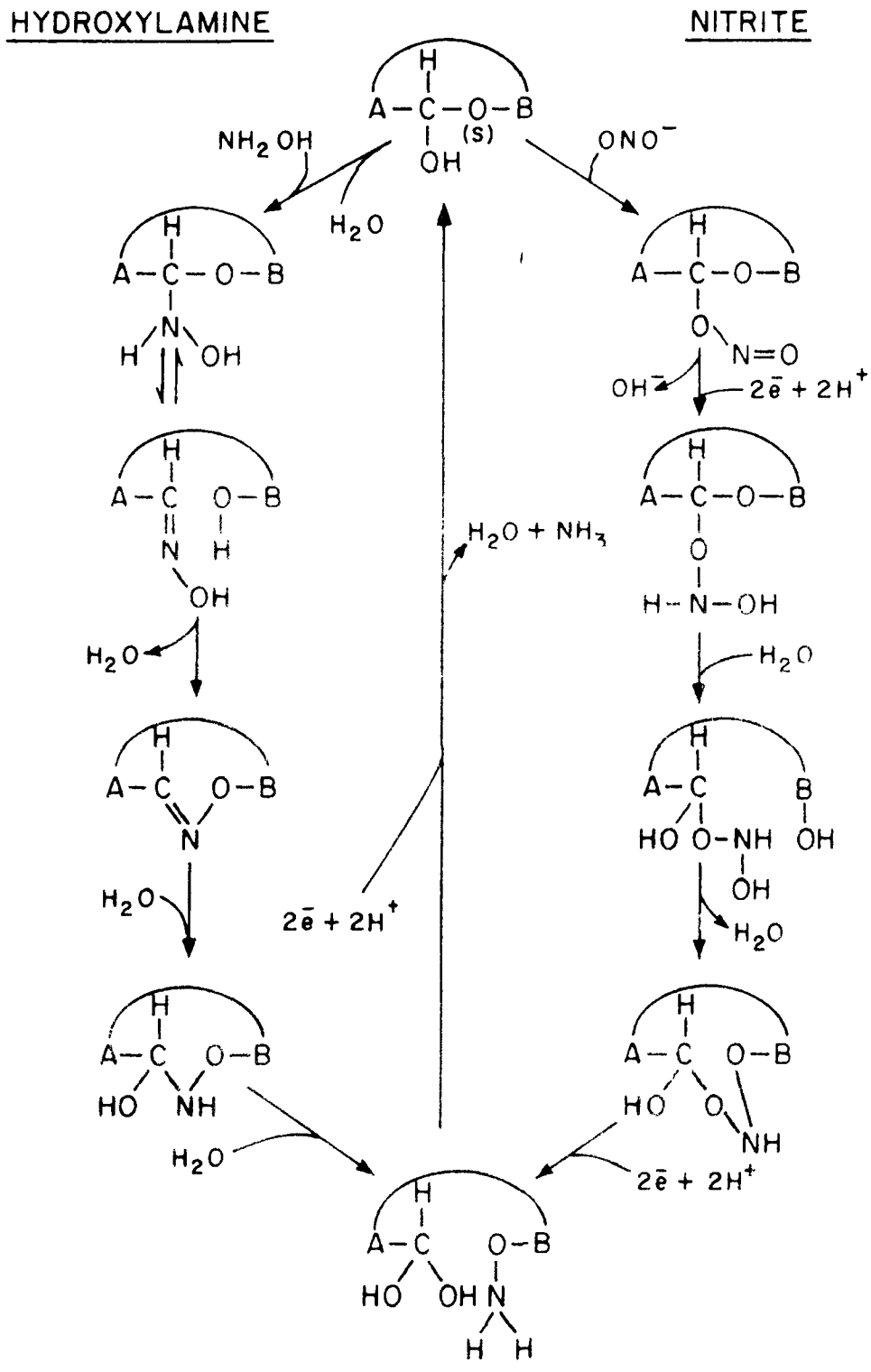


FIG 1 TENTATIVE SCHEME FOR REDUCTION OF NITRITE OR HYDROXYLAMINE BY SAME ENZYME SYSTEM PRODUCING NO FREE INTERMEDIATES

(Prabhakararao and Nicholas, 1969; Siegel and Kamin, 1968).

Although in plants, sulfite- and nitrite reductions are catalyzed by distinct enzymes (Asada et al., 1969; Mauzerall, 1962), it is interesting to note that in bacteria and fungi, sulfite reductases of both assimilatory (Lazzarini and Atkinson, 1961; Kemp and Atkinson, 1966; Siegel et al., 1971; Siegel and Kamin, 1968; Yamanaka and Okunuki, 1963) and dissimilatory (Trudinger, 1970) types are capable of catalyzing the reduction of nitrite. With E. coli sulfite reductase the product of nitrite reduction by NADPH was identified as ammonia (Lazzarini and Atkinson, 1961). The possibility that some structural features may be common to both sulfite- and nitrite reductions in nature, was supported by the studies of Zumft (1972) who demonstrated striking similarities between nitrite reductases (from Chlorella and spinach) and sulfite reductases (from spinach, yeast and E. coli) suggesting that the two enzymes might share a common heme chromophore. The heme chromophore has been isolated from E. coli sulfite reductase and identified as a new type of heme prosthetic group, an octacarboxylate iron-tetrahydroporphyrin of the isobacteriochlorin-type which has been given the name "siroheme" (Murphy et al., 1973; Murphy and Siegel, 1973). Murphy et al. (1974b) reported that the heme-like prosthetic group of spinach ferredoxin-nitrite reductase is identical in its spectral and chromatographic properties to the siroheme of E. coli sulfite reductase. The presence of this new type iron-porphyrin, siroheme in the assimilatory sulfite

reductase of E. coli and spinach ferredoxin-nitrite reductase suggests that both types of multielectron reduction processes (sulfite to sulfide and nitrite to ammonia) may share common mechanistic features. The association of siroheme with the reduction processes involved in the metabolism of two of the major elements of the biosphere, nitrogen and sulfur, suggest that this novel heme may have played a key role in the evolution of redox metabolism.

Nitrite reductase (ferredoxin-nitrite oxido-reductase, EC 1.7.7.1) of higher plant leaves catalyzes the six-electron reduction of nitrite to ammonia with reduced ferredoxin as electron donor. The enzyme contains heme iron of a similar type to the siroheme of bacterial sulfite reductase (Murphy et al., 1974b; Aparicio et al., 1975; Hucklesby et al., 1976) and labile sulfur (Zumft, 1972; Hucklesby et al., 1974; Aparicio et al., 1975; Vega and Kamin, 1977) indicative of an iron-sulfur centre (Aparicio et al., 1975; Vega et al., 1976). Optical absorption measurements indicate that nitrite and inhibitors such as cyanide and CO bind to the heme-iron (Aparicio et al., 1975; Hucklesby et al., 1976; Vega and Kamin, 1977) and it has been proposed that the iron-sulfur centre serves to transfer electrons from ferredoxin to heme.

The detailed mechanism of the reduction of nitrite to ammonia is complex. Cammack et al. (1978) has drawn the following conclusions about the early steps of the reduction cycle on the basis of the EPR detectable intermediates:

Step I: Spectrophotometric measurements indicate that nitrite

(formal oxidation state + 3) binds to the heme. The reaction of nitrite with the oxidized form of the enzyme is slow, requiring several minutes at 0°C for complete disappearance of the signal around g = 6.8 and 5.0. Such a reaction could not be part of the enzyme reaction under turnover conditions, and therefore the heme must first be reduced to the ferrous state, as concluded from spectrophotometric observations (Aparicio et al., 1975; Hucklesby et al., 1976; Vega and Kamin, 1977).

It would be expected that the iron-sulfur centre may be in the oxidized state initially, since its midpoint potential is extremely low. Therefore, the initial one electron reduction of the heme would probably occur directly by ferredoxin. After substrate is bound, the iron-sulfur centre is probably more easily reduced by analogy with the effect of cyanide and CO.

Step 2: In the second stage nitrite already bound to the ferrous heme is reduced to a heme-NO complex (oxidative state + 2). The electron for this process might be provided either by the iron-sulfur centre or by ferredoxin. Some evidence that the siroheme-NO species is a intermediate in the reaction, rather than a dead and complex, is provided by the observation that it disappeared at least within a few seconds of the completion of the enzyme reaction. However, its true status may only be conclusively demonstrated by more rapid freezing techniques.

Further reduction: Four more electrons are now required to be added to the nitrogen ligand while it is bound to the heme of

the enzyme. EPR signals from the intermediates of this reaction have not been seen, but observations of the reactions with different electron donors are relevant to this topic. With nitrite as substrate and $Na_2S_2O_4$ alone as reductant the reaction proceeds slowly to ammonia, reaching an apparent equilibrium with a relatively small heme-NO signal. This indicates that most of the NO bound to heme has been reduced to a further stage but not as far as ammonia. (Alternatively, the NO might have dissociated. However, the Fe-S centre is fully reduced, suggesting that a ligand is still bound to the heme.) Assuming that Fe-S centre still has a comparatively low midpoint potential under these conditions, why does it not become reoxidized? A possible explanation of the block in activity in the absence of methyl viologen or ferredoxin is that one of the later stages of reduction requires two electrons to be added, simultaneously, one by the Fe-S centre and the other by the electron donor directly. $Na_2S_2O_4$ either does not react sufficiently rapidly or can not do so at two sites simultaneously, but may function slowly in the slightly dissociated form, $SO_2^{\cdot-}$, as a single electron donor.

An alternative possibility for the origin of NO-heme intermediate

An alternative hypothesis, which can not be excluded at this stage, is that NO generated by reaction of nitrite at the nonheme centre subsequently reacts with siroheme. This possibility is suggested by studies of the reaction between nitrite and ferredoxin, which may be in this respect characteristic of iron-sulfur proteins in general. Thus, whereas

$Na_2S_2O_4$ does not react appreciably with NO_2^- at pH values above 6.0, NO is produced in stoichiometric amounts at pH 6 - 7 when $Na_2S_2O_4$ and nitrite are incubated with ferredoxin (Garrioch and Hewitt, 1976). At pH 7 - 8.5, NO is formed together with ammonia, which predominates at higher pH. If a similar reaction occurs during enzymatic nitrite reduction, the NO generated might then combine with the reduced siroheme. Nitrosylation of reduced heme has previously been described (Paul and Kumta, 1975) and was used by Garrioch and Hewitt (1976) as a sensitive detector of NO produced from nitrite and ferredoxin. They also showed that myoglobin—NO was formed with $Na_2S_2O_4$-reduced Mb in the presence of nitrite and that the comparatively slow reduction of Mb-NO to ammonia by $Na_2S_2O_4$ was accelerated about 25% by the presence of ferredoxin. Ferredoxin and myoglobin may be viewed as an interesting model system for the Fe-S and heme component of nitrite reductase, although functioning at only approximately 1% of the rate obtained by a ferredoxin-nitrite reductase. It appears that the nitrite reductase reaction involves concerted changes in the redox potentials of the electron-transfer components that react with the various nitrogenous intermediates.

In denitrifying prokaryotes, at least two types of dissimilatory nitrite reductases i.e. enzymes catalyzing the reduction of nitrite to gaseous nitric oxide or nitrous oxide, can be distinguished; a cytochrome cd containing

enzyme and another heme-free Cu-containing enzyme. The literature about the mechanism of nitrite reduction by these organisms is very limited. A recent ^{15}N NMR study (Timcovich and Cork, 1982) has proposed that the sequence of events in nitrite reduction by P. aeruginosa nitrite reductase is: first reduction of cytochrome cd_1 by an appropriate electron donor and then interaction of reduced cytochrome cd_1 with substrate nitrite. They proposed that there is a weak association between nitrite and ferric reductase with a value of 1.3 M^{-1} for the association constant.

PRESENT INVESTIGATIONS

Nitrate is metabolized by A. fischeri giving ammonia as the end product (Prakash and Sadana, 1972). There are two enzymes which are required to catalyze the reduction of nitrate to ammonia: (1) nitrate reductase, which catalyzes the two-electron reduction of nitrate to nitrite, and (2) nitrite reductase which catalyzes the six-electron reduction of nitrite to ammonia. Both the enzymes have been studied.

Nitrate reductase

It is evident from the literature reviewed that nitrate reductase has been purified and characterized from various sources ranging from prokaryotes to eukaryotes and revealed important differences regarding subunit composition and prosthetic groups. Nitrate reductase from A. fischeri was partially purified by Sadana and McElroy (1957). The purified enzyme so obtained was strongly coloured and showed absorption maxima at 550, 520 and 419 nm in the reduced state. Although a certain amount of indirect evidence (inhibitor studies) indicated that the nitrate reductase does not contain iron porphyrin, it was not certain whether the characteristics of a cytochrome-like component were due to nitrate reductase or to an associated impurity. Later on the enzyme was obtained free from cytochrome (Sadana et al., 1963). The nitrate reductase so obtained was relatively unstable and generally lost about 20% of its activity on storage at 4 °C for 24 h. Ultracentrifugal studies indicated the presence of two components in the purified enzyme (Sadana et al., 1963).

The aim of the work presented in this thesis (Chapter III) was to obtain A. fischeri nitrate reductase in a homogeneous state and having better stability. The physicochemical properties and subunit structure of the enzyme was also studied.

Nitrite reductase

A. fischeri nitrite reductase has been purified to homogeneous state in this laboratory previously (Husain and Sadana, 1972). The enzyme has also been studied for molecular weight, subunit structure, amino acid composition and hydrodynamic properties (Husain and Sadana, 1974a; 1974b). The amino acid residues which are essential for the activity of the enzyme have not been studied previously.

The aim of the work, described in Chapter V was to determine the essential amino acid residues which are required for the activity of the enzyme. This aspect of the study was carried out by chemical modification studies. The pK values of the essential amino acid residues were also determined.

A. fischeri nitrite reductase utilizes various electron donors including $FADH_2$ and $FMNH_2$ but the K_m values for these are much higher than for reduced benzyl viologen. The part of the work in Chapter VI was aimed to determine the likely physiological electron donor for the enzyme. Studies were also undertaken to understand the status of hydroxylamine as an intermediate in the reduction of nitrite to ammonia.

Electron paramagnetic resonance (EPR) spectroscopic studies were also carried out to detect intermediates during the enzymatic reduction of nitrite to ammonia and also to understand its mechanism of earlier steps of reduction.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

The following chemicals were purchased from the suppliers indicated: Bacto peptone was obtained from Bengal Immunity Co. Ltd., Calcutta; bacto agar (Difco) from Difco Laboratories, USA; and beef extract from Oxoid, England. Crystalline bovine serum albumin, crystalline ovalbumin, gamma-globulin, transferrin, deoxyribonuclease I, yeast alcohol dehydrogenase, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, spinach NADPH-ferredoxin oxidoreductase, Coomassie Brilliant Blue, protamine sulfate (Salmine) and p-hydroxymercuribenzoate were obtained from Sigma Chemical Co., USA. Acrylamide, N,N'-methylenebis-acrylamide and N,N,N',N'-tetramethylethylenediamine were the products of Eastman Organic Chemicals, USA. Benzyl viologen, methyl viologen were purchased from British Drug House Ltd., England. Sephadex G-200, Sephadex G-25 and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P-150 was obtained from Bio-Rad Laboratories, USA. 2-Mercaptoethanol, diethylpyrocarbonate and imidazole from Fluka, Switzerland; sodium dodecyl sulfate from BDH Products Pvt. Ltd.; iodoacetamide from Koch-Light Laboratories, U.K.

Argon gas and sodium nitrite ($Na^{15}NO_2$, 99.7 atom % excess, were purchased from British Oxygen Co. Ltd., London, SW19 3UF, UK. ^{14}NO gas was obtained from Cambrian Gases Croydon, Surrey, and passed through 1% sodium hydroxide solution to remove higher oxides of nitrogen. Hydroxylamine

hydrochloride of 95 - 98% purity was obtained from E. Merck and used after recrystallization. Chondrus crispus flavodoxin was kindly supplied by Dr. L.J. Rogers, School of Biological Sciences, University of Wales, Aberystwyth, U.K.

All other chemicals used were from commercial sources and were of analytical grade. Prior to use p-HMB was recrystallized by the procedure of Boyer (1954). The analytical grade urea was recrystallized from aqueous ethanol and stored dry over P_2O_5 under vacuum. Dry ethanol was prepared by reaction of water (in ethanol) with magnesiumethoxide (Fieser, 1955).

Hydroxylapatite gel was prepared according to the procedure of Tiselius et al. (1956). The gel was equilibrated with 1 mM potassium phosphate buffer, pH 6.8.

Glass distilled water was used in all enzymatic studies.

METHODS

Organism: The salt-water luminous bacterium Achromobacter fischeri used in the present investigations was obtained from Dr. W. D. McElroy (McCollum-Pratt Institute, Johns Hopkins University, Baltimore, USA). The culture appeared as gram-negative rods, sometimes slightly curved.

Maintenance and propagation of culture: A. fischeri was propagated on nutrient agar slants of the following composition:

Peptone	0.5 g	Glycerol	0.3 ml
Sodium chloride	3.0 g	Agar	2.0 g
Beef extract	0.3 g	Calcium carbonate	0.3 g

Distilled water to make 100 ml.

The first four constituents were dissolved in water and the pH was adjusted to 7.4 with 4N KOH. The final volume was made to 100 ml. Agar and calcium carbonate were then added and the mixture steamed for 1 h. For preparation of slants, 7 - 8 ml aliquots were distributed into 19 x 150 mm tubes and autoclaved at 15 psi (120°C) for 20 min. The tubes were shaken while hot in order to distribute calcium carbonate uniformly, immediately slanted and allowed to solidify. These slants were inoculated from the stock culture and incubated at 28°C for 24 h.

The organism was maintained at 4°C and subcultured routinely every month.

Basal liquid medium for growth: To obtain large amounts of A. fischeri cells, the organism was grown in the following basal liquid medium:

Sodium chloride, NaCl	30.00 g
Ammonium phosphate, $(NH_4)_2HPO_4$	0.50 g
Potassium dihydrogen phosphate, KH_2PO_4	2.10 g
Disodium hydrogen phosphate $Na_2HPO_4 . 12\ H_2O$	7.08 g
Magnesium sulfate, $MgSO_4$	0.10 g
Ferric chloride, $FeCl_3 . 6H_2O$	0.01 g
Glycerol	3.00 ml
Peptone	10.00 g

Distilled water to make one litre.

All the constituents, except ferric chloride and magnesium sulfate, were dissolved and pH adjusted to 7.4 by

4 N KOH. Ferric chloride and magnesium sulfate were dissolved separately and then mixed. The medium was autoclaved at 15 psi (120°C) for 20 min.

Growth conditions and collection of cells: Inocula from 24 h cultures, grown on agar slants, were transferred into 500 ml conical flasks containing 100 ml basal liquid medium and grown for 20 h at 28°C on a rotary shaker, 210 rpm. The organism was subcultured through two transfers in liquid media (without nitrate) under aerobic conditions. The inoculum was then transferred to 15 L of the basal liquid medium in glass carboys containing 0.1% potassium nitrate. Antifoam (0.2 to 0.3 ml, Alkaterge C, Commercial Solvent Corporation, USA: one part antifoam mixed with four parts of liquid paraffin) was added to each carboy in order to prevent excess frothing. The culture was kept at 28 - 30°C and purified air was continuously passed through the cultures from sintered glass units at 550 ml/min. After growing for 18 to 20 h the cells were harvested in a Sharples centrifuge (2,000 rpm) at a flow rate of 10 L/h. The cells were washed free of nitrite by suspending in 3% sodium chloride solution and centrifuging. The cells were stored as paste at -20°C until used.

Definition of unit of nitrate reductase activity and specific activity: The unit of nitrate reductase activity is defined as the amount of enzyme required to produce 1 µmol of nitrite from nitrate in 1 min at 28°C, pH 7.5, using reduced benzyl viologen as the electron donor under

these experimental conditions. The specific activity is defined as the activity per mg of protein.

Estimation of nitrate reductase activity: Nitrate reductase activity was measured in Thunberg tubes with chemically reduced benzyl viologen as electron donor. The rate of reaction was measured by determining the amount of nitrite formed in the reaction mixture by the diazo-coupling procedure of Snell and Snell (1949). The details of the assay procedure are as follows:

The incubation mixture contained in a final volume of 1.5 ml, 200 µmol of potassium phosphate, pH 7.5, 10 µmol of sodium nitrate, and enzyme protein in the main arm of the Thunberg tube. Benzyl viologen, 0.5 ml (10 mg/ml in water) and one ml of freshly prepared dithionite solution (1 mg/ml in 200 mM potassium phosphate buffer, pH 7.5) were placed in the side arm of the Thunberg tube and the tubes were evacuated immediately. The reaction was started by the addition of reduced benzyl viologen. The final pH of the reaction mixture was 7.5. After 8 - 10 min of incubation at room temperature (28°C), the reaction was terminated by opening the Thunberg tubes and shaking for few seconds to oxidize all the reduced benzyl viologen. One ml of sulfanilamide reagent (1% w/v in 1 M HCl) followed by 1 ml of N-(1-naphthyl)-ethylenediamine dihydrochloride (0.02% w/v in water) was added to 1 ml aliquot of reaction mixture. The resulting red colour was read at 540 nm after 10 min, after making the volume to 9.5 ml with water. The

absorbance of 0.5 for 10 mm light path is equivalent to 0.1 µmol of nitrite produced. A blank with all the assay constituents except enzyme was always run.

Definition of unit of nitrite reductase activity and specific activity: The unit of nitrite reductase activity is defind as the amount of enzyme required to cause disappearance of 1 µmol of nitrite in 10min, at 28°C, pH 7.5, using reduced benzyl viologen as the electron donor under the experimental conditions. The specific activity of the enzyme is defined as the activity per mg protein.

Estimation of nitrite reductase activity: Nitrite reductase activity was measured in Thunberg tubes under anaerobic conditions. The rate of reduction of nitrite was measured by determining the decrease in nitrite concentration in the reaction mixture by the diazo-coupling procedure of Snell and Snell (1949). The details of the assay procedure are as follows:

The main arm of the Thunberg tube contained 200 µmol of potassium phosphate, pH 7.5, 0.67 µmol of sodium nitrite and the enzyme sample in a final volume of 1.5 ml. The side arm of the Thunberg tube contained 0.5 ml of benzyl viologen (10 mg/ml in water) and 1 ml of freshly prepared dithionite (1 mg/ml in 0.2 M potassium phosphate buffer, pH 7.5). The tubes were evacuated immediately and the reaction was started by adding reduced benzyl viologen from the side arm of the Thunberg tubes to the main arm. After 4 to 6 min of incubation at room temperature (28°C), the reaction was

terminating by opening the Thunberg tubes and shaking for few seconds to oxidize all the reduced benzyl viologen. The final pH of the reaction mixture was 7.5. One ml of sulfanilamide reagent (1% w/v in 1 M HCl) was added to 1 ml aliquot of the reaction mixture followed by addition of 1 ml of N-(1-naphthyl)-ethylenediamine dihydrochloride (0.02% w/v in water). The resulting red colour was read at 540 nm after 10 min, after making the volume to 9.5 ml. The amount of enzyme used was adjusted so that the nitrite utilized was between 0.2 - 0.3 μmol. A blank with all the assay constituents except enzyme was always run.

Estimation of catalase activity: Catalase was assayed by the method of Beers and Sizer (1952). The enzyme (0.1 ml) was added to 2.9 ml buffered solution of hydrogen peroxide (0.2 ml of 30% H_2O_2 in 50 ml of 0.05 M potassium phosphate buffer, pH 7.0) taken in 3 ml silica cuvettes. The decrease in absorbance at 240 nm per 1 - 2 min was recorded.

Estimation of alcohol dehydrogenase: Alcohol dehydrogenase was assayed by the method of Vallee and Hoch (1955). The assay mixture contained 2.5 ml potassium phosphate, 20 mM, pH 8.8, 0.1 ml of NAD^+ (10 mg/ml in 0.02 M phosphate, pH 6.5) and 0.2 ml of 95% ethanol in 3 ml silica cuvettes. The reaction was started by adding 0.2 ml of the enzyme sample and the increase in absorbance was measured against the reagent blank at 340 nm.

Protein determination: The following methods were adopted for the determination of protein in the enzyme samples:

(a) Turbidimetric method: The protein content in the crude extracts was estimated by turbidity measurements of trichloroacetic acid - precipitated proteins as described by

Stadtman et al. (1951). Samples containing 0.1 - 0.2 mg of protein were diluted with water to a volume of 2 ml and 3 ml of 5% trichloroacetic acid solution was added. The resulting suspension was allowed to stand for 1 min and the turbidity measured at 540 nm. Crystalline bovine serum albumin was used as the standard.

(b) Method of Lowry et al. (1951): Protein determination in the subsequent purification steps was carried out with the Follin-Ciocalteau reagent as described by Lowry et al. (1951). Crystalline bovine serum albumin was used as the standard and the final solution was read at 750 nm. Samples free of ammonium sulfate and tris and containing only low levels of phosphate were used to avoid interference from these substances.

(c) Optical method: Protein determination by the optical method of Warburg and Christian (1941) was carried out by using the empirical equation of Jagannathan et al. (1956) to correct for light absorption due to nucleic acids, the light path being 10 mm:

$$\text{mg protein/ml} = 4/7\left[2.3(A_{280} - A_{340}) - (A_{260} - A_{340})\right]$$

This method was used to obtain rapid comparative estimates of protein content in the various fractions during column chromatography, although this method was somewhat inaccurate. The concentration of bovine serum albumin was calculated from its molar extinction coefficient, 39.6×10^3 $M^{-1}cm^{-1}$ at 280 nm (Tanford and Roberts, 1952).

Ammonium sulfate fractionation: Ammonium sulfate saturation refers to 0°C and the quantity required for changing the degree of saturation was calculated from the following equation (Jagannathan et al., 1956):

For solid ammonium sulfate

$$X = \frac{50\ (S_2 - S_1)}{1 - 0.28\ S_2}$$

For saturated ammonium sulfate solution

$$Y = \frac{100\ (S_2 - S_1)}{1 - S_2}$$

where X equals g of solid ammonium sulfate to be added to 100 ml of a solution of saturation S_1 in order to change its saturation to S_2, and Y equals to ml of saturated ammonium sulfate solution to be added to 100 ml of solution to change its saturation from S_1 to S_2, S_1 and S_2 being expressed in fraction of saturation at 0°C. Ammonium sulfate, solid or saturated solution, was added slowly with gentle stirring to avoid frothing. The suspension was allowed to stand for 30 min to 40 min and then centrifuged at 4,000 x g for 45 min.

Gel filtration studies: A column of Sephadex G-200 (40 - 120 μ) or Bio-Gel P-150 was equilibrated at 4°C with 50 mM potassium phosphate buffer, pH 7.0. The hydrated gel and buffer were routinely deaerated under vacuum prior to use. When the bed had settled to a constant height, the sample

solution (0.5 to 1.0 ml) containing 10% sucrose was carefully layered under the buffer solution at the top of the gel. Eluate fractions of 1 ml were collected at a flow rate of 10 - 12 ml/h and assayed for protein and/or enzymic activity. Dextran Blue 2000 (Pharmacia) was used to determine the void volume (Vo) and phenylalanine to measure the inner volume (Vi). The total volume (Vt) of the gel column was determined directly with water. The elution volume (Ve) of a given solute zone was taken in all cases as the effluent peak position of the solute. The column was calibrated with the proteins of known molecular weights (Andrews, 1964) or Stokes' radii (Ackers, 1964). Gel filtration data are presented in terms of Ve/Vo, Kd, and Kav, the parameters involved in several mathematical correlations of elution volume with Stokes' radius and molecular weight (Ackers, 1964; Porath, 1963; Laurent and Killander, 1964). The parameters Kd and Kav are calculated as defined by the following equation (Siegel and Monty, 1966):

$$Kd = \frac{Ve - Vo}{Vi} = \frac{Ve - Vo}{Vt - Vg - Vo}$$

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where Ve, Vo, Vt and Vi have the same meaning as described above, Vg, the volume occupied by the gel grains, is estimated from the following equation:

$$Vg = Vt/B.d$$

where B = bed volume per g of dry Sephadex G-200 (approximately 35 ml/g) and d is the density of the dry Sephadex G-200 (1.65 g/ml) (Laurent and Killander, 1964). For the columns used in the present work Vg = 1.74 and Kav = 0.973 Kd.

Polyacrylamide gel electrophoresis: Analytical polyacrylamide gel electrophoresis was carried out at pH 8.9 and 4.3 for testing the homogeneity of the enzyme.

Electrophoresis at pH 8.9 was performed according to Davis (1964) using 7.5% acrylamide gel polymerized with 0.14% ammonium persulfate. The discontinuous buffer system consists of Tris-glycine buffer, pH 8.3 (electrode buffer) and Tris/HCl buffer, pH 8.9 (separation gel). Bromophenol Blue was used as a marker dye.

Electrophoresis at pH 4.3 was performed according to Maurer (1971) using 7.5% acrylamide gel polymerized with 0.14% ammonium persulfate. The discontinuous buffer system consists of β-alanine and acetic acid buffer, pH 5.0 (electrode buffer) and KOH-acetic acid buffer, pH 4.3, (separation gel). Aqueous solution of methyl green was used as a marker dye.

Sample and stacking gels were omitted. After 2 h of preliminary electrophoresis to remove residual persulfate ions, 50 - 200 µl sample (made dense with 20% sucrose) was applied through the upper buffer on the surface of the gel. Electrophoresis was carried out in the cold room (4 °C) at 3 mA per tube for 3 - 4 h until the dye reached the bottom of the gel. After the electrophoresis, protein bands were

stained with 1% amido schwarz in 7% acetic acid or 0.1% Coomassie Brilliant Blue. The destaining of the gel was performed by diffusion in 7% acetic acid.

The molecular weight of the native nitrate reductase, using polyacrylamide gel electrophoresis, was determined according to the method of Hedrick and Smith (1968). Separation gels with various concentrations of acrylamide (6 - 12%) were prepared according to Ornstein and Davis (1964) except that the ratio of acrylamide to bis (N,N'-methylene-bisacrylamide) was 30:1 which was maintained constant in all the gels. The use of spacer gel was found unnecessary. Samples (100 µl) in 5 mM Tris-glycine buffer, pH 8.3 containing 50% glycerol and 0.05% Bromophenol Blue were layered on top of the gels. Electrophoresis was carried out at 2 mA for 30 min and 4 mA for 2 h in a cold room (4 °C). At the end of the run the dye front was marked by inserting 25 gauge copper wire. The staining and destaining of protein bands was performed as already described. The migration of dye and protein bands was measured on a illuminated box using a magnifying glass mounted on the top of the light box. Measurements were accurate to ± 0.5 mm.

SDS-gel electrophoresis containing 0.1% SDS was carried out as described by Weber and Osborn (1969) and Shapiro et al. (1967) except that samples after treatment with 1% SDS, 1% 2-mercaptoethanol and 1% iodoacetamide at pH 7.0 were incubated at 100 °C for about 10 min and were not dialyzed prior to electrophoresis. The electrophoresis was carried out at 8 mA for 3 h at room temperature (28 °C).

Preparative polyacrylamide gel electrophoresis: A simplified procedure of preparative polyacrylamide gel electrophoresis was adopted as the final step of purification for A. fischeri nitrate reductase and nitrite reductase as described by Husain and Sadana (1972). The details of the procedure are as follows:

The apparatus used for carrying out the preparative electrophoresis was fabricated in this laboratory and resembled to that described by Davis (1964). The cathode and anode vessels were provided with platinum electrode. Cathode vessel was also provided with two ground glass joints (19B) at the bottom for attaching the gel columns. The electrophoretic columns (1.2 x 10 cm) were also provided with ground glass joints.

The gel columns were filled with 7.5% acrylamide gel solution, pH 8.9, having 0.14% ammonium persulfate for polymerization, leaving the required space for loading enzyme samples. After polymerization of gels, columns were attached to the cathodic compartment. Cathodic and anodic compartments were filled with Tris-glycine buffer, pH 8.3. Both the compartments were connected to the respective terminals of a power supply. A current of 10 mA per gel was applied and electrophoresis was continued for 4 h for removal of residual persulfate from the gels.

The apparatus was switched off and enzyme samples (1 - 2 ml) were made dense by adding 20% glycerol and layered on top of the gel columns. The electrophoresis was carried

out at 4 °C at a constant current of about 6 mA per gel column. In order to avoid excessive heating during electrophoretic run, gel columns were kept immersed in lower bath buffer.

After appropriate time a dialysis sack containing one ml bath buffer (Tris-glycine, pH 8.3) was attached to the bottom of the gel column. Trapped air bubbles, if any, were removed by introducing a plastic capillary into the dialysis sack by the side of the column and pushing the sacking upward. The electrophoresis was continued for some time and then the apparatus was switched off. Another dialysis sack was attached after removing the first one. In this manner a number of fractions were collected and tested for enzyme activity and protein.

CHAPTER III

PURIFICATION AND CHARACTERIZATION OF *Achromobacter fischeri* NITRATE REDUCTASE

SUMMARY

A. fischeri nitrate reductase has been purified by a modification of an earlier procedure described by Sadana *et al.* (1963). The enzyme has been purified to a state which is homogeneous in polyacrylamide disc gel electrophoresis at pH 8.9 and 4.3 and at different gel concentrations (6, 8, 10, 12%), and in SDS-gel electrophoresis. The overall recovery of the enzyme was 26% as compared to 4.8% obtained by the earlier procedure. The purification procedure involved crude extract preparation of bacterial cells by osmotic lysis, homogenization and centrifugation to get clear supernatant, concentration of the crude extract by ammonium sulfate precipitation (0.90 saturation), protamine sulfate treatment to remove nucleic acids, fractionation with ammonium sulfate, two successive hydroxylapatite gel column chromatography steps, and final purification by a simplified preparative polyacrylamide gel electrophoresis.

The purified *A. fischeri* nitrate reductase has a specific activity of about 3.65 µmol nitrite formed from nitrate per mg protein per min. The enzyme showed better stability as compared to the enzyme purified by the earlier procedure (Sadana *et al.*, 1963) when stored at 0 - 4 °C.

The relative molecular mass of the nitrate reductase, determined by different methods, viz. polyacrylamide gel electrophoresis, gel filtration on Sephadex G-200 and Bio-Gel P-150, and SDS-gel electrophoresis, was an average value of 54,000. The Stokes' radius, calcu[illegible] gel

filtration data by methods of Ackers, Porath, and Laurent and Killander, was an average value of 3.1 nm. The purified enzyme, on treatment with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol in presence of 1% iodoacetamide, does not dissociate in SDS-gel electrophoresis indicating that the A. fischeri nitrate reductase is comprised of a single polypeptide chain.

INTRODUCTION

During studies on the luminescent system of A. fischeri, W.D. McElroy and J.R. Klinenberg (cited in Sadana and McElroy, 1957) observed that this organism requires an ammonia nitrogen source for growth, and that when nitrate is added to the growth medium, nitrite accumulates. From this observation it was concluded that A. fischeri can form a nitrate reductase but not nitrite reductase. Prakash et al. (1966) reported that A. fischeri can form both nitrate reductase and nitrite reductase. Sadana and McElroy (1957) purified and characterized nitrate reductase from A. fischeri. Although purified enzyme was strongly coloured and showed absorption bands at 550, 520 and 419 nm in the reduced state indicative of a heme; however, a certain amount of indirect evidence (inhibitor studies) was obtained which indicated that the terminal nitrate reductase from A. fischeri did not contain iron porphyrin. Sadana et al. (1963) further purified this enzyme by a modified procedure. This purified enzyme showed no absorption peak, which is specific for heme except that of protein in the vicinity of 280 nm. The absorbance decreased gradually over the entire near ultraviolet and visible region with increasing wavelength indicating none of the characteristics of a heme enzyme. The enzyme was relatively unstable and lost about 20% of its activity on storage at 4 °C for 24 h. Ultracentrifugal studies of the purified enzyme indicated the presence of at least two components, though it was not determined, which of the two components represented the enzyme.

The work presented in this Chapter describes a modified procedure for the purification of A. fischeri nitrate reductase. The second step in the purification procedure of Sadana et al. (1963), i.e. precipitation at pH 4.5, was replaced by 90% ammonium sulfate precipitation as considerable enzyme losses (60 - 70%) were observed during precipitation at pH 4.5. The column chromatography on cation-exchange resin, Amberlite IRC-50 (NH_4^+-form), was eliminated from the present procedure. The final purification of the nitrate reductase was carried out by a simplified preparative polyacrylamide gel electrophoresis. This has resulted in obtaining an enzyme which is homogeneous in the disc gel electrophoresis with an overall yield of about 26% as compared with 4.8% obtained by the procedure of Sadana and McElroy (1957), and the enzyme obtained was not homogeneous. The specific activity of the enzyme was 3.65 µmol nitrite formed/mg protein/min.

Unless otherwise indicated, all purification steps were carried out at 0 - 4 °C.

1. Preparation of crude extract:

The frozen cells (100 g wet weight) were thawed overnight at 4 °C and extraction was carried out by osmotic lysis of cells in cold distilled water (1 g wet weight of cells/20 ml water). The suspension was stirred for 30 min, homogenized in a Potter-Elvehjem glass homogenizer and stirred again for 30 min. The cell-free supernatant fluid was collected by high speed centrifugation in a RC-5 Sorvall centrifuge at 20,000 x g for 30 min. The sedimented pellet was again

suspended in distilled water (1 g wet weight/15 ml water), stirred for 30 min, homogenized and centrifuged as before. The total volume of pooled crude extracts was 3200 ml and contained 41.6 units of nitrate reductase activity. The specific activity of the enzyme at this step was 0.005 and the ratio of absorbance at 280 nm to that at 260 nm was 0.6.

Sadana and McElroy (1957) reported that although most of the proteins were extracted by the first water lysis, the supernatant solution contained little or no nitrate reductase activity. The second extract, obtained by resuspending the sediment in water and extraction, showed most of the nitrate reductase activity.

The first crude extract contained most of the nitrite reductase activity. It was, therefore, not possible to estimate nitrate reductase activity (which was determined from the accumulation of nitrite) in the first extract as whatever nitrite was formed by nitrate reductase got converted to ammonia by nitrite reductase.

Nitrate reductase can be heated for 10 min at 50°C without any detectable loss of activity, whereas nitrite reductase is completely inactivated under these conditions. Therefore, nitrate reductase activity was estimated in the first crude extract after heating the sample at 50°C for 10 min. It was observed that both the extracts (first and second) contained almost the same units of nitrate reductase activity.

2. Ammonium sulfate precipitation

The pH of the combined crude extracts was adjusted to 7.4 by adding 1 M K_2HPO_4 solution and solid ammonium sulfate was added, with gentle stirring, to the clear supernatant to bring it to 0.90 saturation. The quantity of ammonium sulfate required was calculated as mentioned in 'Materials and Methods'. The suspension was stirred for 30 min and centrifuged at 20,000 x g for 15 min in a RC-5 Sorvall centrifuge. The sediment was suspended in 300 ml of 50 mM potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight with three changes of buffer. The enzyme sample was tested for enzyme activity and protein content. It is evident from Table 3 that very little purification is achieved by this step but it helped in reducing the large volume of the crude extracts for easy handling in further purification steps. The ratio of absorbance at 280 nm to that at 260 nm slightly increased from 0.6 in the crude extract to 0.68. About 90% of the enzyme activity was recovered in this step.

3. Protamine sulfate treatment

The ammonium sulfate precipitate contained large amounts of nucleic acids, as observed from the absorbance ratio at 280 nm to 260 nm, and approximately 10 mg protein/ml. The pH of the enzyme was adjusted to 6.0 by adding 0.2 M acetate buffer, pH 4.3. The protamine sulfate solution (15 mg/ml, pH 5.0) was then added to the clear supernatant from the second step to precipitate nucleic acids. The

addition of the protamine sulfate solution was continued till no further precipitate was observed. Usually 300 ml of protamine sulfate solution (15 mg/ml, pH 5.0) was required to precipitate most of the enzyme activity present in the extracts from 100 g (wet weight) of bacterial cells. The precipitate was collected by centrifuging the suspension at 4,000 x g for 15 min. The clear supernatant, which had very little nitrate reductase activity, was discarded.

4. Extraction of nitrate reductase from protamine sulfate precipitate

Protamine sulfate-precipitated sediment from step 3 was suspended in 50 ml of 0.2 M potassium phosphate buffer, pH 7.5, homogenized in a Potter-Elvehjem glass homogenizer, stirred for 45 min and centrifuged at 14,000 x g for 15 min. The extraction was repeated five to six times in a similar manner until the final extract showed negligible nitrate reductase activity. The extracts were combined, centrifuged and the inactive precipitate discarded. The absorbance ratio at 280 nm to 260 nm increased from 0.68 in the second step to 1.0 or more in the phosphate extracts of the protamine sulfate precipitate. This step provides three-fold purification with 87% recovery in the enzyme activity from the previous step.

5. Ammonium sulfate fractionation

Further purification of the enzyme was carried out by fractional precipitation with ammonium sulfate. The combined phosphate extracts (260 ml) of the protamine sulfate

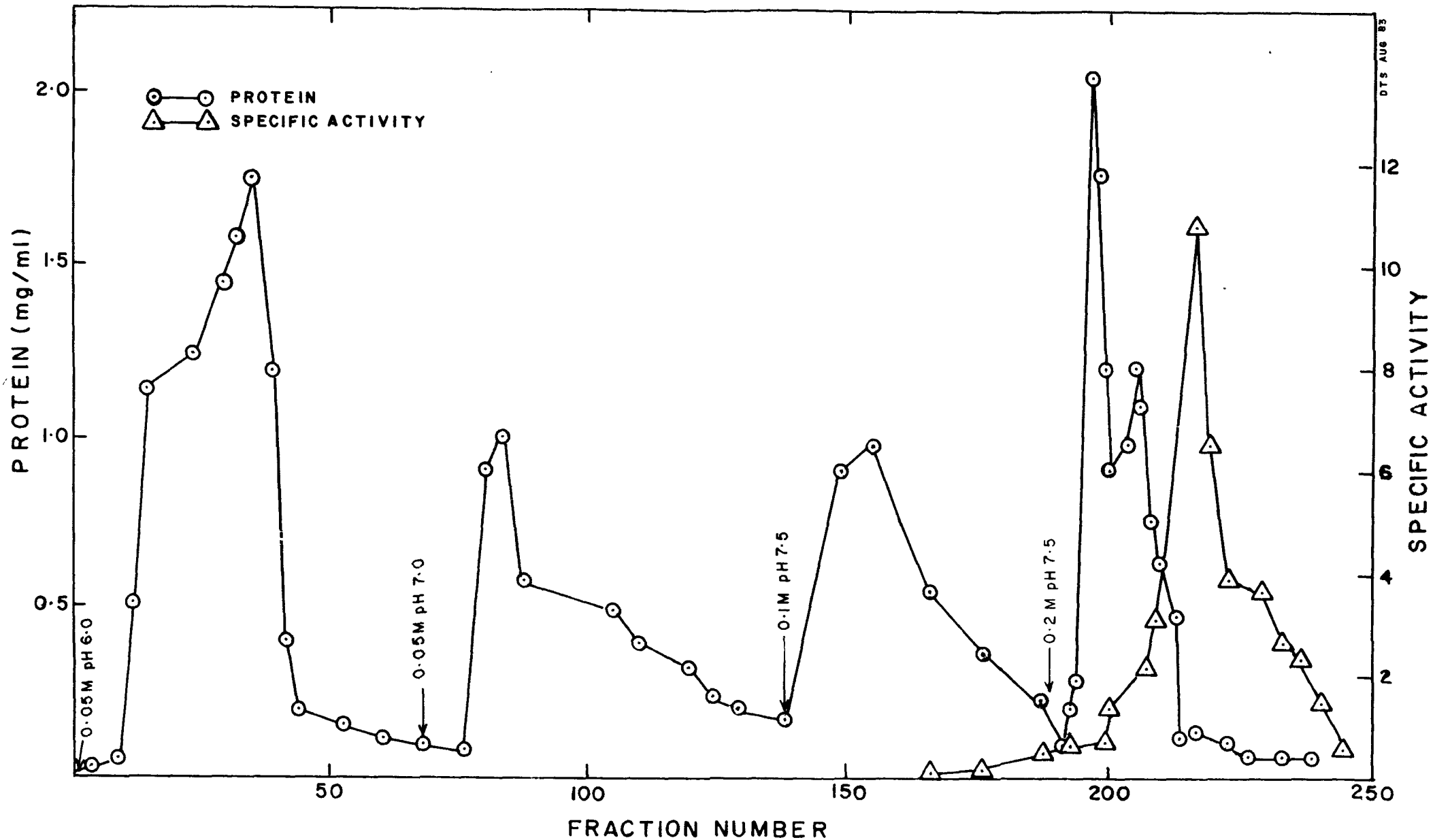
precipitate from step 4 were brought to 0.60 saturation by adding the required amount of solid ammonium sulfate with gentle stirring. The suspension was centrifuged at 14,000 x g for 30 min in a RC-5 Sorvall centrifuge. The sediment was suspended in 20 ml of 50 mM potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight with three changes of buffer. No significant inactivation of the enzyme activity was noticed during ammonium sulfate fractionation, since 90% of the activity was accounted in the various fractions. About 1.6 fold purification with an overall recovery of 65% of the enzyme activity was obtained in this step. The ratio of absorbance at 280 nm to 260 nm increased from 1.05 in protamine sulfate extracts to 1.60 in the 0 - 0.60 saturation ammonium sulfate fraction. No attempt was made to maintain the pH of the solution during the addition of ammonium sulfate.

6. First hydroxylapatite column chromatography

The partially purified enzyme obtained from step 5 was further purified by hydroxylapatite gel column chromatography. A column (1.8 x 50 cm) was prepared as described in 'Materials and Methods' and equilibrated with 50 mM potassium phosphate buffer, pH 6.0. The pH of the enzyme was adjusted to 6.0 by adding 0.2 M acetate buffer, pH 3.5, just before loading on the column. Approximately 864 mg of protein containing 27 units of nitrate reductase activity was loaded on the top of the column. The column was washed with the same buffer until the washings did not show any absorbance at 280 nm.

The enzyme sample was coloured before loading on the column. Coloured components were eluted during washing of the column with 50 mM potassium phosphate buffer, pH 6.0. Approximately 270 mg of protein was eluted during this washing but no nitrate reductase activity appeared in the effluent. About 400 ml of the buffer was required in order to completely wash down the proteins eluted by this buffer. The column was then washed with 50 mM potassium phosphate buffer, pH 7.0, until the washings did not show any absorbance at 280 nm. Nitrate reductase activity could not be detected in this washing step and approximately 180 mg protein was eluted. The column was further washed with 0.1 M potassium phosphate buffer, pH 7.0, and enzyme activity was detected after few fractions. When the enzyme activity started appearing in the eluate, the nitrate reductase was eluted with 0.2 M potassium phosphate buffer, pH 7.5, at a flow rate of 20 - 25 ml per h and the eluate was collected in fractions ranging in volume from 2 to 5 ml. Figure 2 shows the elution pattern of the nitrate reductase activity and protein. The fractions containing nitrate reductase activity were dialyzed against 50 mM potassium phosphate buffer, pH 7.0. The enzyme activity and protein content were determined in each fraction. The most highly active fractions, eluted from the column, contained 30.6 mg of protein with 22 units of enzyme activity representing a specific activity of 0.72 and a final yield of 53%. No significant loss in the activity on hydroxylapatite gel column chromatography was observed.

FIG. 2 Elution pattern of A. fischeri nitrate reductase activity and protein on first hydroxylapatite column (1.8 x 50 cm). Potassium phosphate buffer of increasing ionic strength and pH was used for elution of the proteins.
Sample loaded: 864 mg protein in 30 ml.


PROTEIN
SPECIFIC ACTIVITY
PROTEIN (mg/ml)
SPECIFIC ACTIVITY
FRACTION NUMBER
0·05M pH 6·0
0·05M pH 7·0
0·1M pH 7·5
0·2M pH 7·5
2·0
1·5
1·0
0·5
12
10
8
6
4
2
50
100
150
200
250
DTS AUG 83

It was possible to account for almost 98% of the total activity in different fractions.

7. Second hydroxylapatite column chromatography

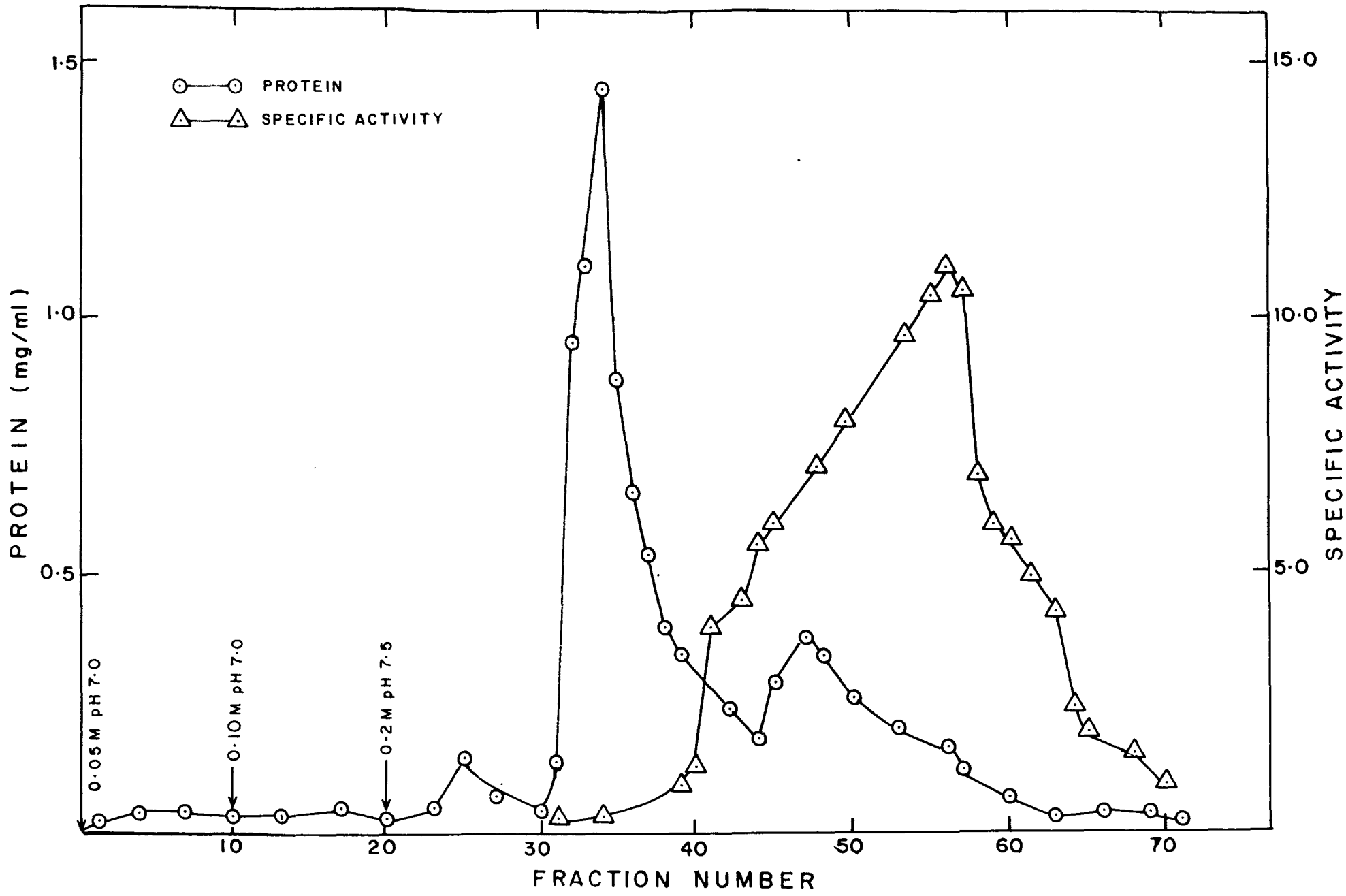
The combined dialyzed fractions, obtained from step 6, were adjusted to pH 6.0 by adding 0.2 M acetate buffer, pH 3.5, just before loading on the top of second hydroxylapatite column (1 x 35 cm). The enzyme sample containing 30.6 mg of protein and 22 units of enzyme activity was loaded on the column. The column was equilibrated, developed and eluted as before. Figure 3 shows the elution pattern of the nitrate reductase activity and protein with 50 mM potassium phosphate buffer, pH 7.0, 0.1 M buffer, pH 7.0 and 0.2 M buffer, pH 7.5. About 10 mg protein was eluted by first three buffers, containing approximately 4 units of nitrate reductase activity. The enzyme was purified 1.5 fold in this step giving a final yield of 40%.

8. Preparative polyacrylamide gel electrophoresis

Final purification of the enzyme was achieved by using preparative polyacrylamide gel electrophoresis. A simplified procedure of preparative polyacrylamide gel electrophoresis of Husain and Sadana (1972) was adopted for the final purification of nitrate reductase, as described under 'Materials and Methods'. Fixed volume of gel solution (6.0 ml) was used for making gel columns.

The partially purified enzyme obtained after second hydroxylapatite column chromatography was dialyzed against Tris-glycine buffer, pH 8.3, for about 6 - 8h, with three

FIG. 3 Elution pattern of *A. fischeri* nitrate reductase activity and protein on second hydroxylapatite column (1 x 35 cm). Potassium phosphate buffer of increasing ionic strength and pH was used for elution of the proteins.
Sample loaded: 30.6 mg protein in 20 ml.


PROTEIN
SPECIFIC ACTIVITY
PROTEIN (mg/ml)
SPECIFIC ACTIVITY
FRACTION NUMBER
0·05M pH 7·0
0·10M pH 7·0
0·2M pH 7·5
1·5
1·0
0·5
15·0
10·0
5·0
10
20
30
40
50
60
70

changes of buffer. The enzyme samples (2.5 ml) containing 8.1 mg protein were mixed with sucrose solution to make it 20% and layered on the top of each column. Electrophoresis was carried out at 4 °C at a constant current of about 5 mA per column.

Nitrate reductase, being colourless, was not visually detectable and, therefore, it was not easy to decide the time of its elution. During preliminary run, under standard conditions of gel volume, current, temperature and pH of the buffer, fractions were collected every hour. It was determined that nitrate reductase enzyme was eluted after 6 - 7 h of electrophoresis. So after loading the enzyme sample on the gel columns, electrophoresis was continued for about 5 h, without interrupting it, then the apparatus was switched off and a piece of dialysis sacking filled with 1 ml of bath buffer (Tris-glycine, pH 8.3) was attached to the lower end of the column, as described in 'Materials and Methods'. The electrophoresis was continued for 20 min and then another dialysis sacking was attached after removing the first one. In this manner 10 to 12 fractions were collected at 20 min intervals and dialyzed overnight against 50 mM potassium phosphate buffer, pH 7.0, with three changes of the buffer. The fractions were tested for enzyme activity and protein. The fractions having the highest specific activity were pooled and tested for homogeneity.

The purification of the enzyme by preparative gel electrophoresis has been repeated 4 to 6 times with reproducible results. A summary of the purification procedure is given

in Table 3. The yield of the finally purified enzyme was about 26% giving 2.97 mg pure protein from 100 g bacterial cells (wet weight), and on a protein basis, represents 2800 fold purification. The specific activity of the enzyme is 3.65 μmol of nitrite produced per mg nitrate reductase per min.

Criteria of purity

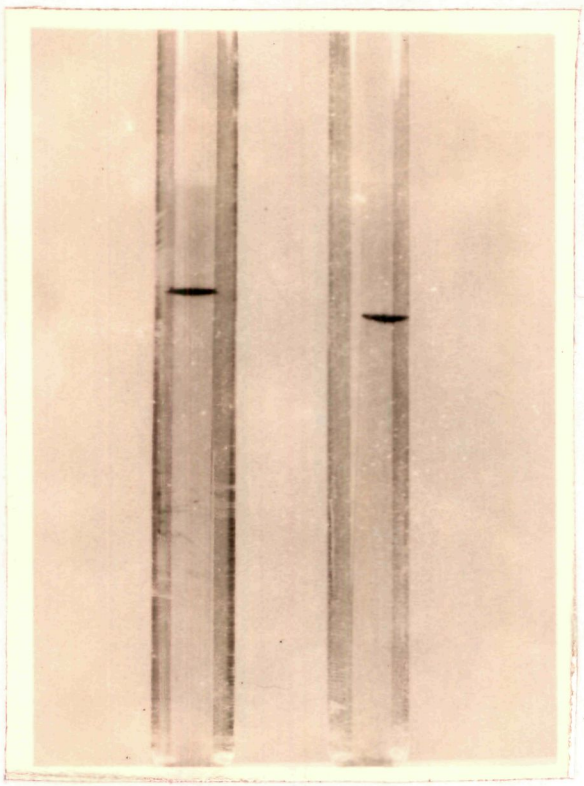
The homogeneity of the purified A. fischeri nitrate reductase of highest specific activity was examined by disc gel electrophoresis in both anodic, pH 8.9 (Davis, 1964), and cathodic, pH 4.3 (Maurer, 1971), systems. In both the systems it revealed only one protein band (Fig. 4). Nitrate reductase also migrated as a single protein band in 6, 8, 10 and 12% polyacrylamide gels at pH 8.9. Only a single protein band of nitrate reductase was detected in SDS-gel electrophoresis.

After running the electrophoresis, gel from one of the tubes was used for detection of the protein band having nitrate reductase activity. The gel was removed from the tube and immediately immersed in 10 ml of a solution containing: 0.07 M potassium phosphate buffer, pH 7.0, 0.16 M KNO_3, 0.5 mg benzyl viologen, 10 mg sodium dithionite and 10 mg $NaHCO_3$ and incubated for 5 min at 30°C. The gels were immediately blotted and immersed in a cold (4°C) mixture of 2.5 ml of 1% sulfanilamide in 2.5 M HCl and 2.5 ml of 0.01% N-(1-naphthyl)-ethylenediamine in distilled water. After a couple of minutes a pink band appeared on the gel. The positic of the protein band coincided with that of nitrate reductase activity band.

FIG. 4 Polyacrylamide gel electrophoresis of purified *A. fischeri* nitrate reductase having specific activity of 3.65 μmol nitrite produced from nitrate per mg protein per min, was loaded on 7.5% gel rods.

(a) Cathodic run, pH 4.3

(b) Anodic run, pH 8.9



a b

Table 3: Summary of purification procedure of A. fischeri nitrate reductase from 100 g (wet weight) of cells.

Fraction	Volume	Activity Units/ml	Total activity Units	Protein mg/ml	Total protein mg	Specific activity	Yield	A_{280}/A_{260}
Crude extract	3200	0.013	41.6	2.6	8320	0.005	100	0.6
0 - 0.90 saturated $(NH_4)_2SO_4$ ppt	530	0.071	37.6	10.1	5353	0.007	90	0.68
Phosphate extracts of protamine sulfate treated sediment.	260	0.125	32.5	6.25	1625	0.02	78	1.05
0 - 0.6 saturated $(NH_4)_2SO_4$ ppt	30	0.9	27.0	28.8	864	0.032	65	1.6
First hydroxylapatite eluate	20	1.1	22.0	1.53	30.6	0.72	53	-
Second hydroxylapatite eluate	5.0	3.32	16.6	3.24	16.2	1.03	40	-
Preparative polyacrylamide gel electrophoresis.	2.2	4.92	10.8	1.35	2.97	3.64	26	-

Determination of relative molecular mass of purified nitrate reductase from A. fischeri

Five different methods were used for the determination of relative molecular mass of the purified enzyme:

1. Polyacrylamide gel electrophoresis

The relative molecular mass was determined by polyacrylamide gel electrophoresis according to the method of Hedrick and Smith (1968). Standard proteins and the enzyme (nitrate reductase) were subjected to electrophoresis in a series of gels which varied in acrylamide concentration from 6 - 12%. All other conditions of electrophoresis were the same (see 'Materials and Methods'). Under these conditions proteins migrate into the gel as a function of the size, charge and the acrylamide concentration of the gel. Plots of log relative mobility (100 log (Rm x 100)), of marker proteins (bovine serum albumin: monomer 68,000, dimer 136,000, trimer, 204,000, ovalbumin: monomer 46,000, dimer 92,000; DNAase I 31,000) versus gel concentration resulted in straight lines. A linear relationship exists between the slope of these lines and relative molecular mass of the proteins. The results with nitrate reductase and marker proteins are presented in Fig. 5(a) and Fig. 5(b). The relative molecular mass of the enzyme, computed from its slope on the calibration curve, was found to be 54,000, which agrees excellently with the values obtained by other methods.

2. SDS-gel electrophoresis

An estimate of relative molecular mass of nitrate

FIG. 5 Determination of the molecular weight of A. fischeri nitrate reductase by gel electrophoresis (Hedrick and Smith, 1968).

(a) Plots of 100 log (Rm x 100) of marker proteins against gel concentrations. The standard proteins used were:

(A) Deoxiribonuclease I (M_r 31,000)

(B) Ovalbumin monomer (M_r 46,000)

(C) BSA monomer (M_r 68,000)

(D) Ovalbumin dimer (M_r 92,000)

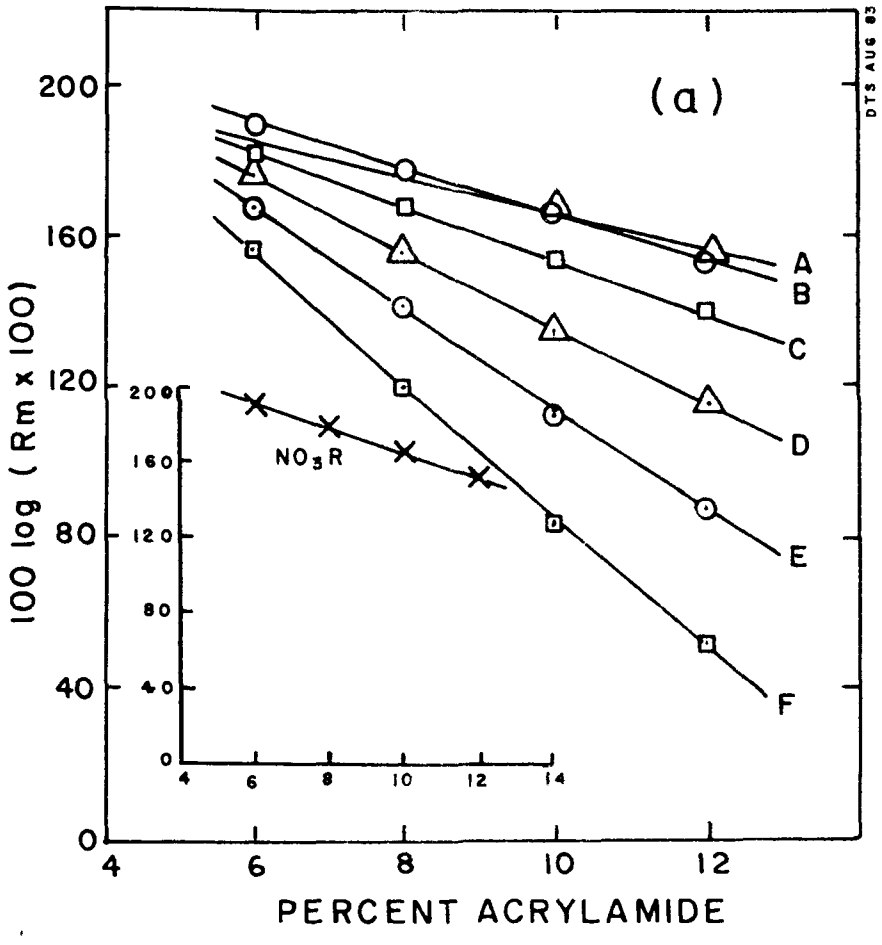
(E) BSA dimer (M_r 136,000)

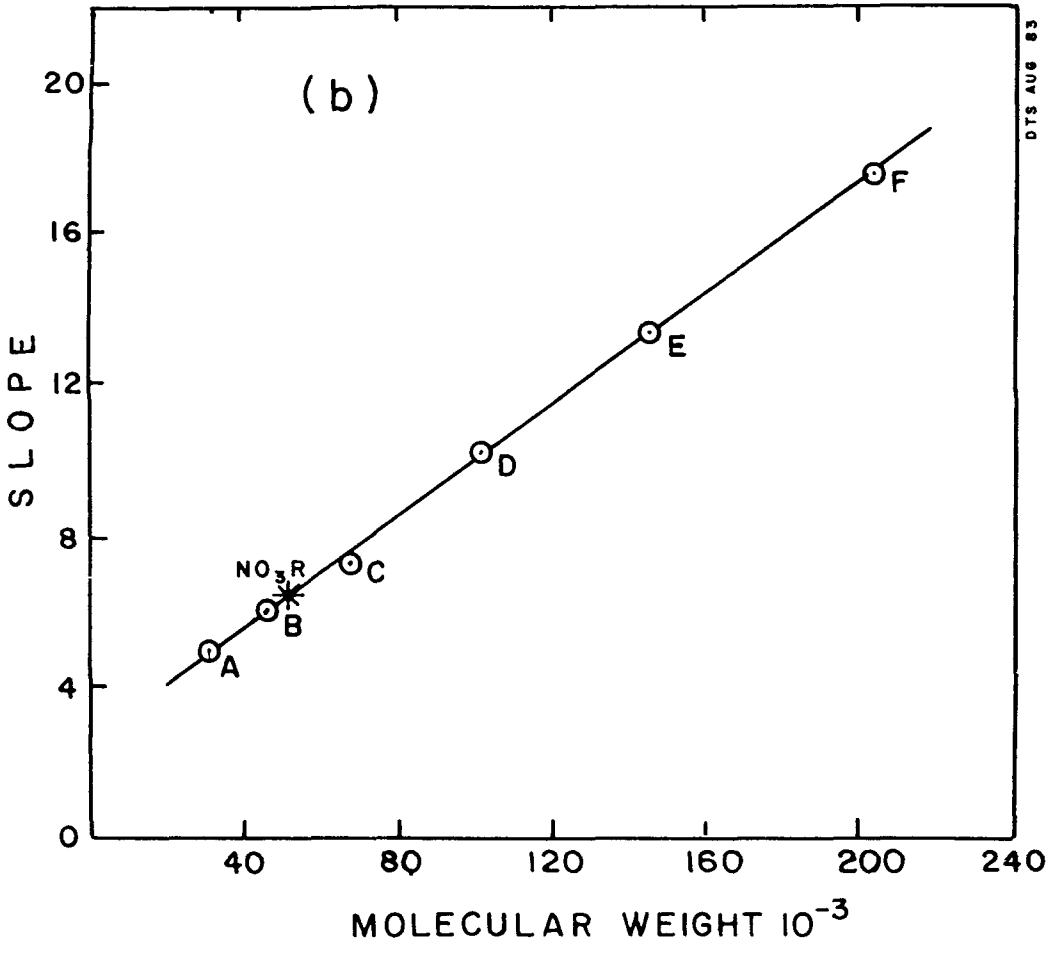
(F) BSA trimer (M_r 204,000)

In the case of deoxiribonuclease I the position of the major band was taken.

In the insert is the plot of 100 log (Rm x 100) of A. fischeri nitrate reductase against gel concentration.

(b) The negative slope of each protein from Fig. 5a was plotted against their molecular weight. The molecular weight of A. fischeri nitrate reductase, computed from the slope on the calibration curve is 54,000.


(a)
DTS AUG 83
100 log (Rm x 100)
PERCENT ACRYLAMIDE
A
B
C
D
E
F
NO3R


(b)
DTS AUG 83
SLOPE
MOLECULAR WEIGHT 10-3
A
B
C
D
E
F
NO3R

reductase was also made by its migration in the SDS-gels (Weber and Osborn, 1969; Shapiro et al., 1967). The enzyme, nitrate reductase and marker proteins were incubated for 10 min at 100°C with 1% SDS, 1% 2-mercaptoethanol and 1% iodoacetamide before subjecting them to SDS-gel electrophoresis. A plot of log molecular weight versus relative mobility of the marker proteins yielded a straight line (Fig. 6) and an estimate of relative molecular mass of A. fischeri nitrate reductase of 52,000.

3. Determination of subunit nature by SDS-gel electrophoresis

Nitrate reductase was treated with (a) 1% SDS, (b) 1% SDS, 1% 2-mercaptoethanol and 1% iodoacetamide at 100°C for 10 min and subjected to SDS-gel electrophoresis. The enzyme showed only one protein band on both the treatments. The relative molecular mass, computed from Fig. 6, was also same for both, corresponding to the native enzyme, i.e. 52,000. This indicated that nitrate reductase from A. fischeri is composed of only one polypeptide chain.

4. Gel filtration (Bio-Gel P-150)

The relative molecular mass of the purified nitrate reductase was estimated by the molecular sieve chromatography method of Andrews (1964). A column (1.5 x 100 cm) of Bio-Gel P-150 was equilibrated with 0.05 M phosphate buffer, pH 7.0 at 4°C. The void volume (Vo) of the column was measured with Blue-Dextran 2,000 (M_r 2,000,000). The column was calibrated using five marker proteins, viz.: (i) gamma-globulin 160,000, (ii) alcohol dehydrogenase

FIG. 6 Molecular weight determination of *A. fischeri* nitrate reductase by SDS-gel electrophoresis. Relative mobility of marker proteins was plotted against their log molecular weight. The marker proteins used were:

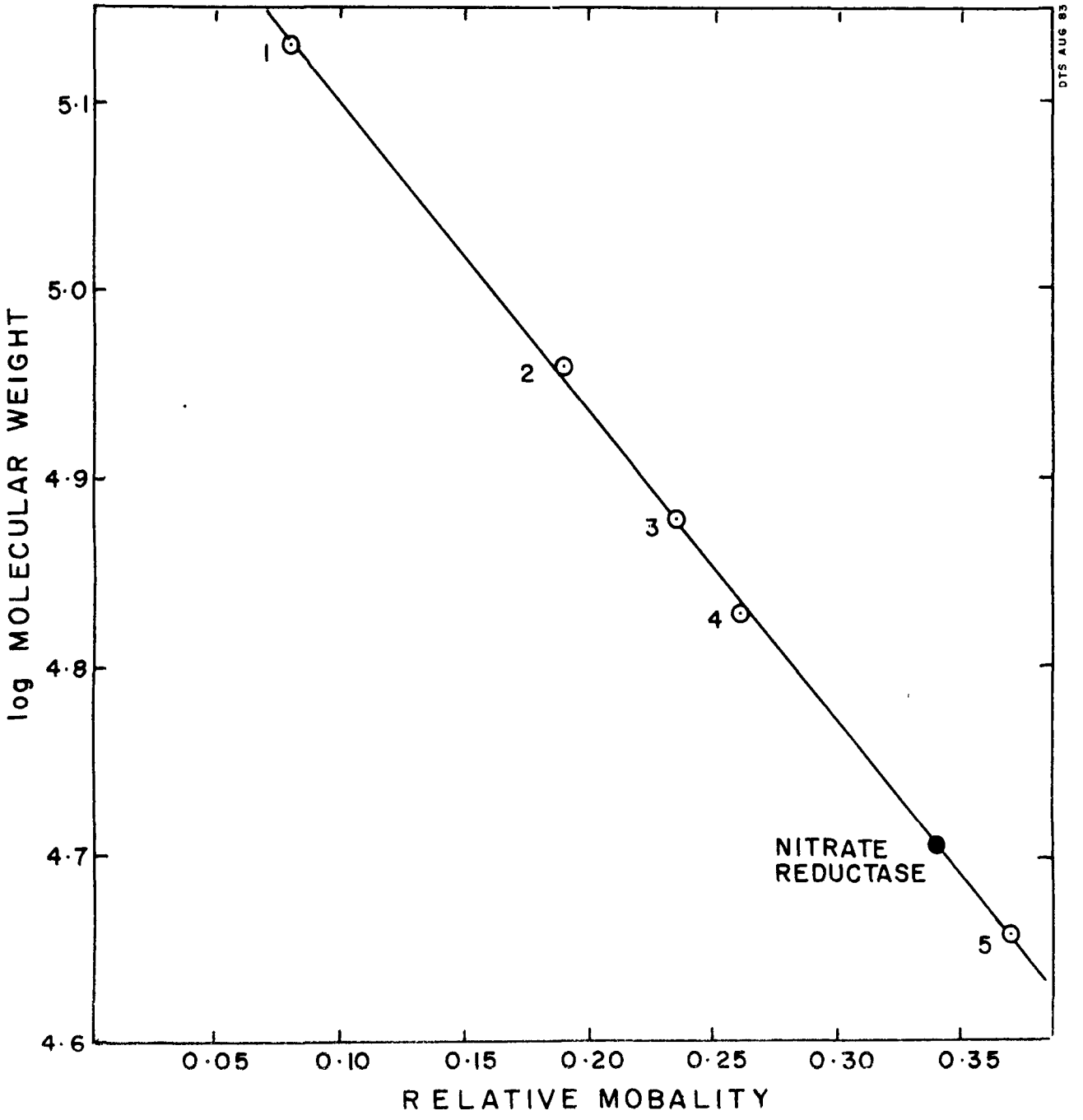
(1) BSA dimer (M_r 136,000)

(2) Ovalbumin dimer (M_r 92,000)

(3) Transferrin (M_r 76,000)

(4) BSA monomer (M_r 68,000)

(5) Ovalbumin monomer (M_r 46,000)


log MOLECULAR WEIGHT
5·1
5·0
4·9
4·8
4·7
4·6
0·05
0·10
0·15
0·20
0·25
0·30
0·35
RELATIVE MOBALITY
1
2
3
4
5
NITRATE
REDUCTASE
DTS AUG 83

141,000, (iii) bovine serum albumin 68,000, (iv) ovalbumin 46,000, and (v) myoglobin 17,600. Nitrate reductase (200 µg) was loaded on a Bio-Gel column along with 2 mg of each marker protein except ovalbumin. It was chromatographed in a separate run with Blue-Dextran. Elution was performed with phosphate buffer, pH 7.0, and fractions of about 1 ml were collected. Bovine serum albumin, ovalbumin and gamma-globulin were determined by measuring the absorbance of the fractions at 280 nm, while myoglobin was determined at 409 nm. Enzyme activities, alcohol dehydrogenase and nitrate reductase, were assayed as described in the text. Blue-Dextran was measured at 625 nm. The elution volume (Ve) for each standard protein and enzyme was calculated from the mid point of the peaks. The elution volumes were found to be reproducible.

A plot of Ve/Vo against log molecular weight (Fig. 7), according to the procedure of Andrews (1964), gave a straight line and the relative molecular mass of A. fischeri nitrate reductase, calculated from the calibration curve, was found to be 57,000.

5. Gel filtration on Sephadex G-200 (40 - 120 µ)

Marker proteins and the enzyme were chromatographed on a Sephadex G-200 column (1.2 x 89 cm) as described for Bio-Gel column. Catalase activity was assayed as described in text. The gel filtration data of the nitrate reductase and marker proteins of known molecular weight and Stokes' radii, in terms of Kd, Kav and Ve/Vo are presented in Table 4. The elution volumes were found to be reproducible.

FIG. 7 Molecular weight determination of A. fischeri nitrate reductase by gel filtration on Bio-Gel P-150 column (1.5 x 100 cm). The marker proteins used for calibration of the column were:

(1) Gamma globulin (M_r 160,000)

(2) Alcohol dehydrogenase (M_r 141,000)

(3) BSA monomer (M_r 68,000)

(4) Ovalbumin monomer (M_r 46,000)

(5) Myoglobin (M_r 17,600)

Ve, elution volume; Vo, void volume

Ve/Vo values were plotted against log molecular weight according to the procedure of Andrews (1964).

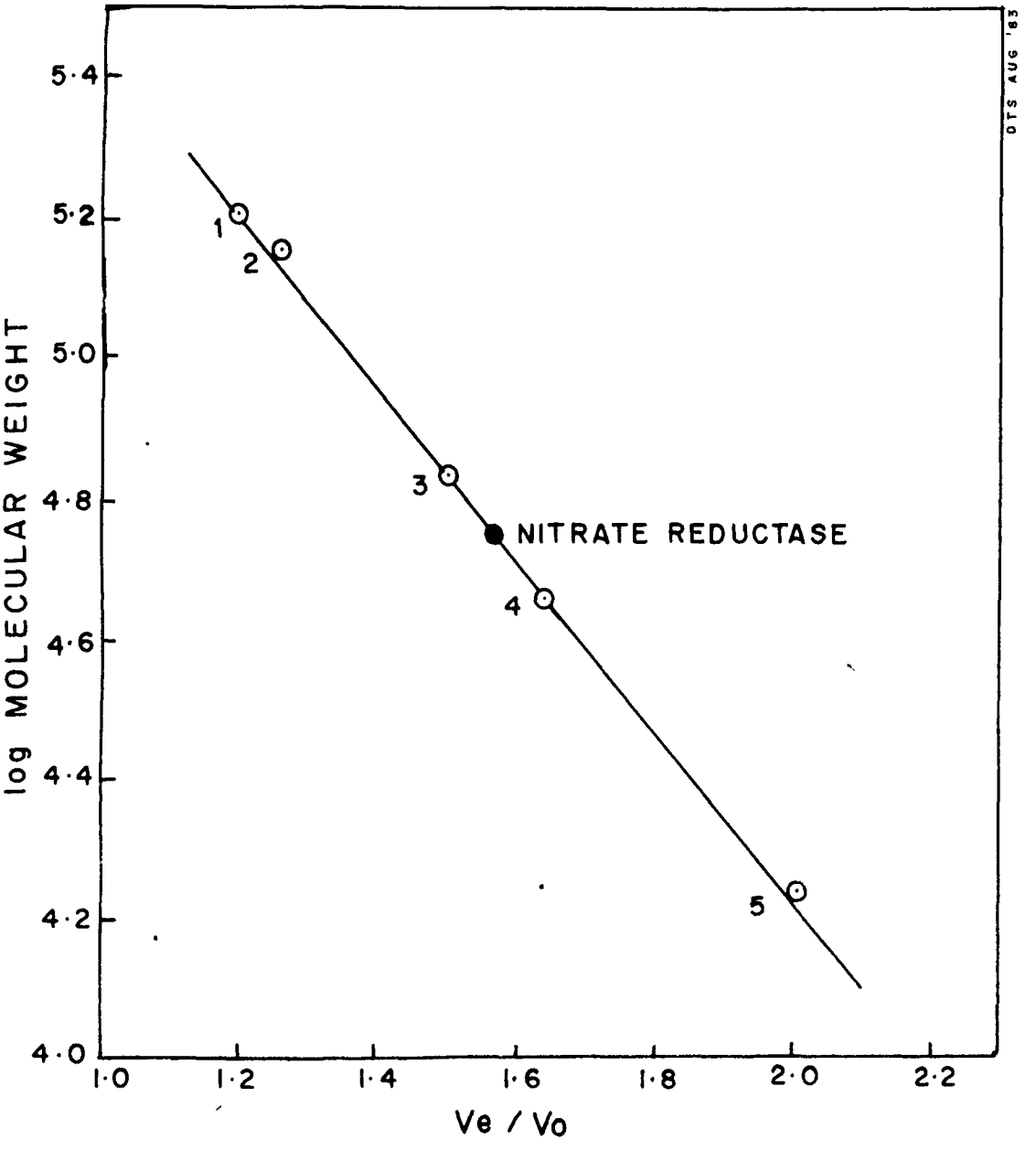

log MOLECULAR WEIGHT
5·4
5·2
5·0
4·8
4·6
4·4
4·2
4·0
1·0
1·2
1·4
1·6
1·8
2·0
2·2
Ve / Vo
1
2
3
4
5
NITRATE REDUCTASE
DTS AUG '83

Table 4: Gel filtration data of A. fischeri nitrate reductase and standard proteins

The Kd and Kav were calculated according to Siegel and Monty (1966). The void volume (Vo) of the column was 36 ml.

Proteins	Stokes' radius nm	Elution volume ml	Ve/Vo	Kd	Kav	r Calculated nm	r Average nm	Reference
Blue Dextran		36.0						
1. Catalase	5.23	47.88	1.33	0.187	0.182	16.7		Rogers et al (1965)
2. Alcohol dehydrogenase	4.6	54.0	1.55	0.286	0.278	18.5		Siegel and Monty (1966)
3. Bovine serum albumin	3.61	60.84	1.69	0.395	0.384	18.6	17.9	Rogers et al (1965)
4. Ovalbumin	2.76	64.80	1.80	0.458	0.445	16.6		Tanford (196
5. Myoglobin	1.9	76.32	2.12	0.64	0.622	19.0		Helwig and Greenberg (1952)
6. A. fischeri nitrate reductase		64.1	1.78	0.447	0.435			

A plot of Ve/Vo versus log molecular weight (Fig. 8), according to the procedure of Andrews (1964), gave a streight line and indicated that the relative molecular mass of the enzyme was 51,000. This value is similar to the relative molecular mass estimated by other methods. There appears to be no interaction between the enzyme and Sephadex polysaccharide matrix because there is not much difference in relative molecular mass values determined on Bio-Gel and Sephadex columns.

Stokes' radius of A. fischeri nitrate reductase:

The Stokes' radius of nitrate reductase was calculated from its distribution coefficients Kd and Kav and the pore radius, r, of the column. A pore radius of 17.9 nm, for the batch of Sephadex G-200 used, was calculated from the known Stokes' radii of the standard proteins (Ackers, 1964). A linear relationship (Fig. 9(a), 9(b)) was obtained when the experimental data are plotted according to Porath (1963) and Laurent and Killander (1964). The validity of the relationship proposed by Ackers (1964) is evident from the agreement obtained for the value of r using different standard proteins. The Stokes' radius of nitrate reductase was calculated by the methods of Ackers, Porath and Laurent and Killander. The three methods yielded similar values (3.05, 3.2, and 3.05 nm) with an average value of 3.1 nm.

FIG. 8 Molecular weight determination of A. fischeri nitrate reductase by gel filtration on Sephadex G-200 column (1.2 x 89 cm). The marker proteins used for calibration of the column were:

(1) Catalase (M_r 230,000) (Andrews, 1965)

(2) Alcohol dehydrogenase (M_r 125,000) (Andrews, 1965).

(3) BSA monomer (M_r 68,000) (Burnett, 1971)

(4) Ovalbumin monomer (M_r 46,000) (Dunker and Rueckert, 1969).

(5) Myoglobin (M_r 17,600) (Dunker and Rueckert, 1969)

The elution data of Table 4 were employed. Ve/Vo values were plotted against log molecular weights according to the procedure of Andrews (1964).

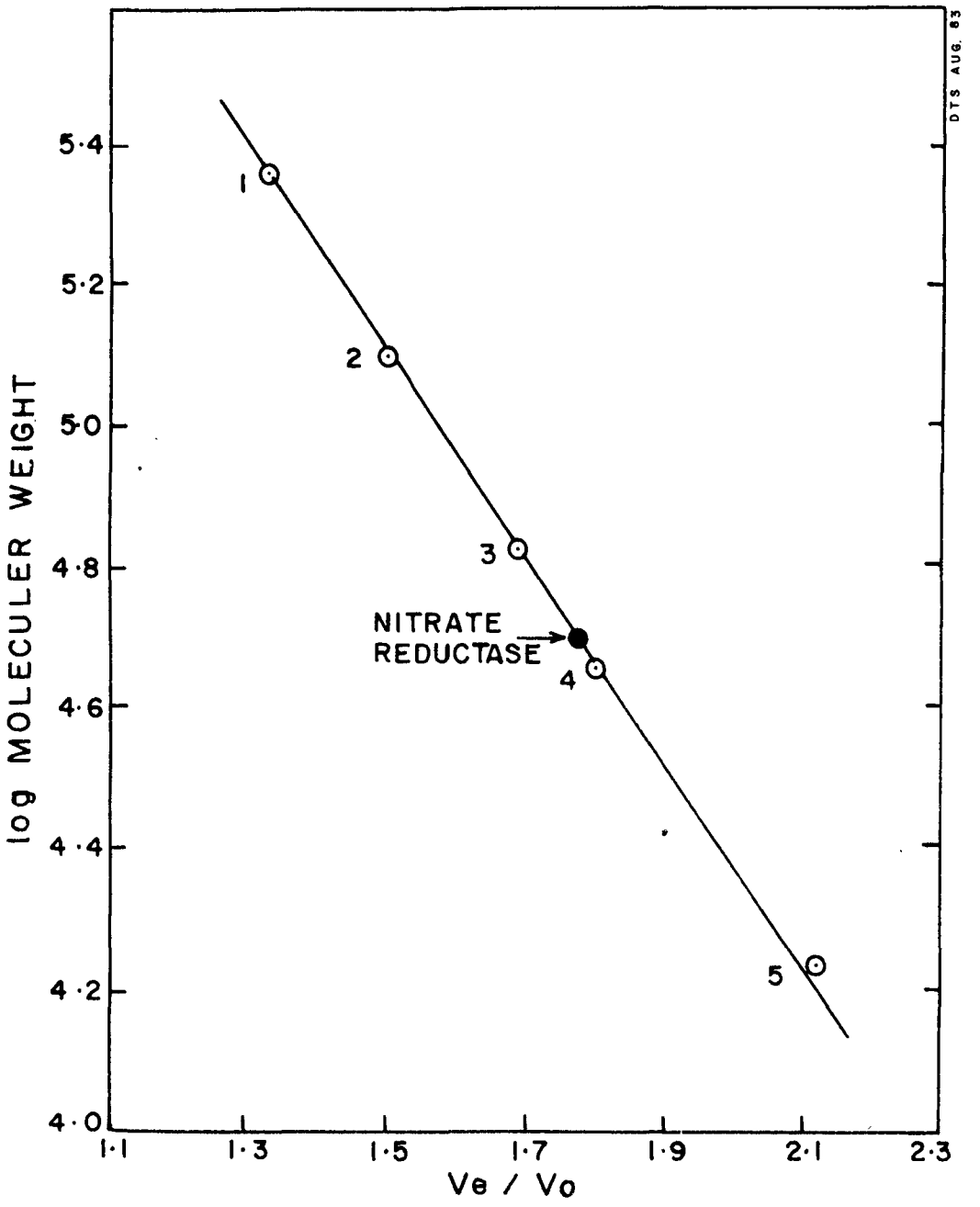
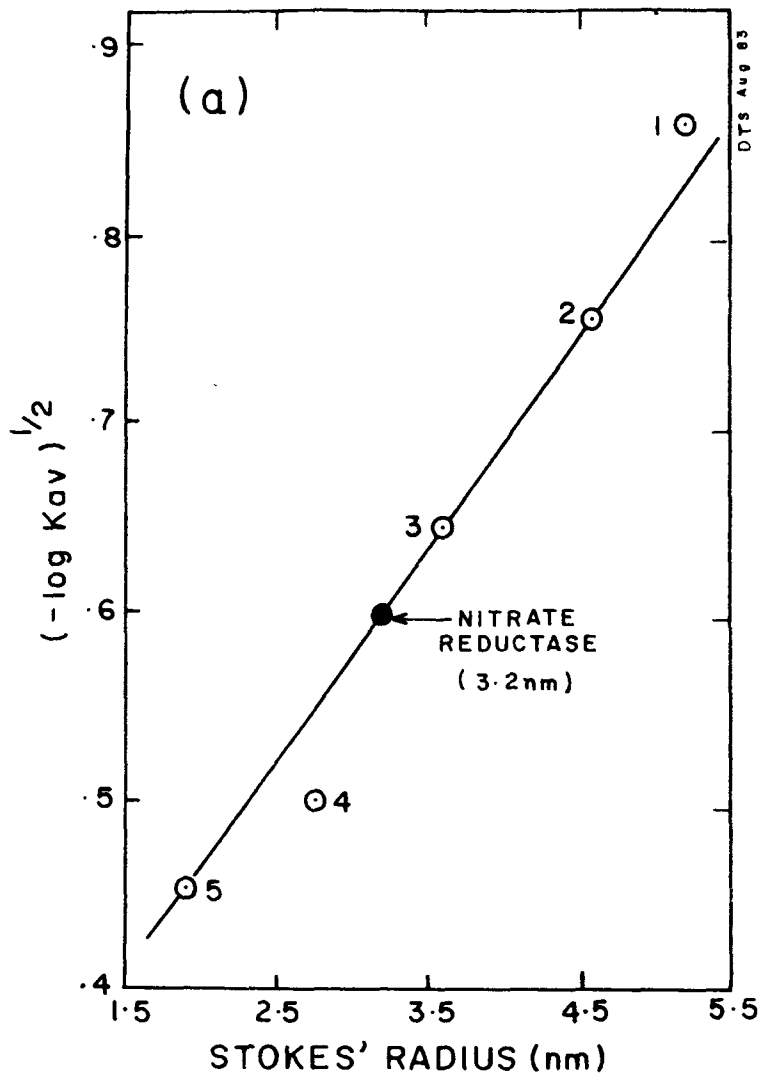
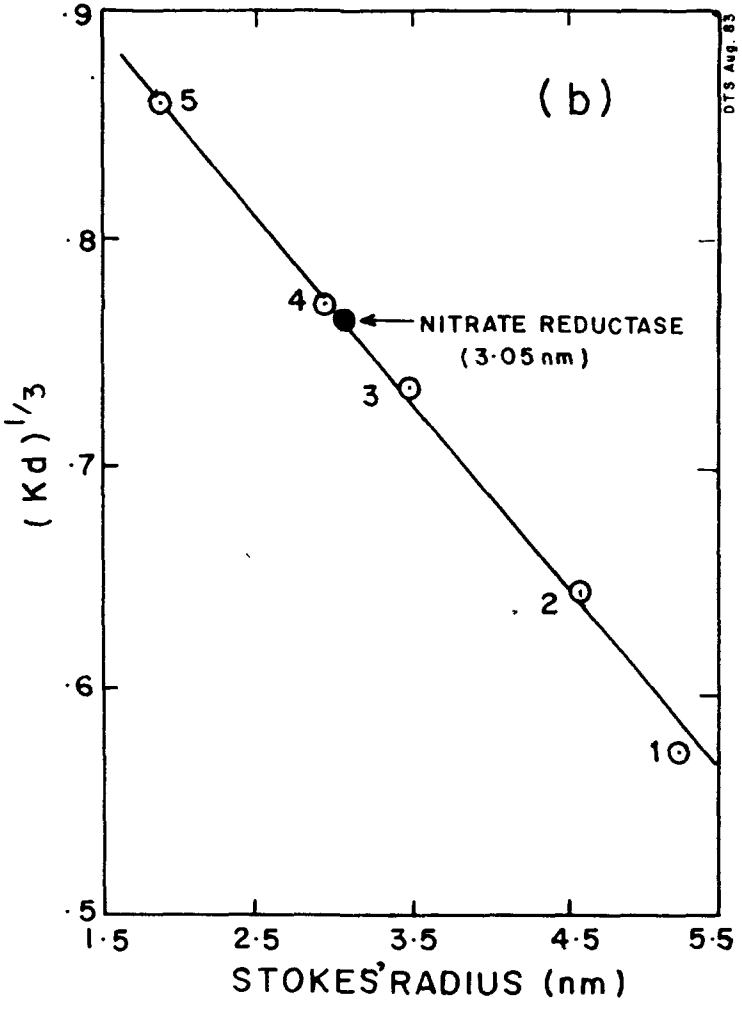

DTS AUG. 83
log MOLECULER WEIGHT
5·4
5·2
5·0
4·8
4·6
4·4
4·2
4·0
1·1
1·3
1·5
1·7
1·9
2·1
2·3
Ve / Vo
1
2
3
4
5
NITRATE
REDUCTASE

FIG. 9 Determination of the Stokes' radius of *A. fischeri* nitrate reductase.
The elution data of Table 4 were used.
(a) The data are plotted according to the correlation of Laurent and Killander (1964).
(b) The data are plotted according to the correlation of Porath (1963).


(a)
DTS Aug 83
(-log Kav)^1/2
·9
·8
·7
·6
·5
·4
1·5
2·5
3·5
4·5
5·5
STOKES' RADIUS (nm)
1
2
3
4
5
NITRATE REDUCTASE
(3·2nm)


(b)
DTS Aug. 83
(Kd)^1/3
·9
·8
·7
·6
·5
1·5
2·5
3·5
4·5
5·5
STOKES' RADIUS (nm)
1
2
3
4
5
NITRATE REDUCTASE
(3·05 nm)

DISCUSSION

The enzyme nitrate reductase which catalyzes the reduction of nitrate to nitrite has been studied in a variety of organisms. It has been purified to varying degrees of purity from different sources, and some of them have also been characterized.

Nitrate reductase from A. fischeri has been purified to homogeneity as shown by analytical polyacrylamide gel electrophoresis and SDS-gel electrophoresis. The purification procedure of A. fischeri nitrate reductase was a modification of an earlier procedure (Sadana et al., 1963), and the yield of the purified enzyme obtained was higher. Preparative polyacrylamide gel electrophoresis was used as the final step for purifying the enzyme. The recent trend for purification of the enzyme is by the use of affinity chromatography and the technique has been used for purification of nitrate reductase from barley leaves and C. vulgaris (Kuo et al., 1980; Solomonson, 1975) using Blue-Dextran-Agarose as matrix, A. braunii (Miguel et al., 1980) using Blue-Sepharose as matrix, and spinach leaves (Nakagawa et al., 1985) using Blue-Sepharose CL-6B as matrix.

The modified purification procedure for A. fischeri nitrate reductase consists of: extraction of bacterial cells by water lysis, concentration by ammonium sulfate, protamine sulfate treatment to remove nucleic acids, ammonium sulfate fractionation, hydroxylapatite gel column chromatography, and finally, purification by preparative

polyacrylamide gel electrophoresis. As nitrate reductase is colourless, the enzyme could not be visualized on the column or on elution during electrophoresis, as is the case with A. fischeri nitrite reductase, which being a heme protein (Prakash et al., 1966) is coloured. Therefore, it was necessary to standardize the gel length, current and time for its elution from the column and collection in the dialysis sack.

Nitrate reductases from R. sphaeroides (Norma and Cardenas, 1982), A. calcoaceticus (Villalobo et al., 1977) and C. perfringens (Chiba and Ishimoto, 1977), and in some other cases, were partially purified. Nitrate reductase from various other sources have been purified to homogeneous state, namely from A. braunii (Miguel et al., 1980), lupine root nodule bacteroid (Alikulov et al., 1980) and P. boryanum (Mikami and Ida, 1984). The purified nitrate reductase from A. fischeri was homogeneous in analytical disc gel electrophoresis and SDS-gel electrophoresis. In analytical polyacrylamide gel electrophoresis the protein band coincided with nitrate reductase activity band on the gel.

The specific activity of the purified A. fischeri nitrate reductase was 3.65 µmol of nitrite formed per min per mg protein. The specific activity of nitrate reductases purified from various other sources were: R. japonicum 0.057 (Kennedy et al., 1975), R. capsulata 0.8 (Alef and Klemme, 1979), D. tertiolecta 0.86 (LeClaire and Grant, 1972), Lupine root nodule bacteroid 0.9 (Alikulov et al., 1980),

barley leaves 8.0 (Kuo *et al.*, 1980), spinach 24.12 (Garrett and Amy, 1978), spinach leaves 80 - 130 (Nakagawa *et al.*, 1985), *N. crassa* 125 (Garrett and Amy, 1978) and *A. nidulans* 875 (Candau, 1979). Recently Mikami and Ida (1984) have purified a nitrate reductase from *P. boryanum* to homogeneous state and showed that the specific activity of the enzyme was 305 and 1020 with ferredoxin and methyl viologen as the electron donors, respectively.

Nitrate reductase from *A. fischeri* showed an absorption maximum at 278 nm in the UV range. The presence of either flavin or cytochrome in the enzyme molecule was excluded. This is very similar to the enzyme from *A. nidulans* (Candau, 1979) which showed only one peak in the UV range at 273 nm and there was no peak in the visible region. The enzymes purified from spinach (Squire, 1964), *R. glutinis* (Guerrero and Gutierrez, 1977), *N. crassa* (Garrett and Nason, 1967), *A. braunii* (Miguel *et al.*, 1980) and *C. vulgaris* (Solomonson and Vennesland, 1972) showed the presence of a cytochrome in the enzyme. The enzymes purified from *C. vulgaris* (Solomonson *et al.*, 1975), *N. crassa* (Garrett and Nason, 1969) and *Aspergillus nidulans* (Downey, 1973) showed the presence of flavin in the enzyme.

The M_r of *A. fischeri* nitrate reductase was determined by four different methods and an average relative molecular mass of 54,000 was obtained. Sadana *et al.*(1963) observed that in the analytical ultracentrifuge the highly purified *A. fischeri* nitrate reductase indicated the presence of two components, a slow moving fraction with an $s_{20,w}$ value

of about 1.5 S and a faster moving component with an $s_{20,w}$ value of about 4.5 S. Bovine serum albumin has an $s_{20,w}$ value of 4.5 S and a relative molecular mass of 67,000. The A. fischeri nitrate reductase relative molecular mass of 54,000 (average of four methods: gel filtration on Sephadex G-200 and Bio-Gel P-150, polyacrylamide gel electrophoresis and SDS-gel electrophoresis) would suggest that the fraction with an $s_{20,w}$ value of approximately 4.5 S (Sadana et al., 1963) represented nitrate reductase.

The enzyme from lupine root nodule bacteroid has a relative molecular mass of 67,000 (Alikulov et al., 1980); R. japonicum 70,000 (Kennedy et al., 1975); A. nidulans 75,000 (Candau, 1979); C. perfringens 90,000 (Chiba and Ishimoto, 1977) and A. calcoaceticus 96,000 (Villalobo et al., 1977). The small size of these nitrate reductases and that from A. fischeri is in striking contrast to the nitrate reductases obtained from higher plants and some other organisms. The spinach enzyme has a relative molecular mass of 500,000 (Relimpio et al., 1971), A. braunii 460,000 (Miguel et al., 1980), soybean 330,000 (Jolly et al., 1976), and spinach leaves 270,000 (Nakagawa et al., 1985). Nitrate reductase from E. coli (Taniguchi and Itagaki, 1960) was reported to have an $s_{20,w}$ value of 25 S, corresponding to a relative molecular mass of about 1,000,000.

Nitrate reductases from different sources show a wide variation in their molecular subunit structure. Carboxyamidomethylation of the reduced form of A. fischeri nitrate reductase on SDS-gel electrophoresis gave one protein band,

having the same relative molecular mass as the native enzyme, indicating that the enzyme is comprised of a single polypeptide chain. In this respect the enzyme from A. fischeri resembles the enzymes from C. perfringens (Chiba and Ishimoto, 1977), R. japonicum (Kennedy et al., 1975) and A. nidulans (Candau, 1979). On the other hand C. vulgaris (Solomonson, 1979) native enzyme has three identical subunits of 100,000 relative mass. The enzyme from R. glutinis (Guerrero and Gutierrez, 1977) has a relative molecular mass of 230,000 and consists of two subunits of 118,000 dalton. The enzyme from N. crassa (Pan and Nason, 1978) is composed of two subunits corresponding to a relative molecular mass of 115,000 and 130,000. The proteolytic mapping and N-terminal amino acid analysis indicated that the two subunits are similar (Pan and Nason, 1978). The respiratory enzyme from B. licheniformis gave two dissimilar subunits having relative molecular mass of 150,000 and 57,000 present in equimolar ratio (Van'T et al., 1979). The nitrate reductase from A. braunii has been shown to be composed of eight identical subunits of 58,000 relative molecular mass (Miguel et al., 1980). The enzyme from spinach leaves (Nakagawa et al., 1985) has a relative molecular mass of 270,000 and consists of two identical subunits of 110,000 - 120,000 dalton.

The A. fischeri nitrate reductase enzyme has a Stokes' radius of 3.1 nm, which is very close with the value of 3.2 nm reported for A. nidulans (Candau, 1979). The enzyme from

other sources showed wide variation in their Stokes' radii: A. braunii (Miguel et al., 1980) 9.8 nm; R. glutinis (Guerrero and Gutierrez, 1977) and N. crassa (Pan and Nason, 1978) 7.0 nm; spinach (Hewitt and Notton, 1980) 6.0 nm; C. vulgaris (Solomonson, 1979) 8.9 nm. The nitrate reductase from R. sphaeroides showed Stokes' radius of 4.1 nm having relative molecular mass of 100,000 (Norma and Cardenas, 1982). The nitrate reductase from spinach leaves has been purified recently (Nakagawa et al., 1985) having Stokes' radius of 6.3 nm.

CHAPTER IV

PURIFICATION OF *A. fischeri* NITRITE REDUCTASE

SUMMARY

Nitrite reductase has been purified from A. fischeri by a modification of an earlier procedure of Husain and Sadana (1972). The enzyme has been obtained in a state which is homogeneous in ultracentrifuge and polyacrylamide gel electrophoresis at pH 8.9 and 4.3 and at different gel concentrations. The overall recovery of the enzyme was 30% which is comparable to that reported earlier. The purification procedure involved: crude extract preparation of bacterial cells by osmotic water lysis, homogenization and centrifugation to get clear supernatant; concentration of crude extract by ammonium sulfate precipitation (0 - 0.90 saturation); protamine sulfate treatment to precipitate the enzyme; fractionation with ammonium sulfate (0.55 - 0.85 saturation); two successive hydroxylapatite gel column chromatography steps; and a simplified preparative polyacrylamide gel electrophoresis as the final step for purification.

The purified enzyme has a specific activity of 150 μmol nitrite reduced $min^{-1}mg^{-1}$ protein with benzyl viologen as electron donor. Methyl viologen also serves as an electron donor and is two times more effective than benzyl viologen.

INTRODUCTION

The purification of *A. fischeri* nitrite reductase was first reported from this laboratory by Prakash *et al.* (1966). The purification procedure consisted of crude extract preparation from *A. fischeri* cells by osmotic water lysis, isoelectric precipitation at pH 4.5, protamine sulfate treatment ammonium sulfate fractionation and chromatography on hydroxylapatite gel and DEAE-cellulose. The purified enzyme was homogeneous in the ultracentrifuge. However, when checked for homogeneity by polyacrylamide gel electrophoresis, two additional bands were noticed by Husain and Sadana (1972).

The procedure for *A. fischeri* nitrite reductase purification was modified by Husain and Sadana (1972). The last step in the purification procedure of Prakash and Sadana (1972), column chromatography on DEAE-cellulose, was deleted, as considerable enzyme losses (40 - 70%) occurred at this step. Further purification of the enzyme was carried out by a simplified preparative polyacrylamide gel electrophoresis. The purified enzyme was homogeneous both in the ultracentrifuge and in disc gel electrophoresis.

The *A. fischeri* nitrite reductase used in this thesis was purified by a further modification of the procedure described by Husain and Sadana (1972). The second step in the earlier procedure i.e. precipitation at pH 4.5, was replaced by 90% ammonium sulfate precipitation, as considerable enzyme losses (60 - 70%) were observed during precipitation at pH 4.5 by me. The ammonium sulfate precipitation step recovered

90% of the enzyme activity. The final product was recovered from the pooled eluates of preparative polyacrylamide gel electrophoresis by precipitation with ammonium sulfate (0 - 0.80 saturation).

Purification procedure

All the steps for purification of A. fischeri nitrite reductase were carried out at 0 - 4 °C. The purification procedure involved following steps.

1. Crude extract preparation

The frozen cells (200 g) were thawed overnight at 4 °C and lysed by suspending them in cold distilled water (1 g wet weight/20 ml water). The suspension was stirred for 30 min, homogenized in a Potter-Elvehjem glass homogenizer, and stirred again for 30 min. The suspension was centrifuged for 40 min at 20,000 x g in a RC-5 Sorvall centrifuge. The clear supernatant was collected as crude extract and the total volume was 4000 ml.

2. Ammonium sulfate precipitation

The pH of the pooled crude extract was adjusted to 7.0 by the addition of 1 M K_2HPO_4 solution. The required amount of solid ammonium sulfate was added slowly with continuous stirrin for 90% saturation. The suspension was stirred for 30 min at 4 °C and centrifuged at 20,000 x g for 20 min in a RC-5 Sorvall centrifuge. The supernatant was discarded. The sediment was suspended in 50 mM potassium phosphate buffer, pH 6.8, and dialyzed overnight against the same buffer with three changes of the buffer. In this step 90% recovery of the enzyme was obtained.

3. Protamine sulfate treatment

The enzyme preparation from second step contained large amounts of nucleic acids as indicated by the absorbance ratio at 280 nm to 260 nm. Protamine sulfate solution (15 mg/ml, pH 5.0) was added slowly to the clear supernatant with continuous stirring. The addition of protamine sulfate solution was continued until no further precipitation was observed. The precipitate was collected by centrifuging the suspension at 4,000 x g for 15 min. The clear supernatant having very little nitrite reductase activity was discarded.

4. Extraction of nitrite reductase from protamine sulfate precipitate

The precipitate from step 3 was suspended in 50 ml of 0.2 M potassium phosphate buffer, pH 7.5, homogenized, stirred for 30 min and centrifuged at 14,000 x g. The sedimented pellet was resuspended in the same buffer and extracted as before. The extraction was repeated 5 to 6 times in a similar manner until the extract showed negligible enzyme activity. The extracts were combined, centrifuged, and the inactive precipitate discarded. The ratio of absorbance at 280 nm to 260 nm increased from 0.69 in the crude extract to 1.2 in this step.

5. Ammonium sulfate fractionation

The combined protamine sulfate extracts (250 ml) were brought to 0.55 saturation by adding appropriate amount of solid ammonium sulfate. The pH of the solution was not adjusted during ammonium sulfate precipitation. The suspension was stirred for 30 min at 4 °C and centrifuged at 14,000 x g

for 15 min in a Sorvall centrifuge. The sediment, having very little activity of nitrite reductase, was discarded. The concentration of the supernatant was then raised to 0.85 saturation by adding the required amount of ammonium sulfate. The suspension was stirred for 30 min and centrifuged again as before. The precipitate was suspended in 20 ml of 20 mM potassium phosphate buffer, pH 6.8, and dialyzed against the same buffer overnight with three changes of buffer.

6. First chromatography on hydroxylapatite gel column

The hydroxylapatite gel column (1.8 x 45 cm) was equilibrated with 20 mM potassium phosphate buffer, pH 6.8. The dialyzed enzyme solution containing 1375 mg protein was loaded on top of the column. The hydroxylapatite gel column was washed with the same buffer until the absorbance of the washing was less than 0.01 at 280 nm. The nitrite reductase was absorbed on the column as a pink diffused band and remained stationary while the column was washed with 20 mM potassium phosphate buffer, pH 6.8. The column was then washed with 50 mM potassium phosphate buffer, pH 6.8, and the washing was continued until nitrite reductase activity started appearing in the eluate. The enzyme was finally eluted with 0.2 M potassium phosphate buffer, pH 6.8, at a flow rate of 20 ml per h and fractions of 2 - 5 ml were collected. The fractions having high specific activity were combined and dialyzed overnight against 20 mM potassium phosphate buffer, pH 6.8.

7. Second chromatography on hydroxylapatite gel column

The combined dialyzed fractions containing 300 mg protein

were loaded on top of the second hydroxylapatite gel column (1.8 x 20 cm). The column was equilibrated and eluted as before. The pink nitrite reductase band was eluted as one major band (8 ml) which contained 165 mg of protein. The enzyme was dialyzed overnight against 50 mM potassium phosphate buffer, pH 6.8 with three changes of the same buffer. The specific activity of the enzyme at this stage was about 680 units (μmol nitrite reduced per ten min/mg protein). The recovery was 46% of the initial activity present in the crude extract.

8. Preparative polyacrylamide gel electrophoresis

A simplified procedure of preparative polyacrylamide gel electrophoresis was adopted for further purification of the enzyme as described by Husain and Sadana (1972). The preparative electrophoresis was performed in 7.5% acrylamide gel at pH 8.9. The electrophoretic gel columns (2 x 15 cm) were filled with acrylamide gel solution, prepared according to the procedure described by Ornstein and Davis (1964), leaving about 2 - 4 ml volume for applying the enzyme samples.

The semipurified enzyme obtained after second hydroxylapatite column chromatography was dialyzed against 10 mM potassiu phosphate buffer, pH 7.5, for 8 h with three changes of the buffer. Enzyme samples containing 20 - 30 mg of protein were made dense by adding 20% glycerol and layered on top of the each column. Electrophoresis was carried out at 4 °C at a constant current of about 6 mA per column. The enzyme migrated as a sharp band and being red colour was easily detectable visually without staining the gel. After about 6 - 8 h,

the apparatus was switched off and a piece of dialysis sacking filled with 1.0 ml of bath buffer (tris-glycine, pH 8.3) was attached to the lower end of the column as described in "Materials and Methods". The electrophoresis was continued till the red band reached the bottom of the column. The main enzyme band was eluted into a fresh dialysis sacking containing Tris-glycine buffer, pH 8.3. At the end of the electrophoresis, fractions were dialyzed overnight against 50 mM potassium phosphate buffer, pH 6.8, with three changes of the same buffer. Three preparative runs using two columns at a time were carried out to purify all the enzyme obtained from second hydroxylapatite column.

A summary of the purification procedure is given in Table 5. The yield of the final purified enzyme is about 48 mg protein from 200 g of A. fischeri cells (wet weight), and on a protein basis represents a 275 fold purification. The overall recovery of the enzyme was 30%. The purified enzyme was homogeneous in ultracentrifuge and polyacrylamide gel electrophoresis at pH 8.9 and 4.3 and at different gel concentrations. The specific activity of the enzyme was 150 - 170 μmol of nitrite reduced per mg protein per min with reduced benzyl viologen as electron donor.

Table 5: Purification procedure of *A. fischeri* nitrite reductase from 200 g (wet weight) of cells.

Fraction	Volume (ml)	Activity (Units/ml) x 10^2	Total activity (Unit x 10^3)	Protein (mg/ml)	Total protein (mg)	Specific activity (Units/mg)	Yield (%)
Crude extract	4000	0.61	244	3.30	13200	18.5	100
0 - 0.95 saturated ammonium sulfate ppt.	800	2.75	220	13.75	11000	20.0	90
Phosphate extracts of protamine sulfate ppt.	380	5.10	194	6.80	2584	75.0	79
0.55 - 0.85 saturated ammonium sulfate ppt.	55	30.00	165	25.00	1375	120.0	68
First hydroxylapatite eluate	20	67.50	135	15.00	300	450.0	55
Second hydroxylapatite eluate	8	140.00	112	20.60	165	680.0	46
Preparative polyacrylamide gel electrophoresis	6	122.00	73	8.00	48	1525.0	30

CHAPTER V

PRESENCE OF ESSENTIAL HISTIDINE RESIDUES AT THE ACTIVE SITE OF *A. fischeri* NITRITE REDUCTASE

SUMMARY

A. fischeri nitrite reductase was inactivated by the histidine-specific reagent, diethylpyrocarbonate (DEPC), at pH 6.8 and 6.5 at 4 °C. The rate of inactivation was faster at pH 6.8 as compared to that at pH 6.5. The inactivation was found to be concentration- and time-dependent. The inactivation rates followed pseudo-first order kinetics. On treatment of the enzyme with DEPC the absorbance around 240 nm increased which is specific for N-carbethoxyimidazole derivative. The absorbance remained unchanged around 280 nm region suggesting that tyrosine residues were not modified. Free sulfhydryl groups were not modified during the inactivation of the enzyme with DEPC. Though four histidine residues have been shown to be modified for the complete inactivation of the enzyme with DEPC, only one histidine residue per molecule of the enzyme has been shown to be essential for loss of activity on the basis of the reaction order (n) which was calculated to be 0.9. The inactivated enzyme was reactivated by 0.75 M hydroxylamine at neutral pH which caused removal of ethoxyformyl groups from the modified enzyme. The substrate of the enzyme, nitrite, considerably protected the enzyme activity against inactivation by DEPC. The maximum protection by nitrite was obtained at a concentration of 5 mM or above. The addition of nitrite at concentrations less than 0.2 mM showed no protection against DEPC inactivation. The pKs of the ionizable groups of the enzyme which are required for the activity of the enzyme were 7.0 and 7.7. These correspond to the imidazole group. These studies suggested that one histidine residue is essential for the activity of the enzyme.

INTRODUCTION

Nitrite reductase from A. fischeri catalyzes the six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). The enzyme has a relative molecular mass of 80,000 and is composed of two subunits of equal size which are covalently bonded by a disulfide bridge. Methionine has been found as the sole N-terminal residue (Husain and Sadana, 1974a; 1974b). It contains two c-type hemes per molecule but no nonheme iron or molybdenum. It is, therefore, distinct from assimilatory nitrite reductases and sulfite reductases, which contain siro-heme and iron-sulfur centres (Lancaster et al., 1979; Siegel et al., 1982; Krueger and Siegel, 1982) and for which electron donor is ferredoxin. Recently Liu and Peck (1981) have reported a new type of nitrite reductase from a strain of D. desulfuricans which contains six c-type hemes per molecule and resembles the nitrite reductase from A. fischeri in having c-type hemes.

Reduction of hydroxylamine is also catalyzed by A. fischeri nitrite reductase. It has been reported that both the substrates, nitrite and hydroxylamine, are reduced at the same active site and no free intermediate has been detected during the six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). Similar data have been reported by Cresswell et al. (1965); Hageman et al. (1962); Lazzarini and Atkinson (1961); Ramirez et al. (1966).

Very little information is available on the amino acid residues which are involved at the catalytic site of nitrite reductase. A. fischeri nitrite reductase was not affected by p-IMB (4 - 5 moles/mole enzyme) or DTNB (50 moles/mole enzyme),

specific reagents for sulfhydryl groups (Husain and Sadana, 1974a). However, in the presence of large excess (400 - 800 fold) of mercurial reagents, p-HMB and p-CMS, the enzyme activit was completely inhibited (Prakash and Sadana, 1972). This was interpreted that inhibition by -SH reagents was not due to mercaptide formation but involved some other interactions.

A mechanism of nitrite reduction which involves a hydroxyl group of an active serine as the substrate binding site, and the existence of bound intermediates of nitrite reduction was proposed by Kemp et al.(1963). Although the enzyme purified from E. coli by Kemp et al.(1963) would reduce NO_2^-, NH_2OH and SO_3^{-2}, its physiological role was that of a sulfite reductase. The models proposed for NO_2^- and SO_3^{-2} reduction were similar. The Achromobacter nitrite reductase, however, did not reduce sulfite (Prakash and Sadana, 1972).

The work of Cresswell et al. (1965) showed that CN^- and p-HMB were inhibitory to nitrite reductase isolated from higher plants. They proposed that hydroxyl of a hemiacetal group could be the binding site for nitrite. The CN^- inhibition was presumed to be competitive and due to the reaction of cyanide with an active carbonyl group to form a cyanohydrin as previously proposed by Mager (1960). However, the lack of inhibition of nitrite reductase from higher plants by phenyl methyl sulfonyl fluoride (PMSF) or fluoride (Dalling et al., 1973), hydroxyl modifying agents, or phenyl hydrazine (Dalling, 1972), a carbonyl modifier, does not support the models proposed by Cresswell et al. (1965) and Hewitt et al. (1968). A histidine residue on the corn nitrite reductase was proposed by Loussart

and Hageman (1974), which reacts with nitrite to form nitrosamine

Histidine as the binding site for nitrite was suggested because of the well known reaction of nitrite with secondary amines that produces relatively stable nitrosamine, and from the pH profile of the nitrite reductase (Hucklesby et al., 1972). There are many reagents known which can modify the histidine residues at the active site of an enzyme and cause inactivation. These include diethylpyrocarbonate (DEPC) (Bosshard, 1984; Gomi and Fujioka, 1983), diazo-1 H-tetrazole (Cohen, 1968) and bromoacetone (Beeley and Neurath, 1968). Whether a histidine residue is involved in the catalytic action of A. fischeri nitrite reductase has been determined by chemical modification of the enzyme using DEPC as the histidine-specific reagent and studying the effect of pH on its kinetic parameters, K_m and V_{max} according to Dixon and Webb (1964).

DEPC has been shown to be specific for histidine residues below pH 7.0 from model studies (Muhlard et al., 1967). It reacts stoichiometrically with histidyl residues (Leskovac and Pavkov-Pericin, 1975; Wallis and Holbrook, 1973). The formation of N-carbethoxy-histidyl derivative is followed spectrophotometrically by the increase in absorbance between 230 - 250 nm (Ovadi et al., 1967; Ovadi and Keleti, 1969). Hydroxylamine removes the carbethoxy groups from N-carbethoxy-histidyl residues (Melchior and Fahrney, 1970). Inactivation of an enzyme by DEPC has been correlated with the modification of histidyl residues at the active site if hydroxylamine reactivates the enzyme (Ovadi and Keleti, 1969; Thome-Beau et al., 1971;

Setlow and Mansour, 1970; Horiike et al., 1979). However, Melchior and Fahrney (1970) have found that the reagent can also react with other nucleophilic residues which occur in proteins.

METHODS

Treatment of A. fischeri nitrite reductase with diethylpyrocarbonate (DEPC)

The carbethoxylation of A. fischeri nitrite reductase was carried out at 4 °C in 50 mM potassium phosphate buffer, pH 6.8.

As diethylpyrocarbonate (DEPC) gets hydrolysed rapidly, its concentration was determined just before its use as described below:

An aliquot of diluted stock solution of DEPC was added to 3 ml of 10 mM imidazole solution at pH 7.5 in a cuvette and increase in absorbance at 230 nm due to the formation of N-carbethoxyimidazole was determined. The concentration of DEPC was calculated from $\Delta\varepsilon = 3 \times 10^3\ cm^{-1}M^{-1}$ (Melchior and Fahrney, 1970).

For studying the effect of DEPC concentration on the inactivation of the enzyme, 0.1 mg of the enzyme was treated with various concentrations of DEPC (1, 2, 3, 5 mM). The enzyme activity of the DEPC-treated enzyme was assayed after different time intervals by drawing an aliquot from the reaction mixture. The extent of inhibition was calculated by comparing the activity of the modified enzyme to the reference enzyme treated in a similar manner but without DEPC. The diethylpyrocarbonate

was used as a solution in cold dry ethanol. The final concentration of ethanol in the reaction mixture was never more than 2%.

For studying the effect of pH on the enzyme inhibition by DEPC, 0.1 mg enzyme in 50 mM potassium phosphate buffer was treated with various concentrations of DEPC (1,2,3,5 mM) at two different pH values of 6.5 and 6.8. The activity of the enzyme was assayed after different time intervals in reaction systems and controls as well.

The apparent first order rate constant of inactivation depends on the concentration of the modifier and can be expressed by the following equation:

$$K_{app} = K(M)^n$$

where K_{app} is the apparent first order rate constant for the inactivation, K is the second order rate constant, M signifies the concentration of the modifier and n is a number equal to the average order of the reaction with respect to the concentration of the modifier. Taking the logarithm of both sides:

$$\text{Log } K_{app} = \text{Log } K + n \text{ Log } (M)$$

K_{app} can be calculated from a semi logarithm plot of the residual enzyme activity as a function of time. The order of the reaction (n) can be experimentally estimated by determining K_{app} at a number of different concentrations of the modifier. A plot of log K_{app} against log (M) should give a straight line with a slope equal to n, where n is the number of molecules of modifier reacting with each active unit of the enzyme to produce

an enzyme-inhibitor complex (Levy et al., 1963; Ramakrishna and Benzamin, 1981; Marcus et al., 1976).

Spectrophotometric study of A. fischeri nitrite reductase inhibition by DEPC

Nitrite reductase (1 mg/ml) in 50 mM potassium phosphate buffer, pH 6.8 was taken in each of the two cuvettes, in the reference and sample compartments of a Gilford 250 spectrophotometer which was cooled with circulating water at 4 °C. A solution of DEPC (0.01 ml/ml of the enzyme solution) was added to the sample cell to give a final concentration of 3 mM and the same amount of ethanol was added to the reference cell. Difference spectra were recorded at different time intervals (5, 10, 15, 20, 30, 45, 60 min) between 300 nm and 235 nm. The number of histidyl residues modified by DEPC was determined using $\Delta\varepsilon = 3200\ cm^{-1}M^{-1}$ at 240 nm (Ovadi et al., 1967). An aliquot (0.01 ml) of the reaction mixture was removed from both the cuvettes after the absorbance at 235 nm was recorded, and assayed for enzyme activity. The time referred to was the time when the spectrum was completed and an aliquot was removed for the assay.

Effect of substrate on inhibition during treatment of A. fischeri nitrite reductase with DEPC

The enzyme (0.1 mg) in 50 mM potassium phosphate buffer, pH 6.8, was preincubated with different concentrations of nitrite (1.8 - 18 mM) for one min at 4 °C, before the addition of DEPC (3.0 mM). The control samples were also treated under identical conditions but without nitrite. The aliquots were

withdrawn from both the treated enzyme and the controls at various time intervals and checked for nitrite reductase activity.

Effect of hydroxylamine on reactivation of DEPC - inactivated A. fischeri nitrite reductase

Initially the enzyme was treated with 3 mM DEPC. Aliquots of the treated enzyme were withdrawn at different time invervals and incubated in 100 mM potassium phosphate buffer, pH 7.0, containing 0.75 M hydroxylamine hydrochloride (adjusted to pH 7.0 with 0.1 N KOH) at 4 °C. The enzyme activity was estimated after 45 min of incubation.

Estimation of free sulfhydryl groups

Free sulfhydryl groups of nitrite reductase, before and after treatment with DEPC, were estimated by titration of the enzyme in the presence of 8 M urea with p-HMB as described by Benesch and Benesch (1962). The solution of p-HMB (sodium salt) was prepared by dissolving 8 - 9 mg of the compound in 1 ml of 0.04 N NaOH and diluted to 25 ml. The p-HMB solution was standardized both spectrophotometrically by recording the absorbance at 232 nm ($\varepsilon_M = 1.69 \times 10^4$) (Boyer, 1954) and by titration against standard reduced glutathione solution as described by Benesch and Benesch (1962). The determinations by the two methods were in good agreement.

The A. fischeri nitrite reductase samples were incubated with 8 M urea for 60 min. An accurately measured aliquot of the enzyme in 50 mM potassium phosphate buffer, pH 7.0 was placed in one ml stoppered silica cuvettes. Small aliquots (10 µl) of standard p-HMB solution were added to the experimental solution and the blank which contained equal volume of the buffer

The contents of the cuvettes were mixed and absorbance recorded at 250 nm after each addition. The p-HMB solution was added till there was no change in the absorbance. The observed absorbances were corrected for dilution and plotted against the volume of the p-HMB added. The end point is obtained from intersection of the two lines.

Kinetics

The kinetic parameters, K_m and V_{max} of A. fischeri nitrite reductase, were determined at different pH values by Lineweaver-Burk method. The final concentration of nitrite in the reaction mixtures varied from 18 mM to 260 mM. The potassium phosphate buffer was used in the pH range 5.5 - 8.5. For each experiment the pH was determined after completion of the reaction. The kinetic parameters, pK_m (- log K_m), log V_{max} and log V_{max}/K_m, were plotted against pH for the determination of pK_m values of ionizable groups according to the procedure of Dixon and Webb (1964).

RESULTS

Inactivation of nitrite reductase with DEPC

During preliminary experiments for the inactivation of nitrite reductase by DEPC, it was observed that nitrite reductase activity was inhibited by low concentration of DEPC (0.2 mM), but the rate of inactivation was very slow. In order to determine the appropriate concentration of the modifying reagent (DEPC) for the inactivation studies, various concentrations of the reagent were tested for the inactivation of nitrite reductase. Inactivation of nitrite reductase as a function of

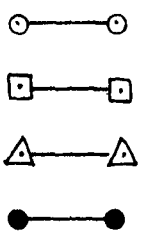
diethylpyrocarbonate concentration (Fig. 10) shows that the enzyme is more or less completely inhibited at 5 mM concentration of the modifying reagent (DEPC) at pH 6.8. The log of percent initial activity versus DEPC concentration is a linear function of the amount of the inhibitor (Fig. 10 inset). Plots of the log of percent initial activity with different concentrations of DEPC against time were linear upto nearly 10% of the initial activity (Fig. 11). This indicated that the inactivation process followed pseudo-first order kinetics with respect to time at any fixed DEPC concentration under these conditions.

Since nucleophiles are reactive in their unprotonated forms, Ovadi et al. (1967) proposed that DEPC should be selective for histidyl residues in proteins at pH 6.0. He showed that this was the case for several proteins. However, the reactivity of the nucleophiles in some proteins may not be the same as in model systems owing to different environments of the residues in these proteins. It is, therefore, considered advisable to determine the inhibition at more than one pH value and to consider the effect of pH on the stability and conformation of the enzyme being studied (Miles, 1977). The inactivation of nitrite reductase was carried out in the presence of different concentrations of DEPC at two different pH values, 6.8 and 6.5. It is evident from Table 6 that the rate of inactivation is at least two times faster at pH 6.8 compared to that at pH 6.5.

Spectrophotometric study of inactivation of nitrite reductase by DEPC

Diethylpyrocarbonate reacts with histidyl residues in

FIG. 10 Effect of DEPC concentration on inactivation of *A. fischeri* nitrite reductase activity



10 min

20 min

30 min

45 min

Insert shows the semilog plot of the same data.

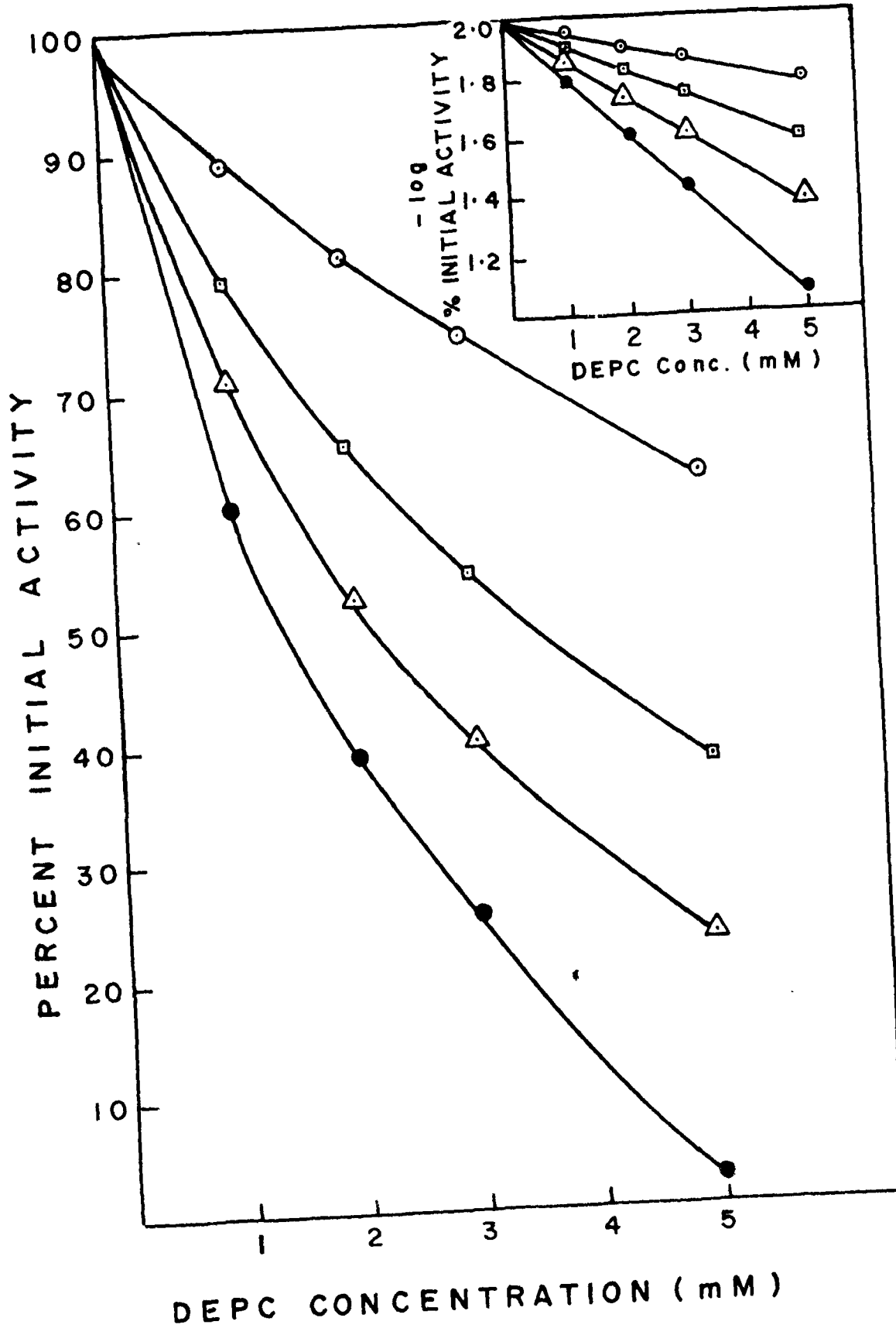
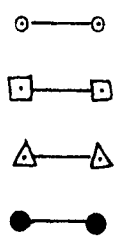

100
90
80
70
60
50
40
30
20
10
PERCENT INITIAL ACTIVITY
1
2
3
4
5
DEPC CONCENTRATION (mM)
2·0
1·8
1·6
1·4
1·2
- log % INITIAL ACTIVITY
1
2
3
4
5
DEPC Conc. (mM)

FIG. 11 Inactivation of A. fischeri nitrite reductase by DEPC.



1 mM DEPC

2 mM DEPC

3 mM DEPC

5 mM DEPC

Insert: Determination of the order of the reaction with respect to DEPC.

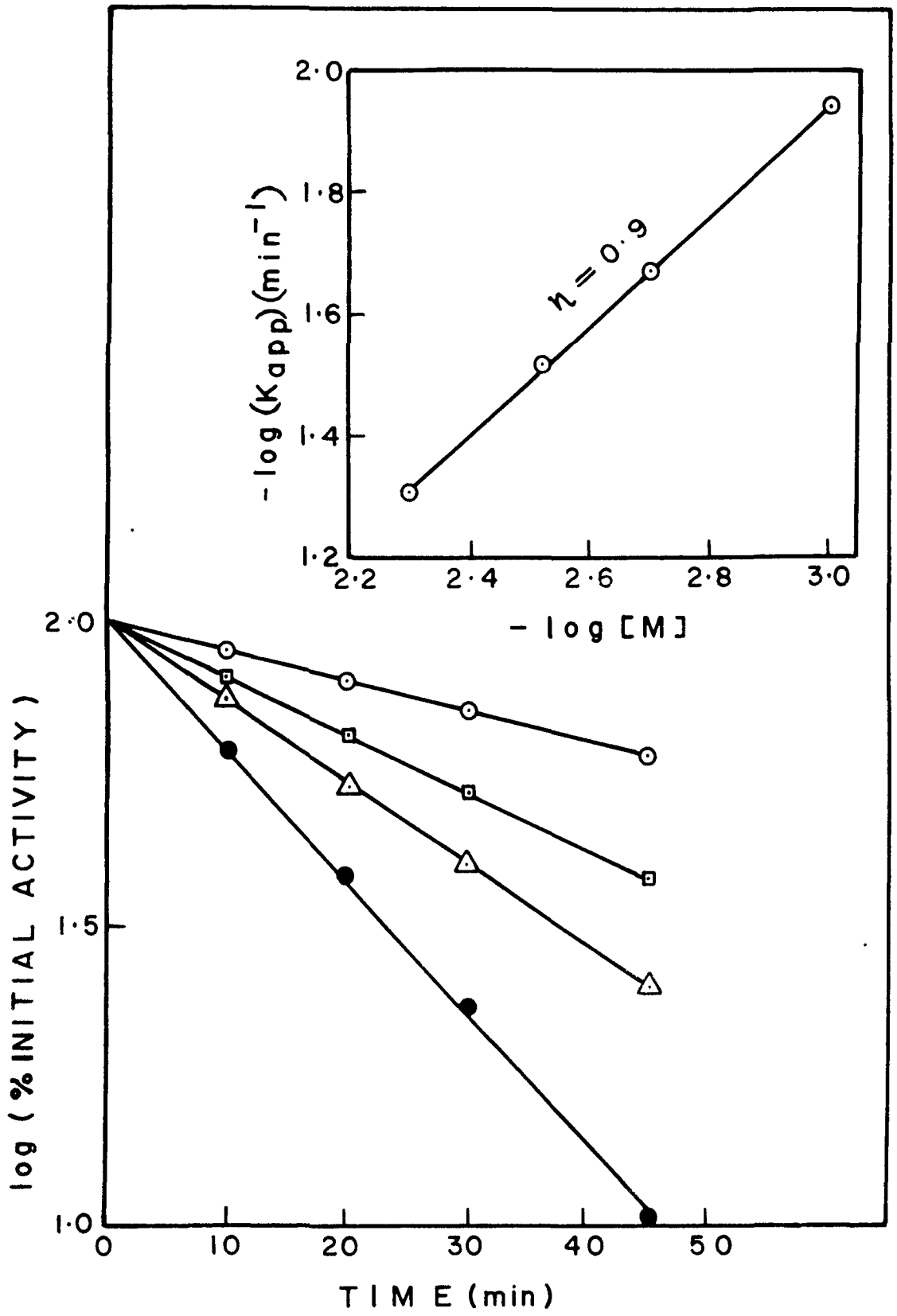

2·0
1·8
1·6
1·4
1·2
- log (Kapp)(min⁻¹)
η = 0·9
2·2
2·4
2·6
2·8
3·0
- log [M]
2·0
1·5
1·0
log (% INITIAL ACTIVITY)
0
10
20
30
40
50
TIME (min)

Table 6: Effect of pH on inactivation of A. fischeri nitrite reductase by DEPC

Concentration of DEPC (mM)	% inhibition of enzyme activity							
	pH 6.5				pH 6.8			
	10 min	20 min	30 min	45 min	10 min	20 min	30 min	45 min
1	4	7	10	16	11	21	29	40
2	5	18	25	33	19	35	48	61
3	7	25	32	40	26	46	60	75
5	10	35	40	48	38	62	77	97

model systems and in proteins to yield an N-carbethoxyimidazole derivative (Ehrenberg et al., 1976; Muhlard et al., 1967; Ovadi et al., 1967; Ovadi and Keleti, 1969). The reaction is conveniently followed spectrophotometrically by the increase in absorbance, which has a maximum between 230 and 250 nm (Ovadi et al., 1967; Ovadi and Keleti, 1969).

Spectrophotometric studies of the inactivation of nitrite reductase by DEPC were carried out at 4 °C in a Gilford 250 spectrophotometer. Figure 12A shows a spectrum of the enzyme before and after treatment with 3 mM DEPC for 60 min (b). There was no change in the spectrum of the DEPC-treated enzyme above 270 nm indicating that tyrosine residues have not been modified, since O-carbethoxytyrosine absorbs between 270 and 280 nm (Muhlard et al., 1967). Figure 12A(a) shows the difference spectra of the enzyme at various time intervals (5, 10, 15, 20, 30, 45, 60 min) during the reaction of the enzyme with the modifying reagent. Large increases in absorbance at 240 nm, characteristic of N-carbethoxyhistidyl derivative, were observed. This is shown in Fig. 12A, 12B. It is evident from Fig. 12B that the plots of percent of initial activity against time, and difference absorbance at 240 nm against time, are mirror-image to each other. The relationship between percent initial activity and modified histidyl residues is shown in Fig. 12C. Extrapolation of the curve to 100% inhibition of the enzyme activity corresponds to carbethoxylation of 4 histidyl residues per molecule of enzyme.

FIG. 12A Effect of DEPC on the spectrum of *A. fischeri* nitrite reductase

(a) Difference spectra of the enzyme with 3 mM DEPC at 5, 10, 15, 20, 30, 45, and 60 min.

(b) Spectra of the enzyme before (——) and after treatment for one hour with 3 mM DEPC (----).

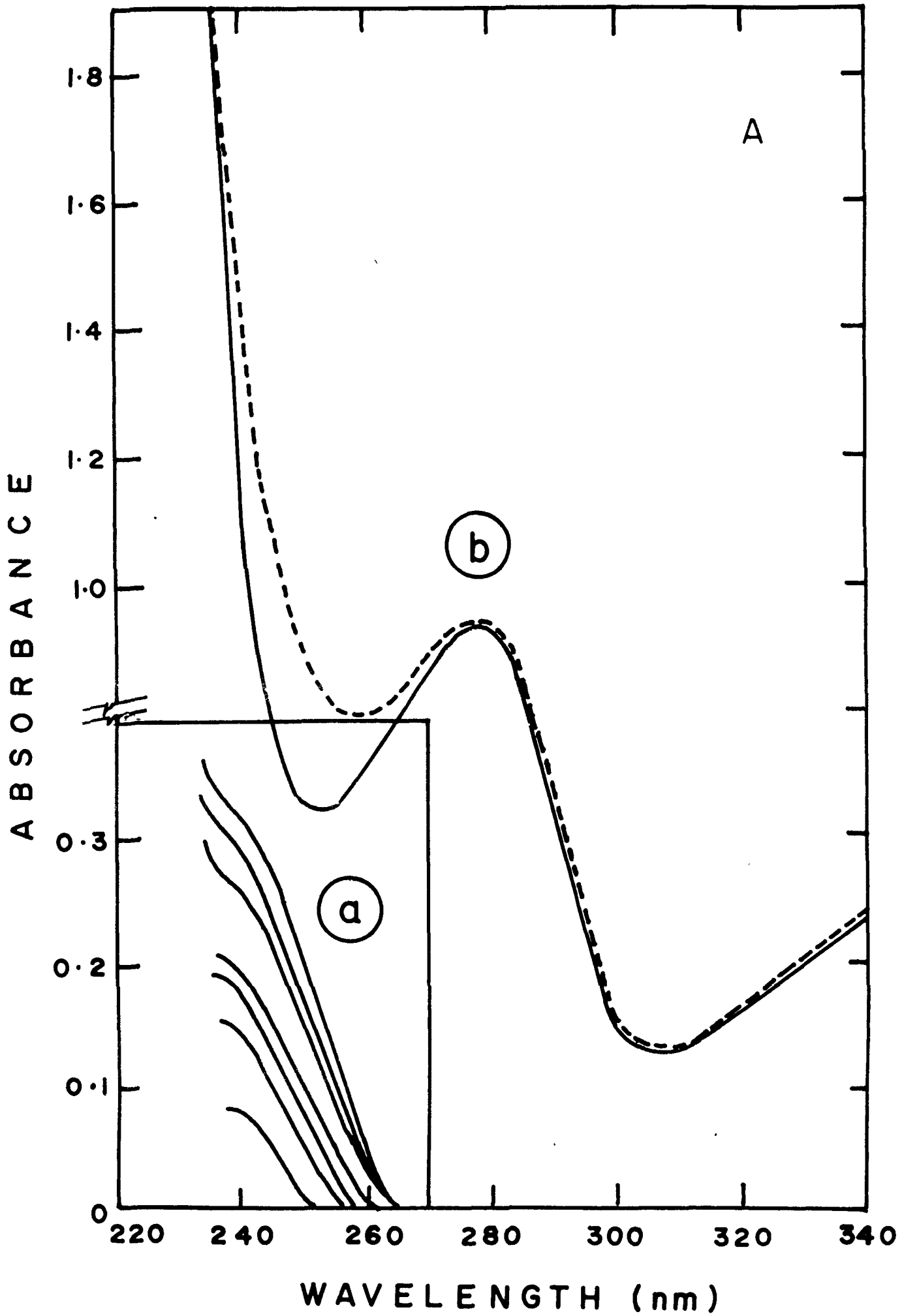

A
b
a
ABSORBANCE
1·8
1·6
1·4
1·2
1·0
0·3
0·2
0·1
0
220
240
260
280
300
320
340
WAVELENGTH (nm)

FIG. 12B Effect of incubation time with 3 mM DEPC on the nitrite reductase activity (⊙——⊙) and difference absorbance at 240 nm (△——△).

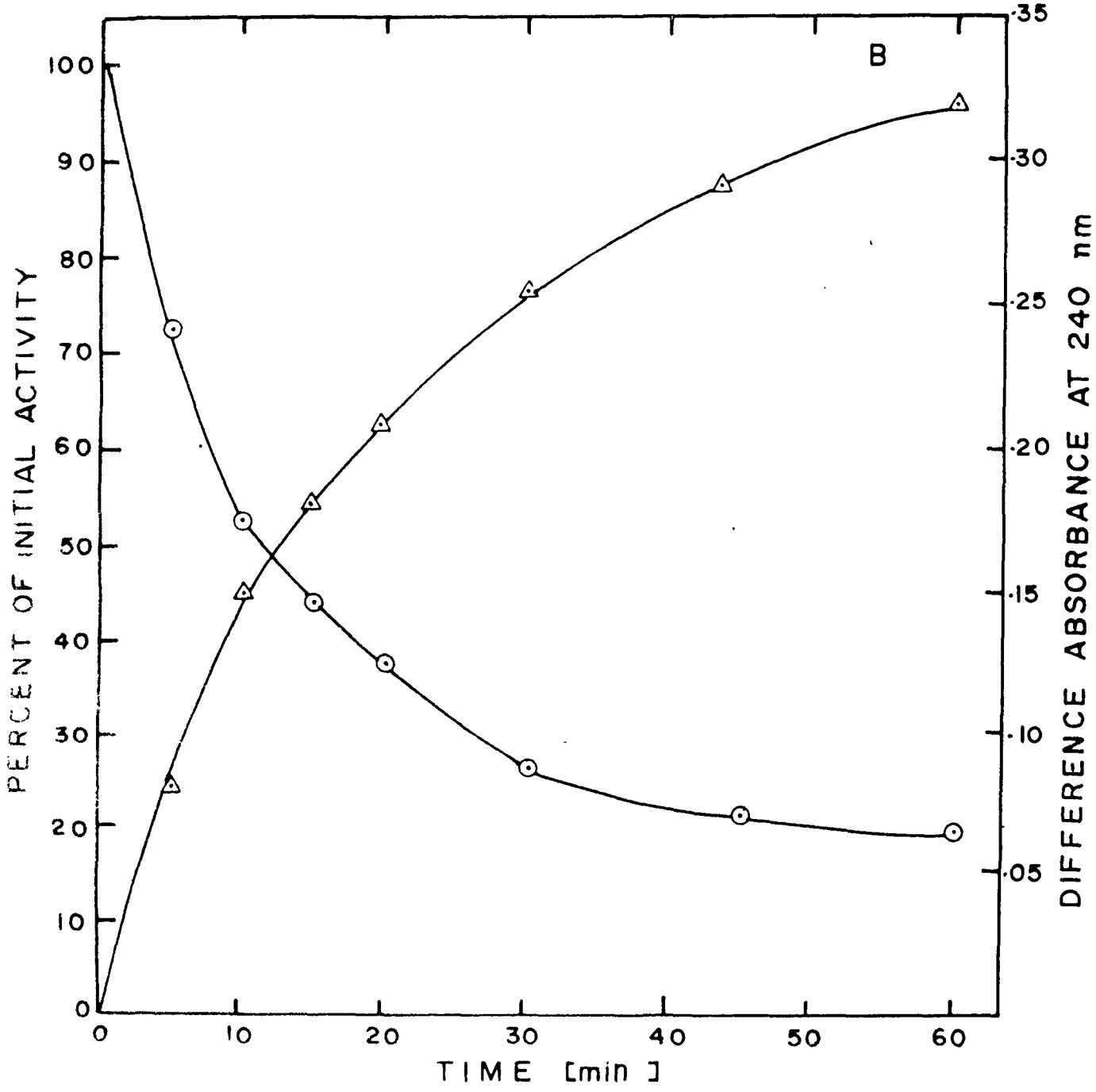
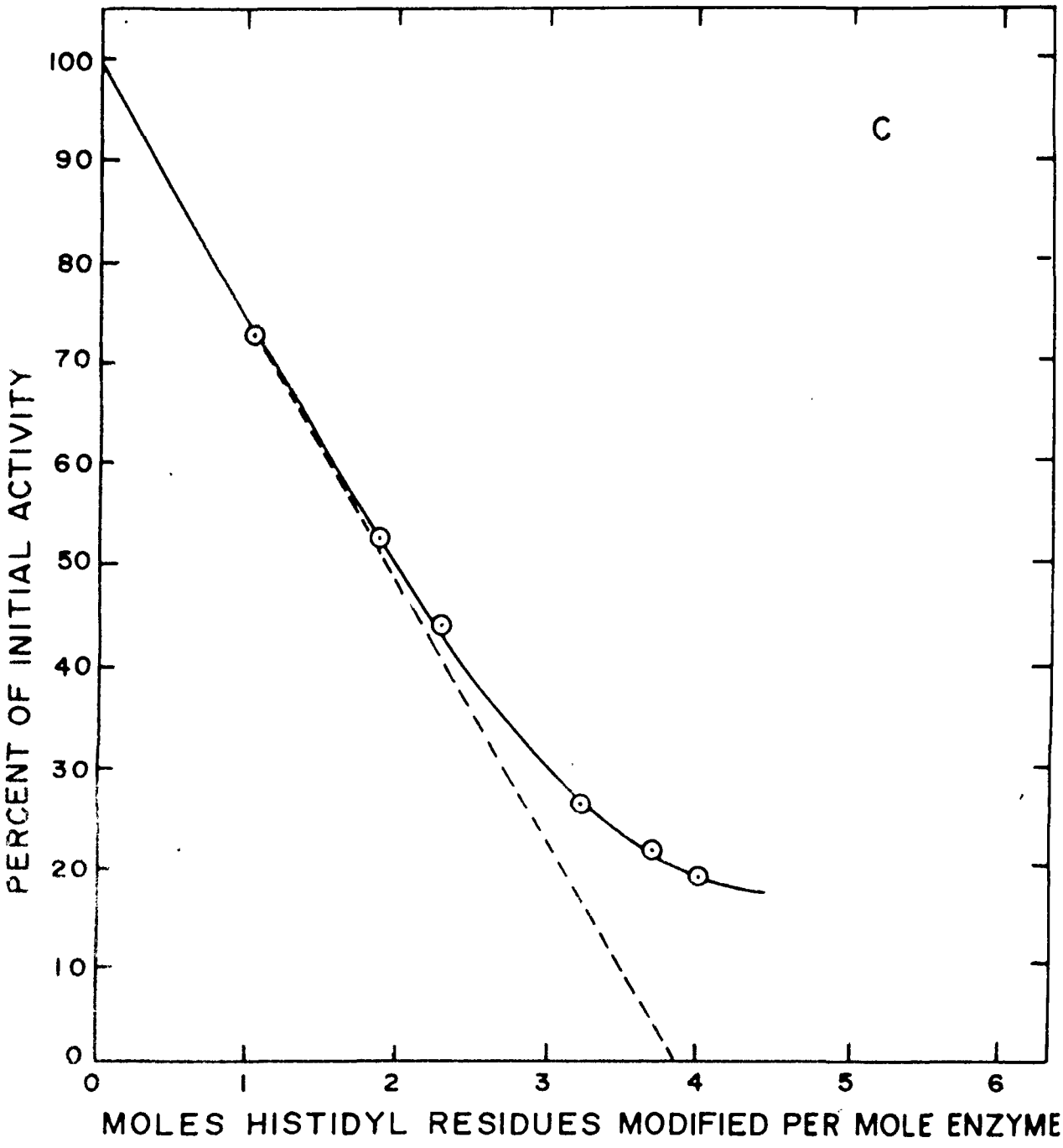

B
PERCENT OF INITIAL ACTIVITY
DIFFERENCE ABSORBANCE AT 240 nm
TIME [min]
0
10
20
30
40
50
60
100
90
80
70
60
50
40
30
20
10
0
·35
·30
·25
·20
·15
·10
·05

FIG. 12C Relationship between the number of histidyl residues modified and the nitrite reductase activity after treatment of the enzyme with 3 mM DEPC for various times.


C
PERCENT OF INITIAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
MOLES HISTIDYL RESIDUES MODIFIED PER MOLE ENZYME

Effect of hydroxylamine on reactivation of DEPC-inactivated enzyme

Melchior and Fahrney (1970) found that hydroxylamine can remove N-carbethoxy group from N-carbethoxyimidazole derivative at pH 7.0 in several minutes. There are several reports where hydroxylamine has been used to reactivate the DEPC-inactivated enzymes (Ovadi and Keleti, 1969; Melchior and Fahrney, 1970; Setlow and Mansour, 1970; Thome-Beau et al., 1971; Huc et al., 1971; Horiike et al., 1979).

Figure 13 shows that A. fischeri nitrite reductase which has 20% or more initial activity can largely be reactivated by treatment with 0.75 M hydroxylamine at neutral pH in 45 min. The enzyme which has been inhibited 50% or less is almost completely reactivated, while the enzyme having less than 50% residual activity is not fully reactivated. For reactivation process, potassium phosphate and Tris-HCl buffers, were tried at the same pH values. Both the buffers gave similar results.

Effect of substrate on inactivation of nitrite reductase by DEPC

A range of nitrite (substrate) concentrations was used to determine if there was any protection in the inactivation of nitrite reductase by DEPC. Figure 14 illustrates that nitrite considerably protects the enzyme from inhibition by DEPC. The maximum protection by nitrite was obtained at a concentration of 5 mM or above; 2 mM nitrite provided some protection against inactivation. There was little or no protection of the inactivation process by 0.2 mM or less nitrite.

FIG. 13 Effect of hydroxylamine on the reversal of DEPC-inactivated A. fischeri nitrite reductase. (⊙—⊙) Enzyme treated with 3 mM DEPC. Aliquots of the enzyme, treated with DEPC were removed at various times and treated with hydroxylamine for 45 min (●—●).

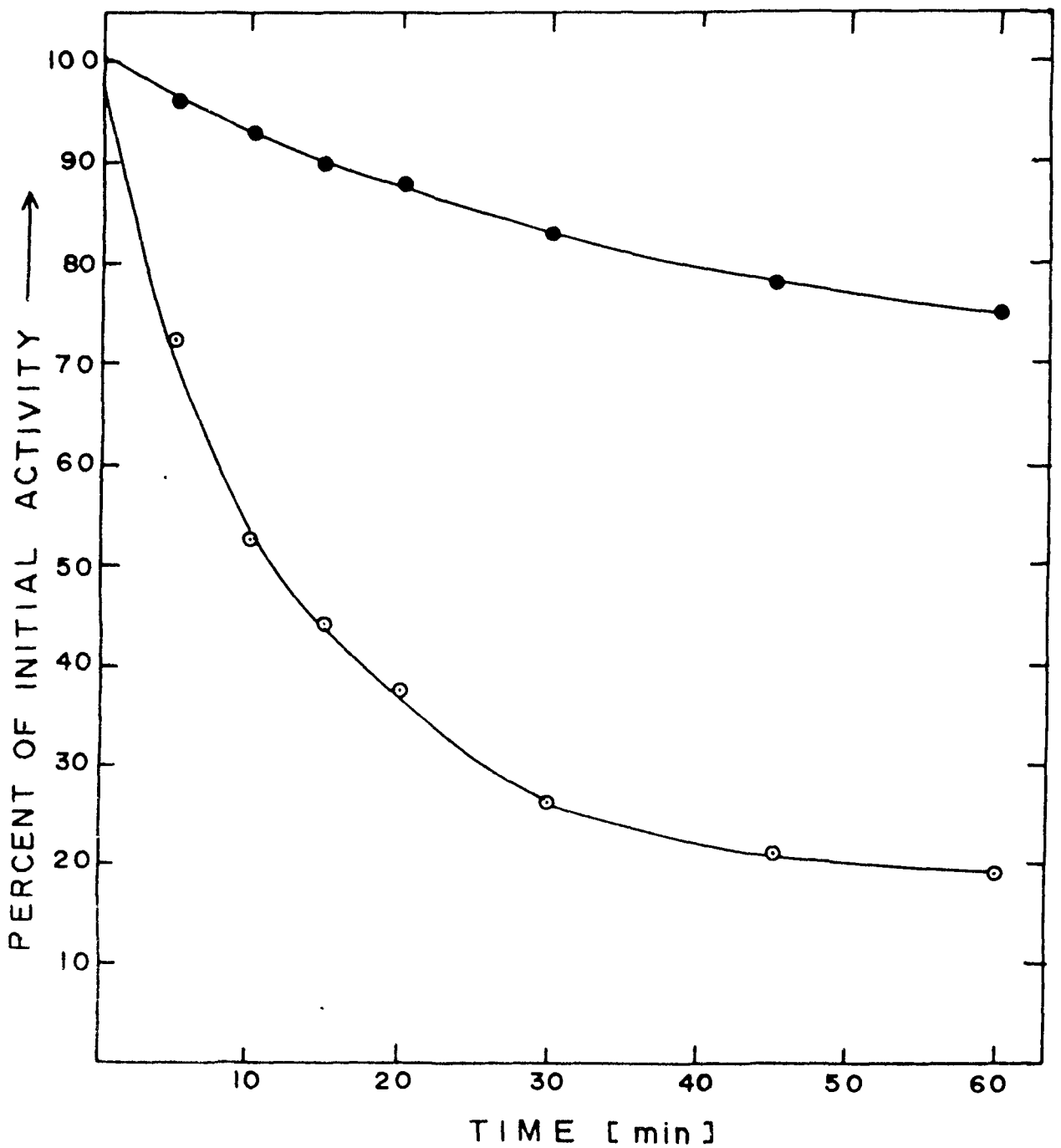
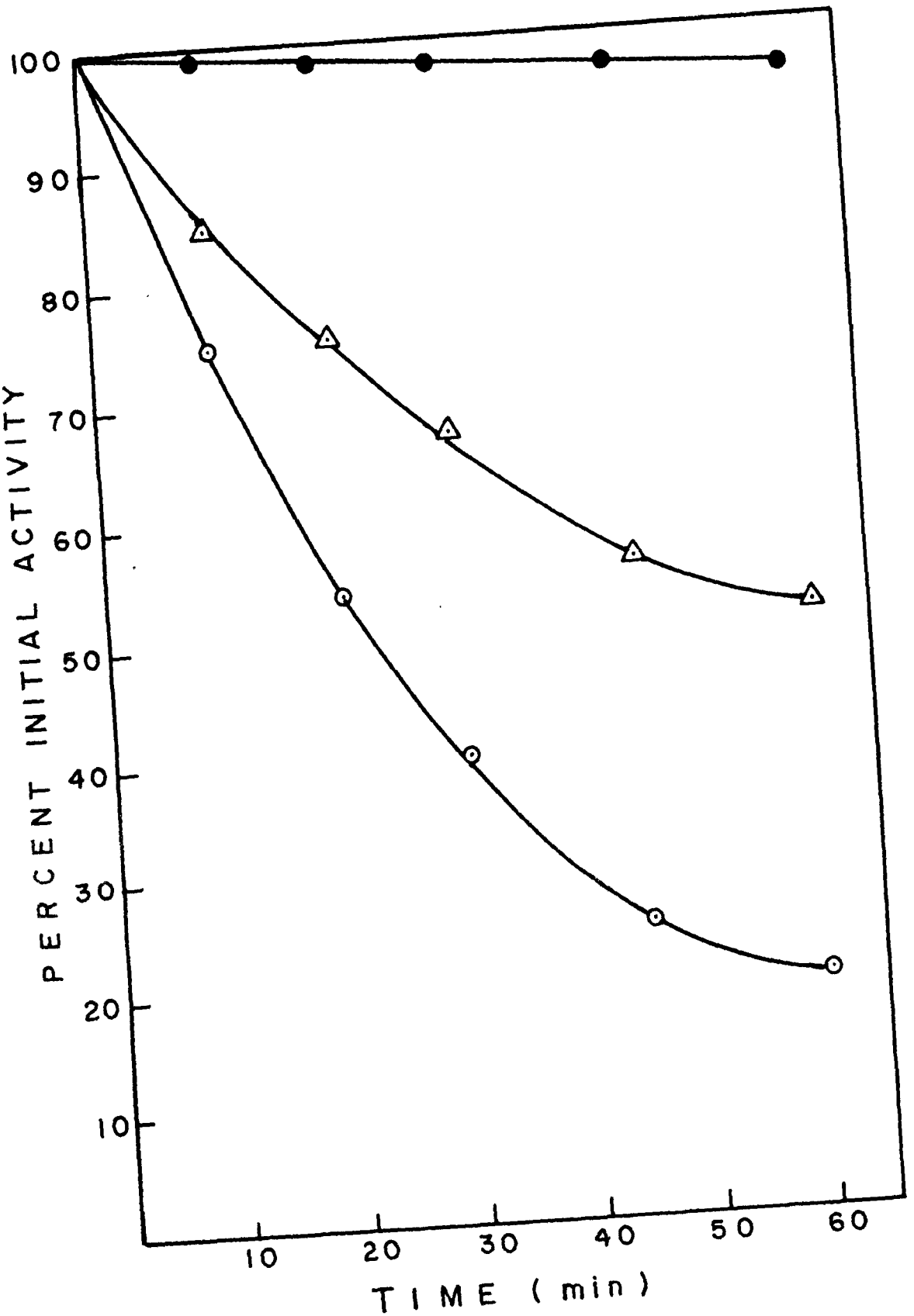

100
90
80
70
60
50
40
30
20
10
PERCENT OF INITIAL ACTIVITY
10
20
30
40
50
60
TIME [min]

FIG. 14 Effect of substrate (nitrite) on inactivation of *A. fischeri* nitrite reductase by DEPC.

●——● Enzyme treated with 3 mM DEPC in presence of 5 mM nitrite.

△——△ Enzyme treated with 3 mM DEPC in presence of 2 mM nitrite.

⊙——⊙ Enzyme treated with 3 mM DEPC in absence of nitrite.


100
90
80
70
60
50
40
30
20
10
PERCENT INITIAL ACTIVITY
10
20
30
40
50
60
TIME (min)

Effect of DEPC on sulfhydryl groups modification

In order to determine if DEPC reacted with free sulfhydryl groups (Muhlard et al., 1967; Melchior and Fahrney, 1970), the total number of free sulfhydryl groups per mol of the enzyme was determined by spectrophotometric titration of the DEPC-treated and untreated enzyme with p-HMB (as described in Methods). Figure 15 represents results which are typical of several experiments performed in the presence of 8 M urea. It was observed that both untreated and treated enzyme having 20% of initial activity contained 3.8 sulfhydryl groups per mole of the enzyme.

Effect of pH on kinetic parameters

In order to obtain additional proof of the involvement of essential histidine residues for the activity of the enzyme, the dependence of the kinetic parameters, Michaelis Menten constant (K_m) and maximum velocity (V_{max}) of the nitrite reductase, on pH was studied. The pH dependence of K_m and V_{max} of the enzyme are shown in Fig. 16. According to Dixon and Webb (1964), the observed pH effects can be interpreted in terms of the pH values of the groups situated in the free enzyme, or in the free substrate, and in the enzyme substrate complex.

The pK_m-pH curve shows a group of two pK values, 6.9 and 7.1 forming a wave. This could correspond to the same group of the enzyme, the ionization of which is affected by substrate binding so that its pK is decreased in the enzyme substrate complex. Further examination of all the three curves shows that there are two ionizable groups showing pK values around 7.0

FIG. 15 Determination of sulfhydryl groups of DEPC-treated enzyme by spectrophotometric titration with p-HMB.

The intercept, a, is due to absorption of the protein.

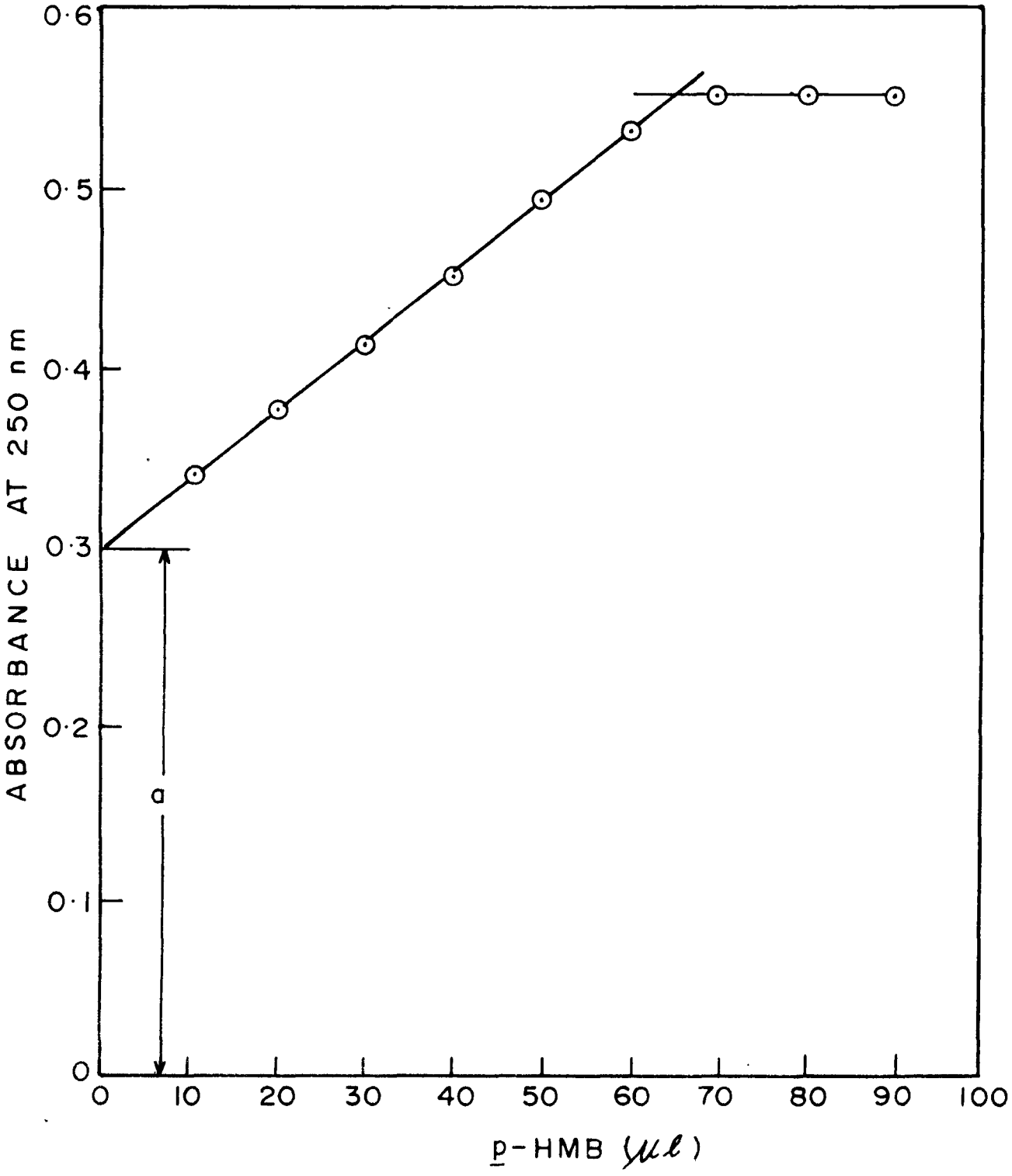
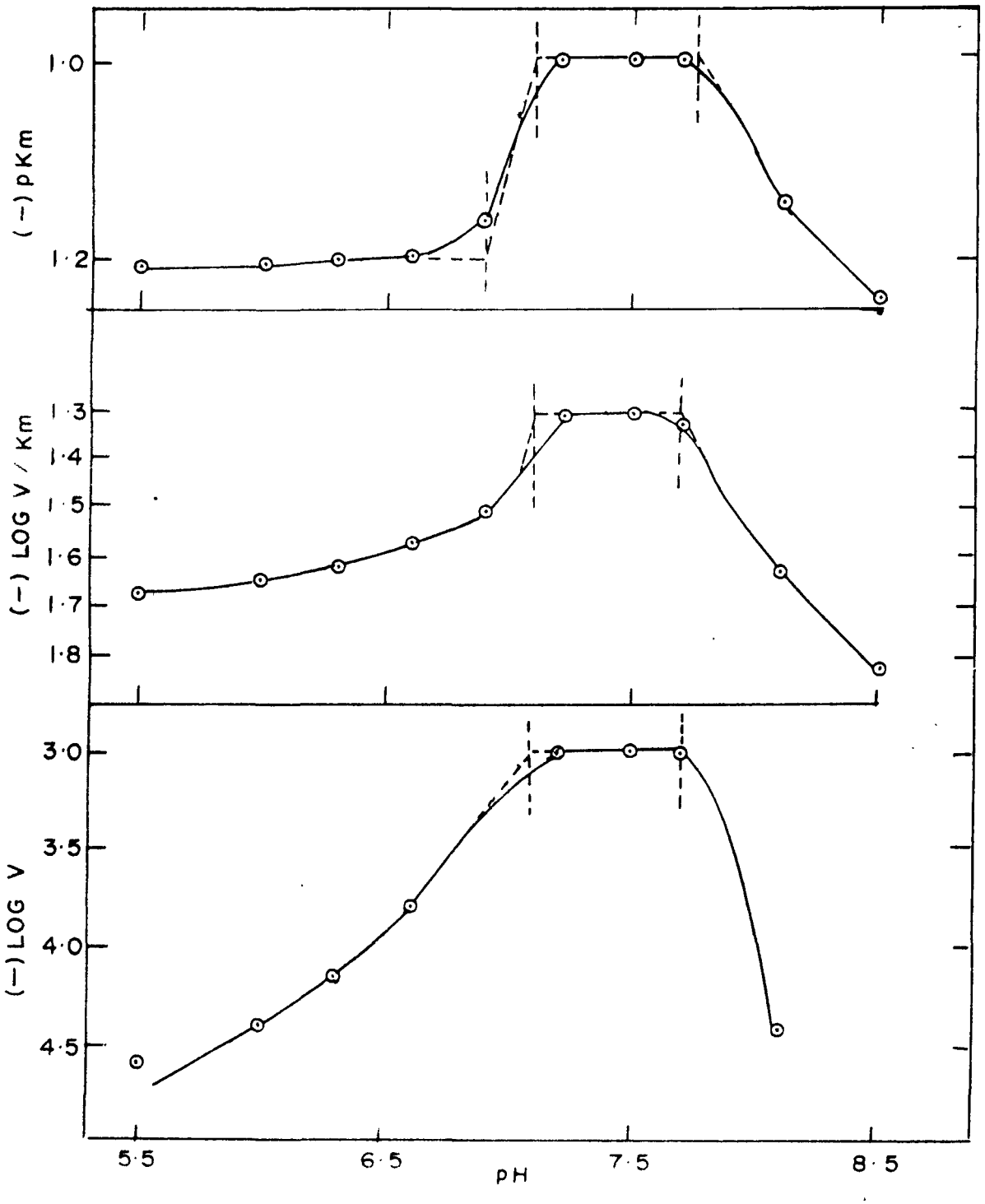

0·6
0·5
0·4
0·3
0·2
0·1
0
ABSORBANCE AT 250 nm
a
0
10
20
30
40
50
60
70
80
90
100
p-HMB (μl)

FIG. 16 Effect of pH on kinetic parameters K_m and V of A. fischeri nitrite reductase. Potassium phosphate buffer was used in the pH range 5.5 to 8.5. Kinetic parameters pK_m (- log K_m), log V and log V/K_m were plotted against pH according to the procedure of Dixon and Webb (1964).


(−) pKm
1·0
1·2
(−) LOG V / Km
1·3
1·4
1·5
1·6
1·7
1·8
(−) LOG V
3·0
3·5
4·0
4·5
5·5
6·5
7·5
8·5
pH

and 7.7. The pK 7.0 corresponds to the intrinsic pK of imidazole group, and is in agreement with the assumption that a histidyl residue is essential for the activity of the enzyme, and is involved either near or at the active centre of the enzyme. Another pK value of 7.7 is also observed in all the three curves. It can be explained either as an imidazole group whose pK is different due to the environment or of a sulfhydryl group. But it has already been stated that free sulfhydryl groups are not required for activity of the enzyme and are also not modified by DEPC. Therefore, both the pK values of 7.0 and 7.7 are assumed for the ionization of imidazole groups.

Number of histidyl residues essential for activity

Under the conditions of Fig. 12C, about four histidyl residues per molecule of the enzyme were modified for the complete inactivation of the enzyme activity. This number of four histidyl residues was obtained by extrapolating the initial linear portion of the plot to zero activity. This method does not usually give the number of residues essential for activity (Tsou, 1962; Horiike and McCormick, 1979).

The number of essential histidyl residues or the residues present at the active site are calculated from the slope obtained from the plot of the logarithm of the apparent first order rate constant, K_{app}, versus the logarithm of the reagent (DEPC) concentration (Fig. 11 inset) (Ramakrishna and Benzamin, 1981). The slope of the curve (n) with respect to DEPC was determined to be 0.9. It suggests that loss of enzyme activity results from reaction of only one histidyl residue per molecule of the enzyme.

DISCUSSION

The inactivation of A. fischeri nitrite reductase was studied using a histidine-specific reagent, diethylpyrocarbonate. This reagent was initially used by Rosen et al. (1966) to inactivate ribonuclease. Pradel and Kassab (1968) used the reagent to show the presence of histidine residues at the active site in creatine- and arginine kinases. Later on Ovadi and Keleti (1969) reported that DEPC reacts only with the histidine residues of glyceraldehyde-3-phosphate dehydrogenase. However, since that time, like many other group-specific reagents, it was shown to react with amino acid residues other than histidine. For instance, Melchior and Fahrney (1970) showed that DEPC can react with an active site serine of chymotrypsin and several amino groups of ribonuclease and only α— amino group of pepsin at pH values as low as 4.0. In addition, it has been shown to react with histidine and tyrosine residues in thermolysine (Burnstein et al., 1974). Despite these limitations, the reagent has been widely used as a specific modifier of histidine at or near pH 6.0 (Ovadi et al., 1967).

When nitrite reductase was treated with DEPC, the enzyme activity was lost. The spectrophotometric studies were conducted to confirm whether inactivation of the enzyme was due to specific interaction of DEPC with histidine residues or not. The difference spectrum between the carbethoxylated and the native enzyme showed a characteristic positive peak at 240 nm which is characteristic of N-carbethoxyimidazole

derivative (Muhlard et al., 1967; Ovadi et al., 1967; Melchior and Fahrney, 1970). The increase in absorbance at 240 nm is concomitant with the loss of enzyme activity. Inactivation of the enzyme activity is stoichiometric with modification of four histidyl residues per molecule of the enzyme.

The specific modification of the enzyme at histidine residue is confirmed by the following observations: The reversal of the enzyme inhibition by 0.75 M hydroxylamine at pH 7.0 rules out the possibility that inactivation of the enzyme was due to the acylation of some of the amino groups of the enzyme (Melchior and Fahrney, 1970). The primary amines (α-amino and ε-amino of lysine) do not constitute a logical binding site for nitrite as the reaction leads to the production of nitrogen and an alcohol rather than a stable bound intermediate. The restoration of enzyme activity by hydroxylamine appears to rule out lysyl and arginyl residues since the more stable amide-like structures formed, when they are carbethoxylated, are not readily reversed by hydroxylamine (Miles, 1977; Muhlard et al., 1967; Melchior and Fahrney, 1970; Horiike et al., 1979). The lack of absorbance changes in 280 nm region during the treatment of the enzyme by DEPC shows that tyrosine residues are not modified by the reagent (Muhlard et al., 1967). It has also been generally believed that lysine and tyrosine side chains do not in any case react with diethylpyrocarbonate below pH 7.0 (Dykes and John, 1977).

Diethylpyrocarbonate is known to react with sulfhydryl residues in model systems (Osterman-Golkar et al., 1974; Berger, 1975). Recently, DEPC has also been shown to react with sulfhydryl groups of β-D-xylosidase from B. pumilus (Hilderson et al., 1984). The possible modification of sulfhydryl residues of nitrite reductase was tested by estimating free sulfhydryl groups in the DEPC-treated and native enzyme. The content of the free sulfhydryl groups of the native and DEPC-treated enzyme was found to be same indicating that -SH groups have not been modified by the reagent.

During the reversal of DEPC-inactivated enzyme by hydroxylamine it was observed that if the enzyme is inhibited for longer times, than the reactivation is not complete. The conformational changes have been implicated by Thome-Beau et al. (1971) in the incomplete regeneration of DEPC-treated arginine oxygenase. If there had been conformational changes in nitrite reductase, it would possibly prevent the access of hydroxylamine modecules to the carbethoxylated histidine residues and there would be incomplete regeneration of the ethoxy-formyl enzyme.

Several examples of the successful use of substrate for preventing or reducing the inhibitory effect of DEPC on enzymes have been cited in literature (Wallis and Holbrook, 1973; Sato and Uchida, 1975; Holbrook and Ingran, 1973; Burnstein et al., 1974) providing evidence that a histidyl residue is a catalytic group or is located at the substrate

binding site. The inactivation of nitrite reductase by DEPC is prevented by 5 mM (or more) nitrite. This provides further evidence that a histidyl residue is the catalytic group or is located at the substrate binding site.

A study of the pH dependence of the kinetic parameters, K_m and V_{max}, gave two main pK values, 7.0 and 7.7, and it is concluded that both the values correspond to imidazole group (Koshland, 1960). The difference in the two pK values may be explained on the basis of different environment of the two imidazole groups. Recently, Bosshard et al. (1984) have shown a histidyl residue with a pK = 8.0. Since K_m varied with pH and was greater on both sides of the optimum pH, apparently the ionizable groups in the free enzyme control the binding of the substrate (Dixon and Webb, 1964).

Though four histidyl residues have been shown to be modified for the complete inactivation of the enzyme (calculated from the difference absorbance spectra), only one histidine residue is shown to be essential as shown by the inactivation reaction order (n). On the basis of kinetic data and the inactivation studies it has been concluded that the enzyme contains one catalytically essential histidyl residue.

Because of the limited stability of ethoxyformyl histidine residues (Holbrook and Ingram, 1973) it has not been possible to isolate the peptide containing the modified histidine residue and to identify the specific histidyl residues whose modification is responsible for loss of enzyme activity.

CHAPTER VI

STUDIES ON (a) THE PHYSIOLOGICAL ELECTRON DONOR OF *A. fischeri* NITRITE REDUCTASE AND (b) INTERMEDIATES DURING ENZYMATIC REDUCTION OF NITRITE TO AMMONIA

CHAPTER VI

SECTION 1

ELECTRON PARAMAGNETIC RESONANCE STUDIES OF HEME C AND ITS NITROSYL DERIVATIVE IN *A. fischeri* NITRITE REDUCTASE AND MECHANISM OF NITRITE REDUCTION

SUMMARY

Low temperature EPR spectra of purified *A. fischeri* nitrite reductase have been presented in this Chapter. The spectrum of the oxidized enzyme is extremely complex and showed signals due to several species of heme. The main features, which were present in all samples of the enzyme, were at g-values 3.7, and 2.88, 2.26 and 1.51. The species at g-value 3.7 is attributed to the active heme species of the enzyme. The species giving this signal was unreactive towards the substrate, nitrite and ligands, CN^- and azide in the oxidized state of the enzyme. Reduction of the enzyme in the presence of excess nitrite produced the spectrum of nitrosyl heme derivative detectable by EPR spectroscopy with a g-value at 2.01, and a ^{14}N hyperfine splitting into three lines separated by 1.6 mT and ^{15}N splitting into two lines separated by 2.3 mT. A similar derivative was observed on treatment of the enzyme with hydroxylamine. Exchange of nitric oxide was observed between the ^{15}N-nitrosyl-heme derivative and ^{14}NO gas.

It is proposed that the reaction cycle involves reduction of the enzyme followed by binding of nitrite to heme 1 and formation of the nitrosyl intermediate. The data suggest that the nitrosyl heme is an intermediate in the reduction of nitrite to ammonia. It seems likely that a bound form of hydroxylamine is also involved in the reduction of nitrite to ammonia.

GENERAL INTRODUCTION TO EPR THEORY

Electron paramagnetic resonance technique is based on the magnetic properties of the matter, more precisely of the electron. The EPR spectroscopic studies have been used in the area of enzyme chemistry concerned with electron transfer, the field of enzymic oxidation-reduction, where compounds with unpaired electrons or transition metals with unfilled electron shells may arise or disappear. However, the usefulness of EPR spectroscopy is not confined to oxidation-reduction; it can also furnish information on changes in the ligand environment of transition metal ions.

A paramagnetic substance may be defined as one that possesses no resultant magnetic moment in the absence of an external field but acquires a magnetic moment in the direction of an applied field. Paramagnetic substances are metal ions with incomplete 'd' shells like those of transition series, free radicals or certain molecules with unpaired electrons.

The electron has a negative charge and a quantum mechanical property that is analogous to spinning on its axis. For a single unpaired electron spin S is 1/2, so there are only two possible spin states, which we can consider as spins in opposite directions. The value that S takes along a specified direction (Ms) is then + 1/2 or - 1/2. A moving charge gives rise to a magnetic moment, and hence the spin of each electron leads to two possible magnetic states. The two orientations have two different energy i.e. each energy state of the electron is split into two parts by the application of an external magnetic field, this is known as Zeeman splitting.

The amount of splitting depends on the strength of the magnetic field. The energy difference (ΔE) between the two spin states is proportional to the applied field H, the value for the magnetic moment of the electron β and another constant g. Thus the basic equation for transition between the two energ levels for unpaired electron is $\Delta E = h\nu = g\beta H$. In principle, resonance absorption by an unpaired electron can occur whenever the ratio of the frequency of exciting energy to the magnetic field strength satisfies the condition, $\nu/H = \beta g/h$. It is this net absorption of electromagnetic energy at resonance that is detected and amplified in electron paramagnetic resonance spectroscopy.

The basic principles of EPR are thus analogous to those of NMR, both being magnetic resonance techniques. However, since the resonance frequency of an unpaired electron is about 658 times larger than that of the commonly used nuclei like the proton in NMR, the NMR experiments are carried out in the megahertz (MHz) range, while EPR experiments require frequencies in the gigahertz (GHz) range.

g-value

The total magnetic moment which is due to the coupling of the electron spin angular momentum and the orbital angular momentum of the unpaired electron gives rise to the tensor 'g'. The g-value is most basic parameter that characterizes an EPR spectrum. At resonance, the magnetic field at the unpaired electron will be resultant of the externally applied magnetic field and the internal magnetic field which it induces within the molecule. Thus in a molecule the value of 'g' will be

altered from that of the free electron spin. The g-value is partly dependent on the polarity of the solvent, but mainly on the orientation of the molecule in the magnetic field. There may be as many as three values for 'g', one for each of the three mutually perpendicular molecular axes of the paramagnetic center in its molecular coordinating system (i.e. g_{xx}, g_{yy}, g_{zz}). In an isotropic system with fast molecular motion, the measured g-value is the average of the g-values along the three molecular axes.

Nuclear hyperfine splitting

Apart from the local fields induced due to the application of the external magnetic field, there are local fields that are permanent and generated by the magnetic moments of nuclei within the same molecule. The interaction between the unpaired electron and the nuclear magnetic moment is termed as the nuclear hyperfine splitting. The EPR spectrum is thus split into a number of lines separated by hyperfine splitting constant A. As in the case of g-values, the value of A depends on the orientation of the molecule with respect to the applied magnetic field giving rise to A_x, A_y, A_z. In biological systems, nuclei with magnetic moments include nitrogen (^{14}N), hydrogen (^{1}H) and deuterium (^{2}H) and carbon (^{13}C) as well as nuclei of the transition metals. The number of possible values of magnetic moment is $2I + 1$ where I is the spin state of nucleus. So if I is 1 (as for ^{14}N) there are three possible spin states (Mi = 1, 0, -1), which lead to three lines in the EPR spectrum. If the unpaired electron interacts with more than one nucleus

a more complex spectrum is obtained.

EPR spectroscopic studies on nitrite reductases

Nitrite reductases from various sources contain transition metals, Fe and Cu. The EPR spectroscopy has been used to understand the mechanism of nitrite reduction to ammonia and to determine the prosthetic groups of the enzymes from various sources.

In the plant nitrite reductase the substrate nitrite and other nitrogenous intermediates of the reaction are presumed to bind to the siroheme and the function of the iron-sulfur center is presumed to be the transfer of electrons to them (Aparicio et al., 1975; Hucklesby et al., 1976; Vega and Kamin, 1977; Cammack et al., 1978). An electron paramagnetic resonance (EPR) signal assigned to nitrosyl-Fe (II) complex of siroheme (Aparicio et al., 1975; Fry et al., 1980) was observed in samples of the nitrite reductase enzymes frozen during turnover (Cammack et al., 1978). This complex was presumed to present the first stage in the reduction of nitrite to ammonia. EPR studies of spinach and E. coli nitrite reductases have shown the presence of a strong magnetic interaction between the siroheme and Fe_4S_4 centers (Janick and Siegel, 1983; Wilkerson et al., 1983). It has also been shown that heme - Fe_4S_4 interaction is maintained on ligation of the heme by a number of compounds. Evidence has also been presented which shows that potential weak field heme ligands can promote interconversion of the S = 1/2 and S = 3/2 type EPR signals characteristic of the exchange coupled heme - Fe_4S_4

center in fully reduced NADPH-sulfite reductase hemo-flavo-protein complex subunit of E. coli (Janick and Siegel, 1983).

Nitrite reductase from Desulfovibrio desulfuricans has been purified and shown to contain six c-type heme groups per molecule of the enzyme rather than siroheme and Fe_4S_4 center which are the prosthetic groups of plants and E. coli nitrite reductases (Liu and Peck, 1981; Murphy et al., 1974b; Siegel, 1978; Lancaster et al., 1979). EPR studies have shown that nitrite reductase from D. desulfuricans also reacts with nitrite to form heme-NO complex, but only a fraction of the c-type hemes reacted with nitrite. In contrast, all the hemes reacted when exposed to NO (Liu et al., 1980). These observations suggested some specificity among the six hemes in their reactivity towards nitrite.

Nitrite reductase from A. fischeri catalyzes the six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). It contains two c-type hemes per molecule of the enzyme, but no non-heme iron or molybdenum (Prakash and Sadana, 1973). The intermediates in the reduction of nitrite to ammonia by nitrite reductase are unknown. The A. fischeri enzyme catalyzes the reduction of both nitrite and hydroxylamine to ammonia and the two substrates are reduced at the same catalytic site (Prakash and Sadana, 1972). The K_m for hydroxylamine, however, is approximately two orders of magnitude greater than that for nitrite (Prakash and Sadana, 1972). The high K_m value seems to preclude hydroxylamine as a free intermediate in the reaction. The same conclusion has also been drawn by us (Sadana et al., 1981), when we tried to trap hydroxylamine

in the form of oxime and were unable to detect it by GC/MS method.

In this Chapter EPR spectroscopic studies were carried out with A. fischeri nitrite reductase for detecting the intermediate and to understand the mechanism of nitrite reduction.

METHODS

Sample preparation for EPR spectroscopy

Except where otherwise stated the enzyme was used at a concentration of approximately 5 mg/ml in 30 mM potassium phosphate buffer, pH 7.5.

Samples for EPR spectroscopy were prepared in quartz tubes of 3 mm internal diameter (Varian Associates, Palo Alto, CA, USA). For experiments in reducing conditions the tubes were flushed with Ar gas through a stainless steel catheter. Reagents such as sodium nitrite, methyl viologen and dithionite were added with 10 µl syringes, fitted with 15 cm needles, while stirring vigorously with a stainless steel wire. Mixing, where it could be observed by mixing of coloured solutions, appeared to be complete within 3 seconds. The enzyme solution for preparation of the nitrosyl derivative and turnover experiments were prepared in oxygen-free 25 mM potassium phosphate buffer, pH 6.8 and kept in stoppered vials under a flow of Ar gas. The samples were frozen by immersing the tubes in a mixture of 2-methyl-butane and methylcyclohexane (6:1 v/v) cooled to approximately 100K with liquid nitrogen. The freezing time for tubes already at 0°C was approximately 2 sec.

EPR spectra were recorded on a E4 spectrometer (Varian) with an ESR 9 liquid-helium transfer system (Oxford Instrument Co., Osney Mead, Oxford, UK) for sample cooling. g-Values were referred to 1, 1-diphenyl-2-picrylhydrazyl as standard. Spectral subtraction was recorded with a [illegible] averaging system.

RESULTS

Spectra of the oxidized enzyme

The EPR spectrum of the A. fischeri nitrite reductase is extremely complex (Fig. 17) and shows signals due to several species of heme, as well as signals at $g = 4.3$ and $g = 2.1$ which probably represent minor contaminants of non-heme iron and copper, respectively. The signal at $g = 4.2 - 4.3$ was also observed by Bray et al. (1964)and was relatively sharp and easily detected. It is of ubiquitous occurrence in biological materials and is often ascribed to impurities. A $g \approx 4.3$ line is characteristic of several non-heme, high-spin, ferric iron complexes. For example, it is found in iron complexes of conalbumins and transferrins (Aasa et al., 1963; Windle et al., 1963), in rubredoxin (Lovenberg, 1967; Bachmayer et al., 1967). The signal at $g = 6$ is typical of high-spin ferric heme. Its intensity varied between samples and did not correlate with enzymic activity. This signal is not due to active enzyme but might be a denatured form. Morton and Bohan (1971) have observed that in the lyophilization process some of the molecules of horse heart ferricytochrome c were

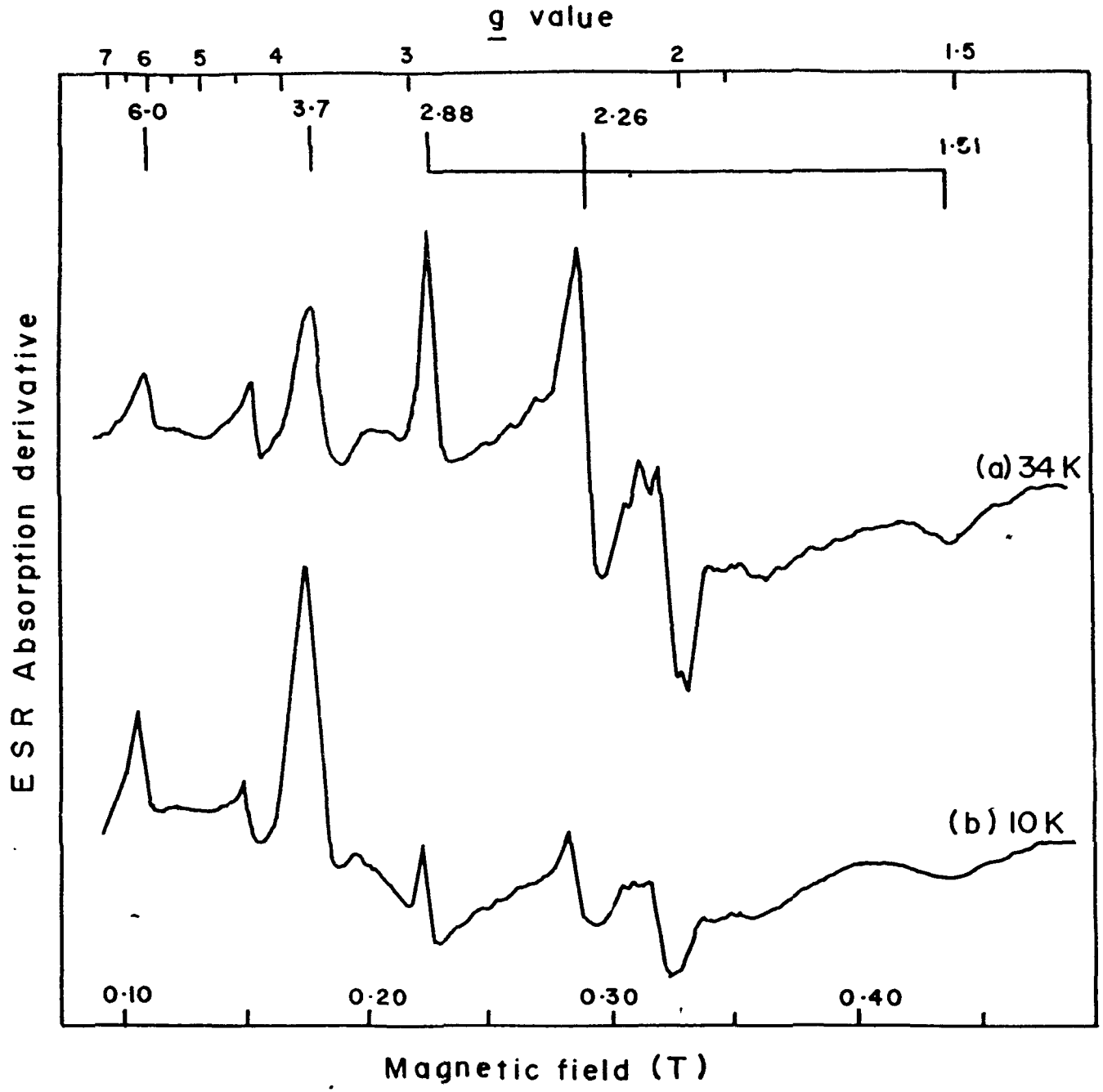
FIG. 17 EPR spectra of the oxidized *A. fischeri* nitrite reductase, approximately 5 mg/ml in 25 mM potassium phosphate buffer, pH 6.5, recorded at (a) 34K (b) 10K

Instrument settings:

microwave power, 2 mW

frequency, 9.25 GHz

modulation amplitude, 1 mT.


g value
7
6
5
4
3
2
1·5
6·0
3·7
2·88
2·26
1·51
(a) 34 K
(b) 10 K
0·10
0·20
0·30
0·40
Magnetic field (T)
E S R Absorption derivative

sufficiently distorted to convert the protein to a high-spin derivative giving a signal at g = 6. It can be presumed that some of the molecules of A. fischeri nitrite reductase might also have been distorted enough to give a high-spin derivative giving a signal at g = 6.

There remain two species in the spectrum of Fig. 17 which were present in all samples of the enzyme. The first, which we will refer to as heme 1, has a feature with a peak at g = 3.7, the rest of the spectrum was too broad to be detected. This feature was most prominent at 10K (Fig. 17 b). The second, which we refer to as heme 2, has a rhombic spectrum with g-values at 2.88, 2.26 and 1.51. These are most clearly seen at 34K at the microwave power used (Fig. 17 a); all of them changed together on reduction and other treatments. Both of these are consistent with low-spin ferric heme species, which is typical of many cytochromes c (Brautigan et al., 1977). Since there are two hemes per molecule of the enzyme it is possible that these are dissimilar and give rise to the two species. The alternative explanation is that one of the low-spin heme signals corresponds to the native enzyme, and the other to a denatured form. On treatment with dithionite-reduced methyl viologen or deazaflavin (photochemically-reduced), the signal at g = 3.7 was found to disappear rapidly while that at g = 2.88, 2.26 and 1.51 remained for upto a minute. This evidence indicates that the signal at g = 3.7 is more likely to represent the active heme species of the A. fischeri enzyme.

The g-value of 3.7 implies a large anisotropy (Palmer, 1979) for heme 1, but is within the range for low-spin ferric

cytochromes. For example, cytochrome b of the mitochondrial respiratory chain has g_z = 3.78 (DerVartanian et al., 1973). It is difficult to draw further conclusions about the ligation to the iron in the A. fischeri enzyme because only the g_z feature was observed, the other g-value features being presumably too broad to be detected. The occurrence of only one prominent g-value feature has also been observed for heme d in nitrite reductase of Pseudomonas aeruginosa (Walsh et al., 1979).

The EPR spectrum of the oxidized Achromobacter enzyme showed the same features at pH values 4.8, 7.0 and 9.0, although there were quantitative differences (Fig. 18). At pH 4 the linewidths of all the features were broader. At pH 9 the g = 6 signal grew while the g = 2.88, 2.26 and 1.51 spectrum was smaller suggesting a low-to high-spin transition. Effect of pH has been studied in a number of hemoproteins (Brautigan et al., 1977). It was shown that EPR spectra of these hemoproteins change when pH is varied from acidic to basic range. At extremes of pH, the horse and bakers' yeast iso-1 cytochrome display several high- and low-spin forms which have been identified, showing that a variety of protein-derived ligands will coordinate to the heme-iron including methionine- and cysteine-sulfur, histidine imidazole and lysine ϵ-amine.

The addition of 5 mM nitrite for 10 min at 20°C had no effect on the spectrum of the oxidized enzyme. It has been observed with other nitrite reductases, such as that from C. pepo and spinach. The reaction of the oxidized enzyme with nitrite is very slow (Cammack et al., 1978; Vega et al., 1976).

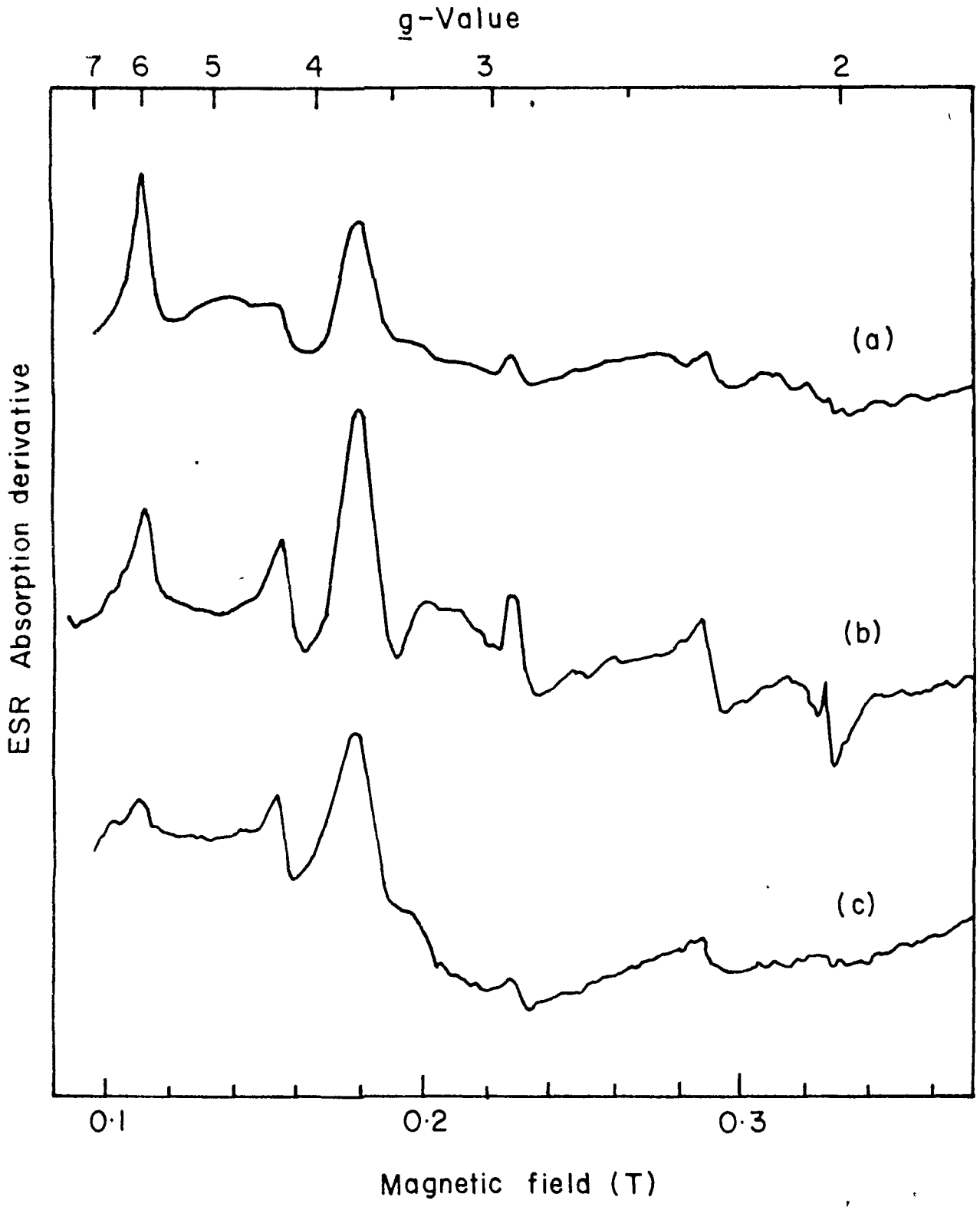
FIG. 18 EPR spectra of the nitrite reductase at (a) pH 9.0 (b) pH 7.0 (c) pH 4.8
Protein concentration approximately 5 mg/ml in 25 mM potassium phosphate buffer.
Conditions of measurement:

temperature, 8K

microwave power, 20 mW

frequency, 9.25 GHz

modulation amplitude, 1 mT.


g-Value
7
6
5
4
3
2
(a)
(b)
(c)
ESR Absorption derivative
0·1
0·2
0·3
Magnetic field (T)

Addition of cyanide, azide or fluoride at concentrations upto 50 mM to the A. fischeri enzyme also had no effect on the spectrum although the signal at $g = 6$ disappeared in the presence of cyanide. This is consistent with a heme in which a low field high-spin ligand such as H_2O is displaced by high field low-spin ligand such as CN^-. The oxidized protein appears to be unreactive to these ligands, which suggests that in the first step of the enzyme reaction, the heme is reduced before nitrite binds to the enzyme.

The enzyme is known to be inactivated by exposure to dithionite, so an attempt was made to reduce it with methyl viologen reduced by limiting quantities of dithionite. On treating the concentrated enzyme in deoxygenated buffer with 2 mM methyl viologen and 0.5 mM additions of dithionite, it was found to be impossible to keep methyl viologen in the reduced state. The blue colour of the reduced viologen disappeared within one or two seconds. Even when excess dithionite was added the colour disappeared within a short time.

EPR spectra of nitrosyl heme

Reduction of the enzyme with limiting amounts of dithionite-reduced methyl viologen in the presence of excess nitrite (^{14}N) induced the EPR spectrum shown in Fig. 19 (a) with a sharply-defined hyperfine splitting into three lines separated by 1.65 mT. This type of spectrum has been seen in a number of nitrosyl Fe (II) heme proteins (Palmer, 1979). The splitting is due to the nuclear spin ($I = 1$) of ^{14}N. A similar spectrum (not shown) was observed on treatment with nitric oxide gas.

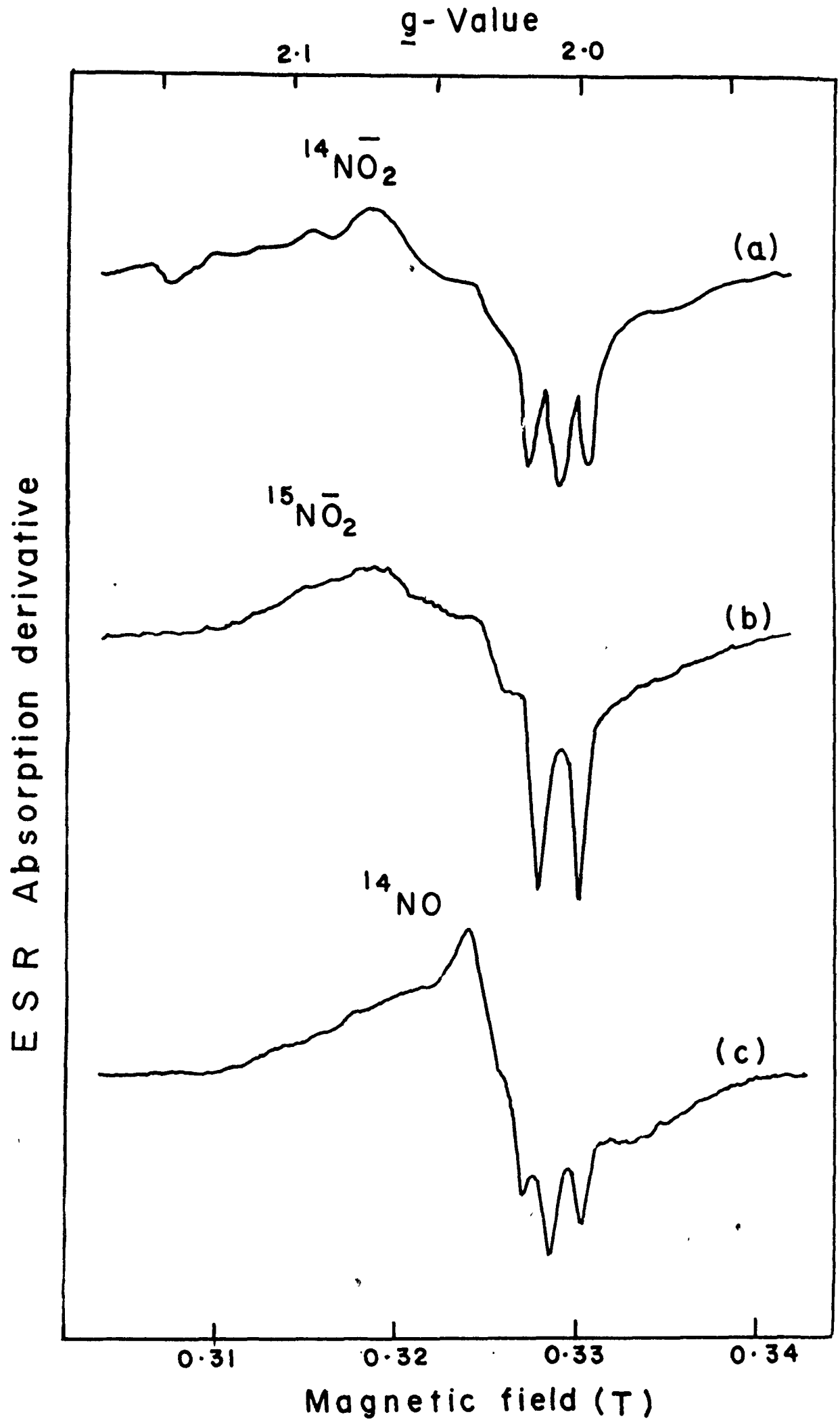
FIG. 19 EPR spectra of nitrosyl-heme nitrite reductase (a) approximately 5 mg/ml enzyme in 25 mM potassium phosphate buffer, pH 6.8 was treated for 1 min at 20°C with 5 mM $Na^{14}NO_2$, 2 mM methyl viologen and 1 mM $Na_2S_2O_4$. (b) as (a) but with $Na^{15}NO_2$ instead of $Na^{14}NO_2$. (c) The ^{15}N nitrosyl derivative, which was prepared as for (b), then passed through a small Sephadex G-25 column as described by Fry et al. (1980) and treated with ^{14}NO gas before freezing. Conditions of measurement:

temperature, 60K

microwave power, 20 mW

frequency, 9.25 GHz

modulation amplitude, 1 mT.


g- Value
2·1
2·0
$^{14}NO_2^-$
(a)
$^{15}NO_2^-$
(b)
^{14}NO
(c)
E S R Absorption derivative
0·31
0·32
0·33
0·34
Magnetic field (T)

An enzyme sample similarly reduced in the presence of excess $Na^{15}NO_2$ (Fig. 19 b) showed a splitting into two lines separated by 2.3 mT, as expected for the nuclear spin (I = 1/2) of ^{15}N.

The ^{15}N-nitrosyl derivative, prepared as for Fig. 19 (b), was passed through a column of Sephadex G-25 to remove excess nitrite, concentrated on an amicon concentrator B 15 (Amicon Corp. Lexington, MA 02773) and then treated with ^{14}NO gas as described by Fry et al. (1980). The EPR spectrum changed to a three-line hyperfine splitting due to ^{14}NO (Fig. 19 c). This indicates chemical exchange of NO in the nitrosyl complex. By contrast the nitrosyl complex of C. pepo nitrite reductase did not show this exchange indicating that it is kinetically more stable than in the A. fischeri enzyme (Fry et al., 1980). Exchange was not observed when the ^{15}N-nitrosyl A. fischeri nitrite reductase was treated, under non-reducing conditions, with $Na^{14}NO_2$ demonstrating that nitric oxide binds more tightly than nitrite.

When the nitrosyl derivative, prepared with nitrite and reduced methyl viologen, was treated with excess dithionite (5 mM) and the sample reoxidized by exposure to air, the spectrum with the characteristic three-line hyperfine splitting disappeared. This result suggests that the nitrosyl group was reduced, presumably to ammonia. This observation is consistent with the interpretation (though it does not prove) that nitrosyl derivative is an intermediate in the reaction cycle of the enzyme.

The nitrosyl heme spectrum was also observed, with decreased intensity, on treatment of the oxidized enzyme with hydroxylamine (Fig. 20). This could be explained by disproportionation of the hydroxylamine to ammonia and the enzyme-bound form of nitric oxide. Lancaster et al. (1979) and Hirasawa-Soga et al. (1982, 1983) have reported, with spinach nitrite reductase, the formation of nitrosyl heme complex on addition of hydroxylamine to the enzyme.

Spextra observed under turnover conditions

As previously noted, the EPR spectrum of the oxidized Achromobacter enzyme did not change when the substrate nitrite was added. However, it changed immediately when a reductant was also added so that nitrite reduction could proceed. In the experiment of Fig. 21, the reaction was started by addition of 10 mM dithionite to the enzyme solution containing 10 mM nitrite and 5 mM methyl viologen at 0°C and frozen within 10 seconds. All of the signals due to oxidized heme were greatly diminished (Fig. 21 b), and the signal near $g = 2$ appeared. This latter signal was saturated with microwave power when measured at 15K, and this part of the spectrum is shown in Fig. 22 (a) recorded on an expanded scale at 60K. It is clearly similar to the nitrosyl heme signal of Fig. 19(a). Therefore, under turnover conditions the heme c in the enzyme are reduced, and some nitrosyl heme is present. No such signals were observed with boiled enzyme under similar assay conditions.

In this experiment, the oxidizing substrate nitrite was

FIG. 20 EPR spectrum of A. fischeri nitrite reductase treated with 10 mM hydroxylamine for 10 min before freezing. Protein concentration, approximately 5 mg/ml in 25 mM potassium phosphate buffer, pH 6.8.

Conditions of measurement:

temperature, 60K

microwave power, 20 mW

frequency, 9.25 GHz

modulation amplitude, 1 mT.

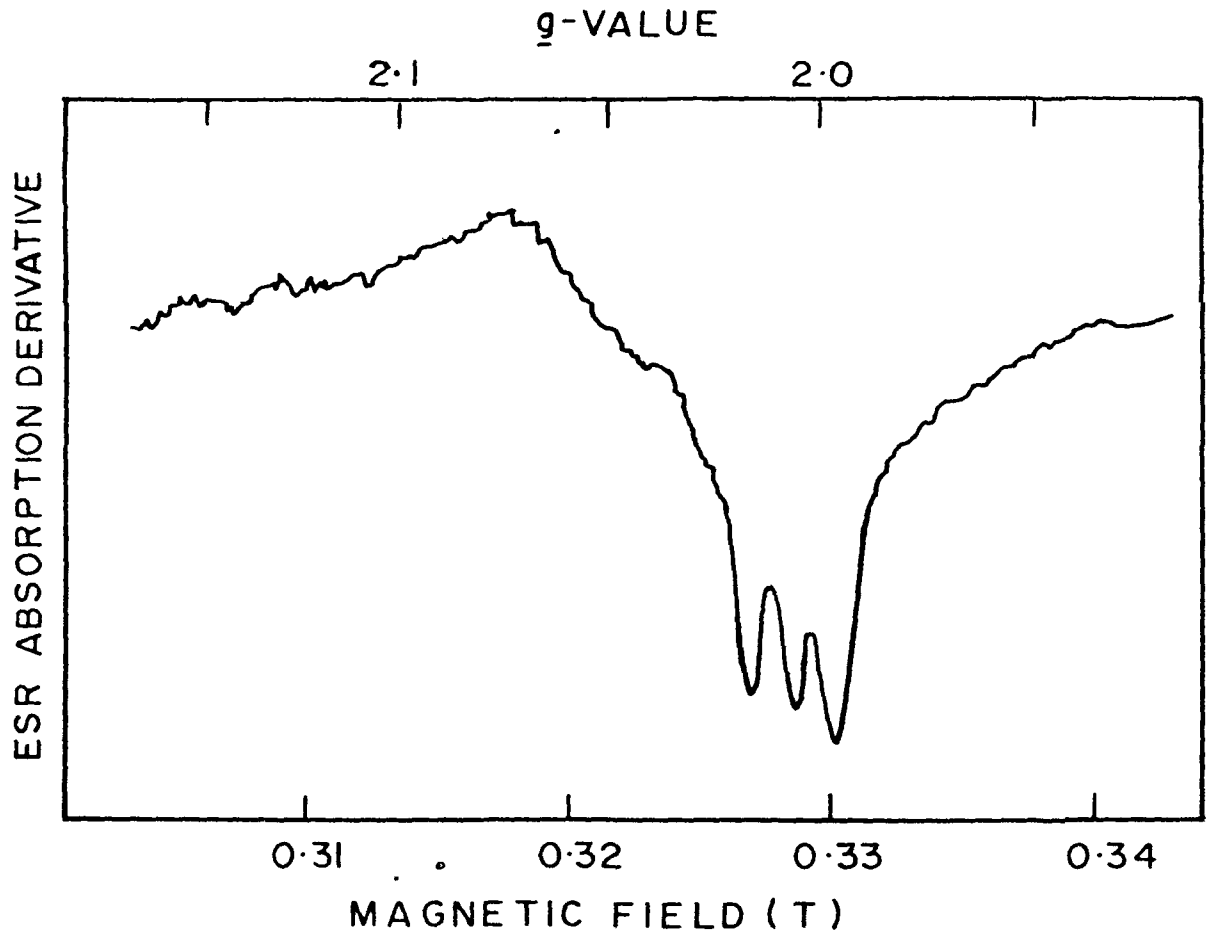

g-VALUE
2·1
2·0
ESR ABSORPTION DERIVATIVE
0·31
0·32
0·33
0·34
MAGNETIC FIELD (T)

FIG. 21 EPR spectra of A. fischeri nitrite reductase before, during and after reaction.

(a) Oxidized enzyme approximately 5 mg/ml in 25 mM potassium phosphate buffer, pH 6.8.

(b) A sample at 0°C containing enzyme, approximately 5 mg/ml, 10 mM $NaNO_2$, 5 mM methyl viologen, was frozen 10 seconds after addition of 10 mM $Na_2S_2O_4$.

(c) A similar sample at 20°C frozen after 5 min.

Conditions of measurement:

temperature, 15K

microwave power, 20 mW

frequency, 9.25 GHz

modulation amplitude, 1 mT.

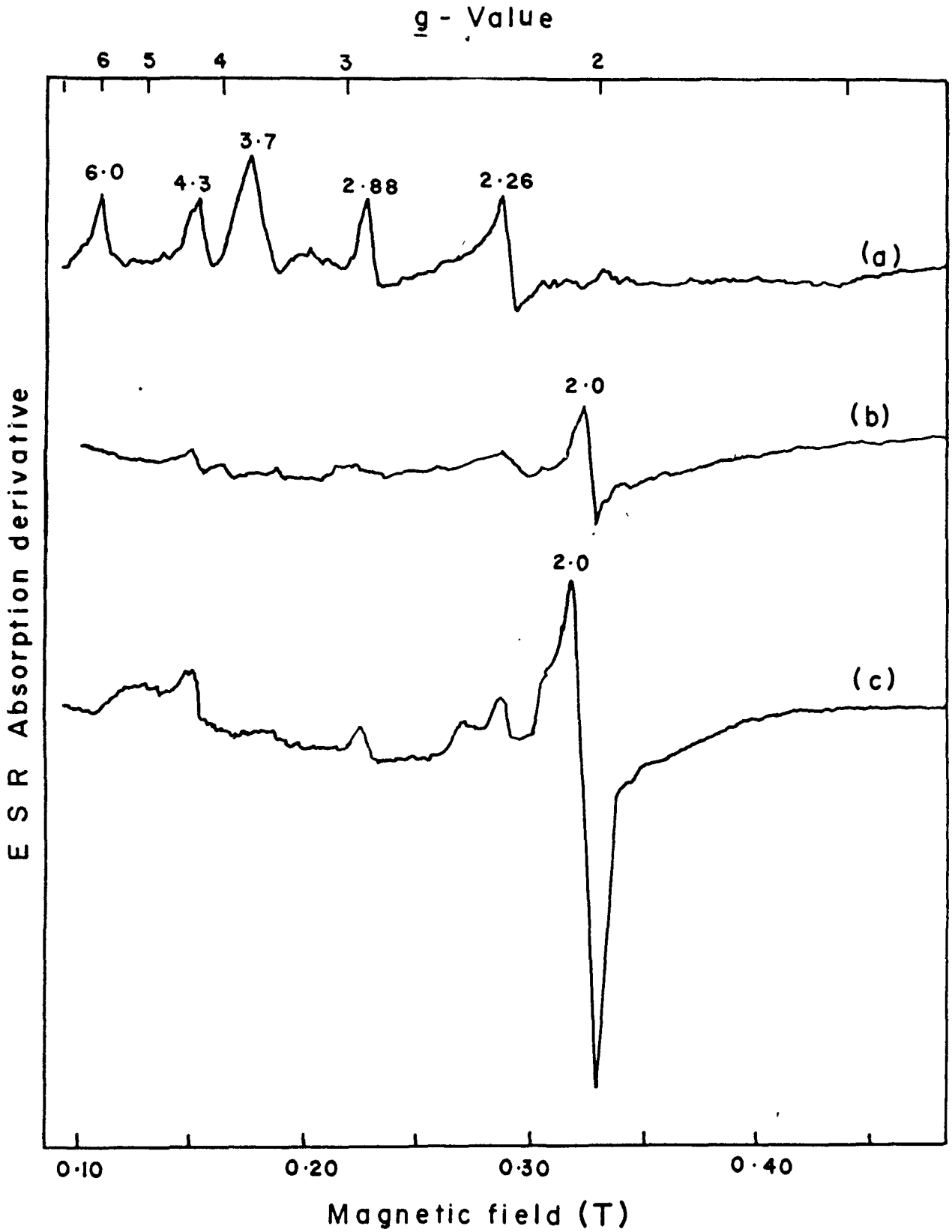
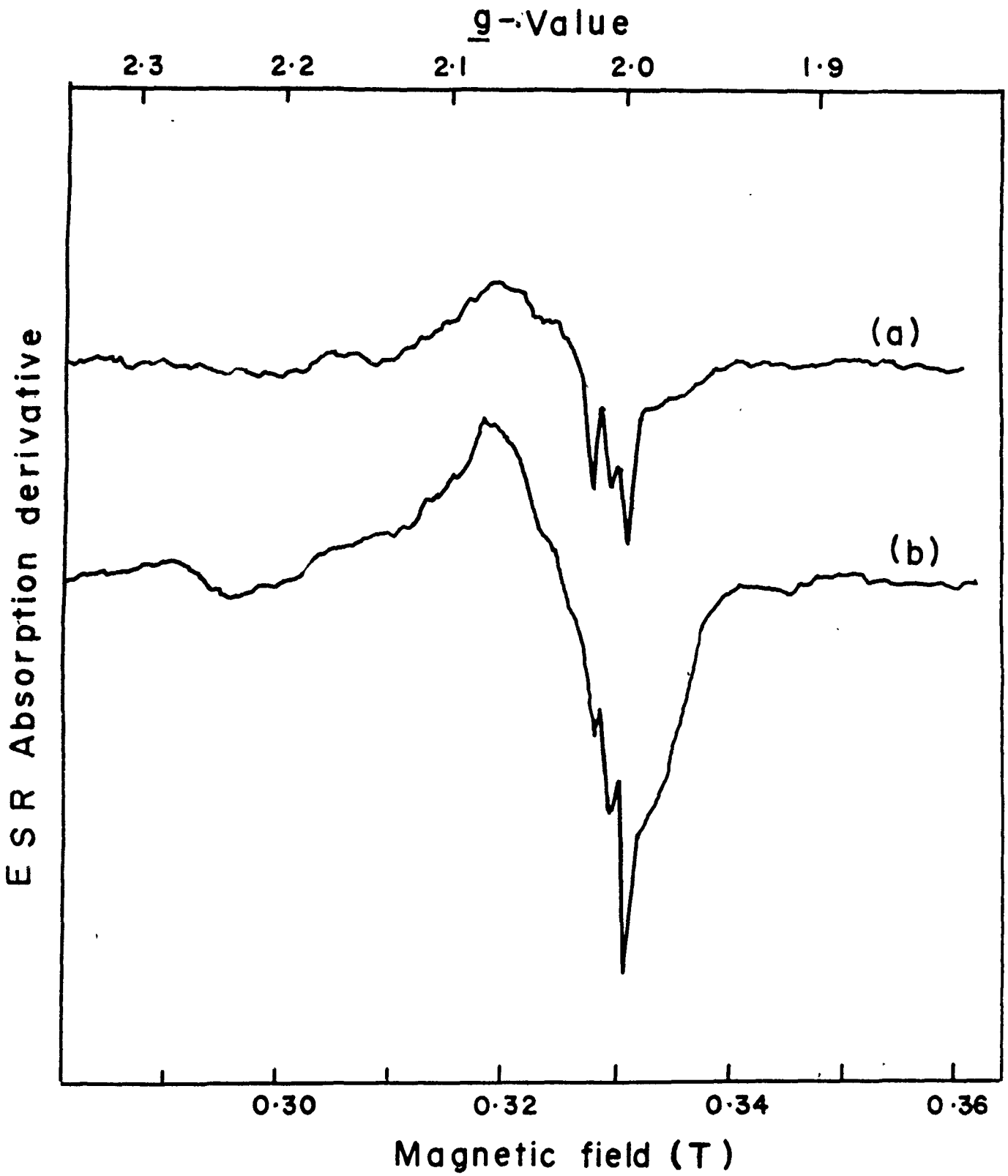

g - Value
6
5
4
3
2
6·0
4·3
3·7
2·88
2·26
(a)
2·0
(b)
2·0
(c)
E S R Absorption derivative
0·10
0·20
0·30
0·40
Magnetic field (T)

FIG. 22 (a) and (b) EPR spectra of the same samples as Fig. 21 (a) and 21 (b) respectively. Recorded at an expanded scale and at 60K.


g-Value
2·3
2·2
2·1
2·0
1·9
(a)
(b)
E S R Absorption derivative
0·30
0·32
0·34
0·36
Magnetic field (T)

present in excess over the reductant, dithionite. After incubating for 5 min and 20°C to ensure that reaction was complete, another sample was frozen for EPR spectroscopy. There was partial reappearance of the signals from oxidized heme 2 but not heme 1 (Fig. 21 c). The signal around $g = 2$ was also more prominent (Fig. 21 c and 22 b). This result indicates that it is heme 1 that forms the nitrosyl derivative, and it is therefore likely to be the active site of nitrite reductase.

DISCUSSION

An unusual feature of the spectra of oxidized *A. fischeri* nitrite reductase is that the heme iron is low spin. This implies that the heme iron is fully coordinated by the heme and two ligands from the protein. Presumably nitrite displaces one of them when it binds. By contrast the iron in the siro-heme-containing nitrite reductases is high-spin in the oxidized state (Cammack *et al.*, 1978; Lancaster *et al.*, 1979; Cammack *et al.*, 1982; Hirasawa-Soga *et al.*, 1982; 1983). The hemes *c* in *D. desulfuricans* nitrite reductase show a combination of high- and low-spin EPR signals (Liu *et al.*, 1980).

Hemoproteins *in situ* and in isolated form can exist in a variety of oxidation- and spin-states, each dependent upon immediate environment of the heme (Peisach *et al.*, 1968). The complex nature of EPR spectra of oxidized *A. fischeri* nitrite reductase may also arise due to change in the heme environment of some of the molecules of the enzyme. It has been observed that conformational changes of the protein

moiety give rise to changes in the EPR spectra (Peisach and Blumberg, 1969; 1971). The effect of pH on hemoproteins has been studied by Brautigan et al. (1977). They have reported that each of the hemoproteins displays upto four low-spin EPR forms that are in pH-dependent equilibrium and can all be seen at near neutral pH. As the pH is raised the predominant pH form is converted into two forms with $g = 3.4$ and $g = 3.6$. A.fischeri enzyme did not clearly show the conversion of one form to the another in the range of the pH studied.

Cammack et al. (1978) have reported that C. pepo nitrite reductase reacts with nitrite in the oxidized state which is detected by EPR detectable signals, but reaction with the oxidized enzyme is very slow, requiring several minutes at 0°C for complete disappearance of the signal at $g = 6.8$ and 5.0. It was thought that such a slow reaction could not be a part of the enzyme reaction under turnover conditions, and suggested that the heme must be first reduced to ferrous state, probably directly by ferredoxin. The EPR spectrum of A. fischeri enzyme in oxidized state did not change when nitrite was added. The enzyme was also unreactive towards CN^-, azide and fluoride. These studies indicated that in the first step of the enzyme reaction, the heme is reduced before nitrite binds. It seems that nitrite binds to the reduced form of the heme which is undetectable by EPR. This supports the hypothesis of Cammack et al. (1978) that heme must be reduced first before nitrite binds to the heme.

The spectrum of the nitrosyl derivative of A. fischeri nitrite reductase is different from that observed in the

siroheme-containing plant nitrite reductase, which shows rather clearly defined g-values at 2.073, 2.060, 2.007 with hyperfine splitting on the latter two g-values (Cammack and Fry, 1980). The spectrum of Fig. 19 (a) shows a prominent triplet splitting at g = 2.01. The other g-values are broad and poorly defined. It may be pointed out that with plants siroheme-nitrite reductase evidence for nitrogen hyperfine splitting was obtained by the use of ^{14}NO-^{15}NO difference spectrum (Fry et al., 1980) or by third harmonic EPR spectroscopy (Cammack and Fry, 1980). The spectrum of nitrosyl derivative of A. fischeri nitrite reductase (Fig. 19 a) shows a prominent triplet splitting at g = 2.01. The other g-values are broad and poorly defined. The unusual broadening of the EPR signals are tentatively attributed to an interaction between the low-spin ferric heme (spin = 1/2) and the ferrous-nitric oxide complexes as spin coupled induced broadening. Broadened metal-nitric oxide complexes arising from such postulated interactions have been reported by Uiterkamp and Mason (1973) and Uiterkamp et al. (1974).

According to the interpretation of Kon and Kataoka (1969) the feature at g = 2.01 corresponds to g_z, normal to the heme plane. The effect of ^{15}N substitution demonstrates that the splitting is due to binding of NO to heme iron. No superhyperfine lines in the g_z region related to the interaction of the iron with the proximal histidine are detected, suggesting a large distance between the metal and N_ε of the imidazole. Similar spectra have been observed in nitrosyl cytochrome P-420 (O'Keef et al., 1978) and nitrosyl hemoglobin in the presence of inositol-hexaphosphate (Rein et al., 1973; Hill et al., 1979)

as well as a number of nitrosyl-heme model complexes (Kon, 1975). It has generally been concluded in these cases that the bond between the iron and the proximal ligand (opposite the NO) is either weak or broken.

The nitrosyl heme spectrum observed after reaction with a relatively large amount of $Na_2S_2O_4$ (Fig. 22 b) is different from the others in having an additional broad component. Such heterogeneity in the spectra of nitrosyl derivative has been observed with other hemoproteins. For example the spectrum of nitrosyl hemoglobin consists of a broad featureless component and a component with a narrow hyperfine splitting. The ratio of these components depends on the pH and concentration of allosteric effectors (Rein et al., 1973). Therefore, the spectrum of Fig. 22 (b) might be due to one nitrosyl heme which can exist in two forms, or alternatively it might be due to two different states of the enzyme.

Although the composition of A. fischeri nitrite reductase is different from those from other sources, it is possible to propose a similar mechanism. Nitrite does not bind to the oxidized enzyme; so presumably the active site heme must be reduced first, which is probably heme 1. The nitrite appears to be reduced in several stages while it is bound to the heme. With the plant enzymes, the first stage appears to be a nitrosyl form (Aparicio et al., 1975; Cammack et al., 1978) and the data presented here are consistent with this possibility for the A. fischeri enzyme. On this interpretation, the observation that the nitrosyl form remains after reaction with reductant and excess nitrite, implies that the nitrosyl form

is a stable intermediate in the reaction cycle or precedes a rate-limiting step.

Hydroxylamine is a weak substrate for the enzyme, and treatment of the oxidized protein with it resulted in the formation of the stable nitrosyl derivative. Lancaster et al. (1979) and Hirasawa-Soga et al. (1982; 1983) have also reported the formation of nitrosyl heme complex on addition of hydroxylamine to the oxidized spinach nitrite reductase. As proposed for the plant nitrite reductase (Vega and Kamin, 1977) it seems likely that hydroxylamine is an intermediate in the reaction, in an enzyme-bound but not a free form. Prakash and Sadana (1972);Sadana et al. (1981) have also reported that hydroxylamine is an enzyme-bound intermediate but not a free form.

CHAPTER VI

SECTION 2

STATUS OF HYDROXYLAMINE AS AN INTERMEDIATE IN THE REDUCTION OF NITRITE TO AMMONIA BY *A. fischeri* NITRITE REDUCTASE

INTRODUCTION

The intermediates during the enzymatic reduction of nitrite to ammonia have not been identified unequivocally. Meyer and Schultze (1894) proposed that nitrite reduction proceeds through a sequence of three 2-electron steps via two intermediates, the first at the oxidation level of hyponitrite and the second being hydroxylamine. The occurrence of hydroxylamine in tissues of higher plants has been reported occasionally. Wood (1953) concluded that free hydroxylamine, which is highly toxic, is unlikely to be present in the appreciable concentration. The presence of oximes was, however, regarded as likely and consistent with the supposed production of hydroxylamine as an intermediate in nitrite reduction by green plants and microorganisms. Nason *et al.* (1954) postulated that nitrite reduction to ammonia probably proceeds through a sequence of two electron steps via an unknown intermediate and hydroxylamine. Nicholas (1959) concluded that a series of enzymes in plants mediate the reduction of nitrite to ammonia via hyponitrite and hydroxylamine as the physiologically important root. McNall and Atkinson (1957) have reported that *E. coli* strain Bn could utilize hyponitrite, hydroxylamine, or nitrous oxide as its sole nitrogen source and provided independent support for the involvement of these compounds as intermediates in nitrite reduction.

The significance of hyponitrite in nitrite metabolism of higher plants was rejected by Frear and Burrel (1958). They pointed out its great instability at physiological pH values and concluded that when introduced into plant leaves it was

first oxidized to nitrite before reappearing as ^{15}N-labelled ammonia.

The purified nitrite reductases from marrow or spinach were unable to reduce hyponitrite or nitric oxide (Hucklesby and Hewitt, 1970; Pickard and Hewitt, 1972) indicating that hyponitrite and nitric oxide are not the potential intermediate during the reduction of nitrite to ammonia. E. coli nitrite reductase catalyzes the reduction of nitrite as well as hydroxylamine to ammonia; the K_m for hydroxylamine being 150 times greater than that for nitrite. The high K_m for hydroxylamine excludes it as a free intermediate (Kemp and Atkinson, 1966). Similar conclusions were reached with ferredoxin-nitrite reductase from Chlorella, spinach, and squash leaves (Beevers and Hageman, 1969; Losada, 1972). Vega et al. (1973) reported that A. chroococcum nitrite reductase catalyzes the stoichiometric reduction of nitrite to ammonia without the formation of hydroxylamine as a free intermediate.

A. fischeri nitrite reductase catalyzes the six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). The enzyme also catalyzes the reduction of hydroxylamine, and both the substrates (nitrite and hydroxylamine) are reduced at the same active site. No free intermediate could be determined during the six-electron reduction of nitrite. The K_m for hydroxylamine was approximately two orders of magnitude greater than that for nitrite and hence excluding hydroxylamine as a free intermediate during the reduction of nitrite to ammonia.

In the present studies an attempt has been made to determine whether hydroxylamine is an intermediate during nitrite reduction by A. fischeri nitrite reductase. The reduction of $Na^{15}NO_2$ to ammonia is carried out in the presence of hydrazine, which has been shown to inhibit the utilization of hydroxylamine in Nitrosomonas (Hollocher et al., 1981). The hydroxylamine is converted to oxime by cyclohexanone, and estimated by using GC/mass spectrometer which can monitor 0.01 nmol hydroxylamine oxime.

METHODS

Preparation and analysis of oxime

For the preparation of standard oxime: hydroxylamine hydrochloride (100 mg, 1.44 mmol) was added to 160 µl of cyclohexanone (1.6 mmol) in 0.15 M potassium phosphate buffer, pH 6.8, in a test tube and shaken by a vortex stirrer at room temperature (30°C) for 90 min. The oxime was extracted from the buffer with 25, 15 and 15 ml of diethyl ether, respectively. The ether extracts were combined and dried over anhydrous Na_2SO_4 for 3 h and then evaporated to dryness. The residue was recrystallized from petroleum ether (b.p. 30 - 40°C), m.p. 90°C. The purity of the oxime was checked by thin layer chromatography on silica gel G using ethyl acetate-benzene (20:80, v/v) as a solvent system.

The oxime was analyzed in a Hewlett-Packard GC/MS model 5992B mass spectrometer fitted with a column (740 mm x 20 mm) containing 2% OV-101 plus 0.2% Carbowax (20 M) on 100 - 200 mesh Chromosorb W. Helium flow was 3.5 ml/min and electron multiplier voltage was 1800 V. Samples, 0.5 to 1.0 µl in

petroleum ether, were introduced by direct injection through a septum. A solvent control was run between each sample to assure that the injection port and column were free of the previous sample. Cyclohexanone eluted with a retention time of approximately 1.4 min at a column temperature of 40°C and appeared shortly after the solvent front. MS data were obtained by means of both the Peak finder programme and the Selective Ion Monitor Programme.

Estimation of $^{15}NH_3$ produced from $^{15}NO_2^-$ by nitrite reductase

The production of $^{15}NH_3$ from $^{15}NO_2^-$ by nitrite reductase was determined by ^{15}N enrichment technique after alkaline hypobromite oxidation of $^{15}NH_3$ to $^{15}N_2$ according to the procedure of Dua et al. (1979).

The ammonia produced was distilled under alkaline conditions into boric acid and concentrated to 2 ml after addition of one drop of 1 N H_2SO_4. The sample was transferred into one of the limbs of a Rittenberg tube and alkaline hypobromite added to the other limb. The tube was affixed to a mass spectrometer vacuum system and rigorously evacuated to 10^{-7} mm Hg. The contents of the Rittenberg tube were then mixed to generate N_2 gas from ammonia. The tube was immersed in liquid nitrogen to freeze out water vapour and nitrogen oxides. The gas was then introduced into an evacuated expansion flask and passed into the mass spectrometer.

The ^{15}N enrichment was determined by measuring mass 28, 29, 30 representing ^{14}N- ^{14}N, ^{14}N-^{15}N, ^{15}N-^{15}N, respectively, using the Micromass 602 C mass spectrometer, AEI Manchester.

RESULTS AND DISCUSSION

There are a number of reagents which can be used for converting hydroxylamine to oxime. Ketones and aldehydes usually need aqueous alcohol to complete the reaction but the recovery of the oxime from the reaction mixture is incomplete. Keto acids also react with hydroxylamine in aqueous solution to form the corresponding oximes, but these compounds aretoo soluble in water to give a good recovery on extraction into hydrophobic solvents. However, cyclohexanone reacts with hydroxylamine in micro quantities in aqueous solution at pH 6.8 to form oxime with a reasonably good recovery in ether.

The design of the experiment for detection of the possible formation of hydroxylamine oxime during the enzymatic reduction of nitrite was similar to that described by Hollocher *et al.* (1981). Thus hydrazine (150 μmol) was used in an attempt to inhibit the further utilization of hydroxylamine and cyclohexanone (1 mmol) was used to convert hydroxylamine to the oxime. In another experiment cyclohexanone (1 mmol) was used with the enzyme in the reaction mixture in an attempt to trap any hydroxylamine as an oxime during nitrite reduction. The oxime formed was extracted and purified in a similar manner as given for the standard oxime. A pH of 7.0 was chosen to maximize the mole fraction of unprotonated NH_2OH (pK = 6.0) and minimize the mole fraction of unprotonated N_2H_2 (pK = 8.1). This was expected to promote oxime formation from cyclohexanone and minimize azine formation.

Using the procedure described above, a reaction mixture containing $Na^{15}NO_2$, cyclohexanone, benzyl viologen and sodium dithionite was incubated under anaerobic conditions for 10 min before extracting in ether. The ether extracts were blown to dryness with nitrogen at room temperature. The residue was suspended in 50 µl of diethyl ether and one µl aliquots were injected into GC/MS. It was observed that cyclohexanone stimulated the reduction of nitrite and was fully accounted for ammonia production.

There was no evidence for the production of $^{15}NH_2OH$ oxime by the GC/MS method which can detect 0.01 nmol of the compound.

In another set of experiments upto 90 mmol hydrazine (NH_2 NH_2) was added to the reaction mixture and after terminating the reaction by exposure to air, cyclohexanone was added in an attempt to form the oxime and then a similar extraction procedure adopted. In these experiments, too, there was no indication that hydroxylamine oxime was produced during nitrite reduction. Indeed, hydrazine did not appear to inhibit the reduction of $^{15}NO_2^-$ to ammonia.

The EPR studies have shown that a nitrosyl heme compound is produced during the turnover of the enzyme indicating that a nitrosyl heme may be an intermediate in the reduction of nitrite to ammonia. The nitrosyl heme spectrum was also observed with hydroxylamine in the absence of reductant (benzyl viologen) which may result from disproportionation of the enzyme bound form of hydroxylamine to free ammonia

and bound nitric oxide. A similar observation for the spinach nitrite reductase was interpreted as indicating that hydroxylamine is enzyme-bound and not a free intermediate in the reduction of nitrite to ammonia (Vega and Kamin, 1977). Our results also indicate that free hydroxylamine does not accumulate. Should a bound form of hydroxylamine be produced then it must be below 0.01 nmol which is the limit of its detection by the GC/MS method employed here.

CHAPTER VI

SECTION 3

FLAVODOXIN AS LIKELY PHYSIOLOGICAL ELECTRON DONOR FOR *A. fischeri* NITRITE REDUCTASE

INTRODUCTION

Plant and algal nitrite reductases are practically specific for single electron donors: ferredoxin and MVH or BVH with certain exceptions and do not utilize NAD(P)H directly (Losada et al.1963; Paneque et al. 1964; Betts and Hewitt, 1966). Reduced flavins are generally ineffective (Zumft, 1972). However, the enzyme from marrow has been shown to reduce nitrite in presence of $FMNH_2$ but the activity was very low as compared with that when BVH or ferredoxin was used as electron donor (Hucklesby and Hewitt, 1970). The Chlorella enzyme was shown to function with illuminated chloroplast and flavodoxin (Zumft, 1972).

The ferredoxins are a group of iron-sulfur proteins present in photosynthetic organisms and in non-photosynthetic anaerobic bacteria. They participate in a number of electron transfer reactions including photosynthetic electron transport and nitrogen fixation. The ferredoxins from higher plants, eukaryotic algae and blue green algae, possess the 2Fe-2S active center, have a relative molecular mass of approximately 11,000 and accept a single electron on reduction. In general bacterial ferredoxins contain two 4Fe-4S active clusters, accept two electrons on reduction and have molecular weight between 6,000 to 15,000 depending on the source (Takruri et al. 1978). The amino acid sequence of C. pasteurianum ferredoxin and the nucleotide sequence of the C. pasteurianum Fd gene have been determined. The amino acid sequence deduced from the DNA analysis agrees exactly with that determined for the protein, with the sole exception of the encoded initiator methionine (Graves et al., 1985).

Flavodoxins are low molecular weight electron transfer proteins which have been shown to substitute for ferredoxins in many of the wide range of reactions in which ferredoxins

can function as electron carrier (Yoch and Valentine, 1972; Mayhew and Ludwig, 1975). The first such protein was isolated from the cyanobacterium A. nidulans (Smillie, 1963) and its physiological role as a low-potential electron transfer agent was indicated by its ability to replace ferredoxin in light-dependent $NADP^+$ reduction by higher plant chloroplasts (Smillie, 1965). One mol of FMN is present per mol of protein. Reduction of flavoprotein proceeds via a blue-flavosemiquinone radical. Flavoprotein carriers can yield reduced flavin semiquinone couple of relatively low potential (- 0.46V) which do not readily equilibrate with the high potential oxidized flavin semiquinone couple and acts effectively as one electron donor (Yoch, 1972).

Flavodoxins have been isolated from a variety of organisms from anaerobic fermentative bacteria to eukaryotic algae. They appear to fall into two groups, one having M_r approximately 15,000 and the other 22,000 (Tanaka et al., 1975).

METHODS

NADPH—flavodoxin linked nitrite reductase assay system

The reaction mixture contained 200 µmol of potassium phosphate buffer, pH 7.5, 75 µmol of glucose-6-phosphate, glucose-6-phosphate dehydronase 0.1 ml (2 units), $NADP^+$ 0.05 µmol, spinach NADPH-flavin diaphorase 0.08 mg, flavodoxin (C. crispus) in the range of 1 nmol to 2.5 nmol, and distilled water to give a final volume of 0.9 ml in the main arm of the Thunberg tubes. After 30 min, when flavodoxin was reduced, 3 - 5 µg of nitrite reductase and 1 µmol of $NaNO_2$ were added

under a stream of O_2-free nitrogen. After 90 min further incubation, the reaction was terminated by adding 0.1 ml of 1 M barium acetate and 2.5 ml of 95% ethanol v/v to precipitate NADPH. The contents of the assay mixture were centrifuged and nitrite estimated in suitable aliquots.

The concentration of C. crispus flavodoxin was determined from its molar extinction coefficient, 10700 $M^{-1}cm^{-1}$ (Fitzgerald et al., 1978).

RESULTS

A. fischeri nitrite reductase has been reported to utilize various electron donors, BVH, $FMNH_2$, $FADH_2$ (Prakash and Sadana, 1972). Reduced ferredoxin from either spinach or C. pasteurianum were unable to donate electron to A. fischeri nitrite reductase although these ferredoxins have been shown to serve as electron donor for assimilatory nitrite reductase (Betts and Hewitt, 1966).

Table 7: K_m values of A. fischeri nitrite reductase for various electron donors.

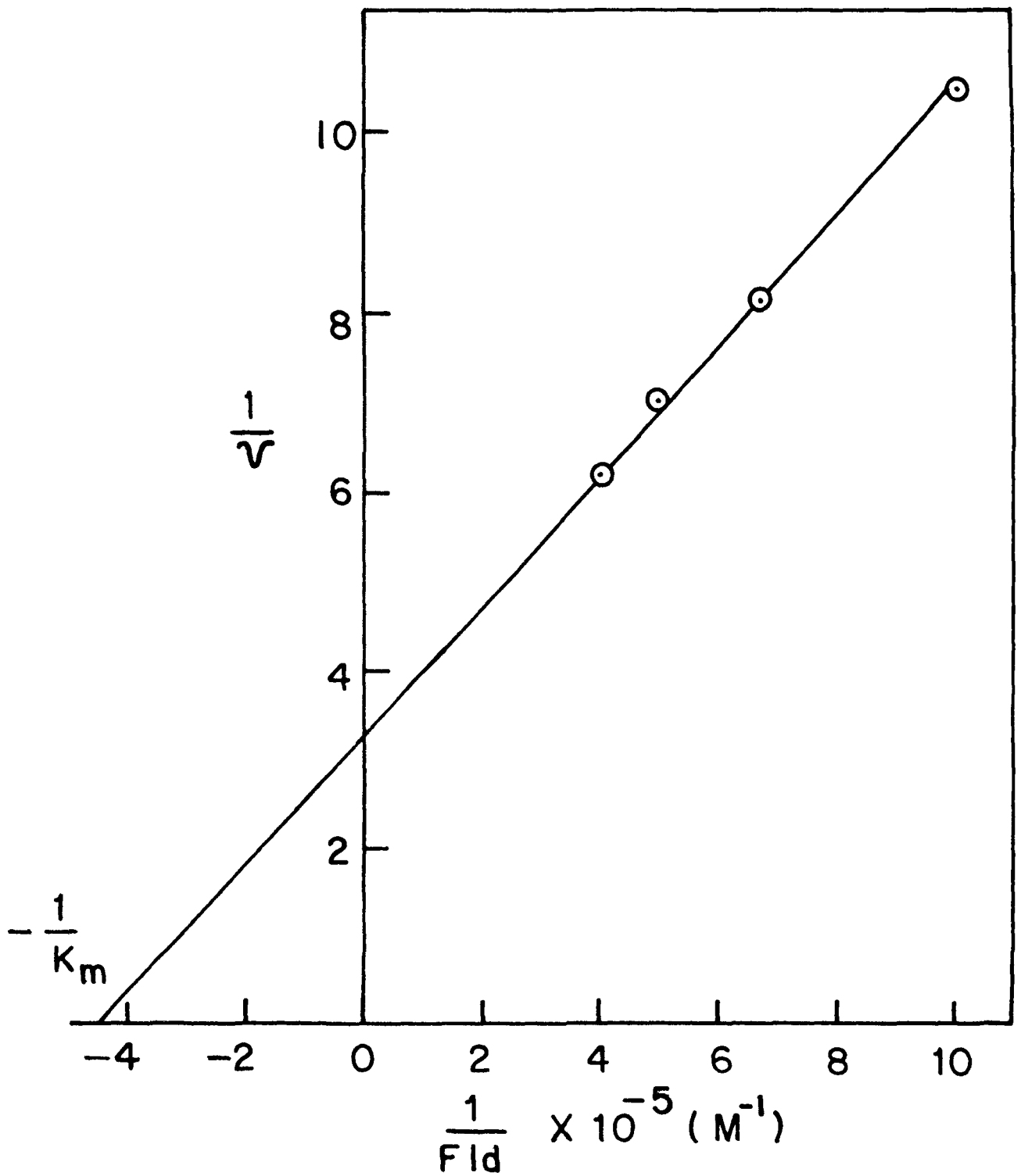
S.No.	Electron donor	K_m
1.	BVH	$2.8 - 4.1 \times 10^{-5}$M
2.	$FMNH_2$	2×10^{-4}M
3.	$FADH_2$	2×10^{-4}M
4.	Flavodoxin (C. crispus)	2.2×10^{-6}M

The K_m values for $FMNH_2$ and $FADH_2$ are much higher than for BVH (Table 7). An attempt was then made to determine the likely physiological electron donor for A. fischeri nitrite reductase. Flavodoxin (Fld) from C. crispus was tried to determine whether it would work as an electron donor for nitrite reduction by A. fischeri nitrite reductase. Nitrite reductase activity was estimated at various concentrations of flavodoxin. The K_m value for flavodoxin, calculated from double reciprocal plot of 1/v versus 1/s (Fig. 23), was approximately 2.2×10^{-6}M (Table 7). This is much less than that of BVH ($K_m = 2.8 - 4.1 \times 10^{-5}$M). Thus, reduced flavodoxin from C. crispus appears to be the likely physiological electron donor for A. fischeri nitrite reductase.

DISCUSSION

Nitrite reductases isolated from various sources show well-defined electron specificity. The assimilatory nitrite reductases from nonphotosynthetic organisms present a marked specificity for reduced pyridine nucleotides as electron donors and require FAD for maximal activity. Three types of enzymes with different specificity for reduced pyridine nucleotide can be distinguished: NAD(P)H- nitrite reductase, which can use either NADH or NADPH as electron donor and is characteristic of the fungus Neurospora (Garrett and Amy, 1978); NADPH- nitrite reductase, with a marked specificity for NADPH as electron donor, characteristic of yeast Torulopsis nitratophila (Rivas et al., 1973); and NADH- nitrite reductase, specific for NADH as electron donor found in

FIG. 23 Lineweaver-Burk plot of the effect of flavodoxin concentration on A. fischeri nitrite reductase activity. The assay conditions are as described in Methods.


10
8
6
4
2
1/v
-1/Km
-4
-2
0
2
4
6
8
10
1/Fld X 10^-5 (M^-1)

prokaryotic organisms (Coleman et al., 1978; Vega et al., 1973).

Photosynthetic nitrite reductases show a marked specificity for ferredoxin as electron donor (Vega et al., 1980). Flavodoxin can substitute for ferredoxin as the immediate electron donor for different nitrite reductases (Manzano, 1977; Vega et al., 1980; Zumft, 1972). Among the artificial substitutes examined for ferredoxin, methyl viologen is the most effective. Reduced flavins are generally ineffective (Zumft, 1972; Hattori and Uesugi, 1968 b). However, marrow preparations showed 5 - 30% activity with flavins as compared to benzyl viologen (Hucklesby and Hewitt, 1970).

A. fischeri nitrite reductase can accept electrons directly from benzyl viologen, methyl viologen and flavins ($FMNH_2$, $FADH_2$). Pyridine nucleotides could not serve as electron donor, and flavins, FMN and FAD, showed 20% activity as compared to that obtained with benzyl viologen (Prakash and Sadana, 1972). The K_m values for flavins are very high and therefore not likely to be the physiological electron donor. Ferredoxins from either spinach or C. pasteurianum were unable to donate electrons to Achromobacter nitrite reductase. Flavodoxin from C. crispus, on the other hand, acted as an efficient electron donor and its K_m value was an order of magnitude lower than benzyl viologen. Flavodoxin thus appears to be the natural electron donor for A. fischeri nitrite reductase.

Fitzgerald et al. (1980) assessed the efficiencies of ferredoxins and flavodoxins from various sources as electron

mediators in systems for hydrogen evolution. They proposed that there was no apparent correlation of efficiency with mid point redox potential (Em) of the mediator. Activity of the mediators therefore primarily reflects differences in their tertiary structure conferring different affinities. The same reasoning can be proposed here also when ferredoxins from either spinach or C.pasteurianum did not serve as electron donor but flavodoxin from C. crispus served as an efficient electron donor for A. fischeri nitrite reductase. Flavodoxins and ferredoxins are known to substitute each other in many biological reactions (Yoch and Valentine, 1972; Mayhew and Ludwig, 1975). In A. nidulans the flavodoxin was equally or more effective in vitro than ferredoxin in supporting nitrite reduction (Bothe, 1969). Flavodoxin has also been shown to replace ferredoxin as electron donor for Chlorella nitrite reductase (Zumft, 1972).

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Nitrate is metabolized by A. fischeri to form ammonia as an end product when it is grown anaerobically or under low oxygen tension. The two enzymes, required to catalyze the reduction of nitrate to ammonia are (1) nitrate reductase, which catalyzes the two-electron reduction of nitrate to nitrite, and (2) nitrite reductase, which catalyzes the six-electron reduction of nitrite to ammonia.

Nitrate reductase from A. fischeri has been purified earlier by Sadana and McElroy (1957) and Sadana et al. (1963). The purified enzyme was relatively unstable and also showed two components on ultracentrifugation. The A. fischeri nitrate reductase has now been purified by modification of an earlier procedure described by Sadana et al. (1963). The enzyme is homogeneous in polyacrylamide gel electrophoresis at pH 8.9 and pH 4.3, at different gel concentrations (6, 8, 10, 12%) and in SDS-gel electrophoresis. The overall recovery of the enzyme was 26% as compared to 4.8% obtained by the earlier procedure, and showed better stability as compared to the enzyme purified by the earlier procedure of Sadana et al. (1963). The purified A. fischeri nitrate reductase has a specific activity of about 3.65 μmol nitrite formed from nitrate per mg protein per min.

The relative molecular mass of A. fischeri nitrate reductase was determined by various methods, and an average value of 54,000 was obtained. There appears to be no interaction between

the enzyme and Sephadex polysaccharide matrix as there was not much difference in the relative molecular mass values determined on Bio-gel and Sephadex columns. The Stokes' radius, calculated from gel filtration data by the methods of Ackers', Porath and Laurent and Killander, was an average value of 3.1 nm. The purified enzyme, on treatment with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol in the presence of 1% iodoacetamide, does not dissociate in SDS-gel electrophoresis, indicating that A. fischeri nitrate reductase is comprised of a single polypeptide chain.

Nitrite reductase from A. fischeri has already been obtained in a homogeneous state and its molecular weight, amino acid composition, subunit structure and hydrodynamic properties have also been studied in this Laboratory earlier (Prakash and Sadana, 1972; Husain and Sadana, 1972; Husain and Sadana, 1974a; 1974b). No information, however, is available on the involvement of amino acid residues at the catalytic site of nitrite reductase. Attempts were therefore made to determine the amino acid residues which are involved at the active site of the enzyme. Diethylpyrocarbonate (DEPC) was used as a specific reagent for modification of histidine residues.

Inactivation of A. fischeri nitrite reductase by DEPC was carried out at pH 6.8 and 6.5 at 4 °C. The rate of inactivation was faster at pH 6.8 as compared to that at pH 6.5. The enzyme was almost completely inactivated at 5 mM DEPC at pH 6.8. The rate of inactivation of the enzyme was

concentration- and time- dependent and followed pseudo-first order kinetics. The difference spectrum between the DEPC- treated (carbethoxylated) and the native enzyme showed a characteristic positive peak at 240 nm which is characteristic of N-carbethoxyimidazole derivative. The increase in absorbance at 240 nm was concomitant with the loss of enzyme activity indicating that the inactivation of the enzyme was due to modification of histidine residues. Four histidine residues have been shown to be modified by DEPC during the inactivation of the enzyme. However, on the basis of the reaction order (n), which was calculated to be 0.9, only one histidine residue per molecule of the enzyme appears to be essential for the activity of the enzyme.

The specific modification of the enzyme by DEPC at histidine residues was confirmed by the following observations: (1) Inactivated enzyme was reactivated by 0.75 M hydroxylamine hydrochloride at neutral pH with removal of ethoxyformyl group from the modified enzyme. (2) Inactivation of the enzyme by DEPC was considerably protected by the substrate (nitrite). The maximum protection by nitrite was obtained at a concentration of 5 mM or above. At 0.2 mM nitrite concentration or less, no effect could be noticed for the protection of nitrite reductase inactivation by DEPC.

The possible modification of some other amino acid residues by DEPC was ruled out on the following grounds: The lack of absorbance changes in 280 nm region during the treatment of the enzyme by DEPC indicated that tyrosine residues

are not modified by DEPC. It has also generally been believed that lysine and tyrosine side chains do not react with DEPC below pH 7.0 (Dykes and John, 1977). The content of free sulfhydryl groups of native nitrite reductase and DEPC-treated enzyme was found to be same indicating that sulfhydryl groups have not been modified by the reagent. The restoration of enzyme activity by hydroxylamine appears to rule out the modification of lysyl- and arginyl- residues since the more stable amide-like structures formed during DEPC treatment are not readily reversed by hydroxylamine (Miles, 1977; Horiike et al., 1979).

The pK values of ionizable groups of the enzyme, which are required for the activity of the enzyme, were found to be 7.0 and 7.7 corresponding to the imidazole group. This lends further support to our conclusion by modification studies that histidine is essential for the activity of A. fischeri nitrite reductase.

The detailed mechanism of the reduction of nitrite to ammonia by nitrite reductase is complex, since it involves six-electron sequential reduction. Electron paramagnetic resonance studies were carried out to detect intermediates of the nitrite reduction reaction and to understand the mechanism of its reduction. Low temperature EPR spectrum of A. fischeri oxidized nitrite reductase was extremely complex and showed signals due to different heme species. The main EPR signals were at g-values 3.7, 2.88, 2.26 and 1.51. The species at g-value 3.7 is attributed to the active enzyme because it disappears immediately

on reduction of the enzyme. The signals at g-values 4.3 and 2.1 were due to contamination of iron and copper, respectively. The EPR spectrum of the oxidized enzyme did not change when treated with substrate, nitrite, and ligands, CN^-, azide or fluoride which suggests that the oxidized protein appears to be unreactive to these ligands and that in the first step of the enzyme reaction, the heme is reduced before nitrite binds.

Reduction of the enzyme with methyl viologen and dithionite in the presence of excess nitrite produced the EPR spectrum of nitrosyl heme derivative with a g-value at 2.01 and a ^{14}N hyperfine splitting into three lines separated by 1.65 mT and ^{15}N splitting into two lines separated by 2.3 mT. Exchange of nitric oxide gas was observed between ^{15}N nitrosyl derivative and ^{14}NO gas. There was no exchange when ^{15}N nitrosyl nitrite reductase was treated under non-reducing conditions with $Na^{14}NO_2$, demonstrating that nitric oxide binds more tightly than nitrite. The nitrosyl-heme spectrum was also observed, though with decreased signal intensity, when A. fischeri nitrite reductase was treated with hydroxylamine. This could be explained by disproportionation of the hydroxylamine to ammonia and the enzyme-bound form of nitric oxide.

It is proposed that reaction cycle involves reduction of heme before binding of nitrite and formation of nitrosyl derivative. The data also suggest that the nitrosyl heme is an intermediate in the reduction of nitrite to ammonia.

A. fischeri nitrite reductase catalyzes the reduction of nitrite and hydroxylamine at the same catalytic site, but the

K_m for hydroxylamine is two orders of magnitude greater than that for nitrite. On the basis of these data it was suggested that hydroxylamine may be an intermediate; however, it was excluded as a free intermediate during the reduction of nitrite to ammonia (Prakash and Sadana, 1972). The EPR studies have also indicated that hydroxylamine is an intermediate in the reduction of nitrite to ammonia. An attempt has further been made here to show conclusively whether hydroxylamine is an intermediate during the reduction of nitrite to ammonia by trapping hydroxylamine (if formed) during nitrite reduction. The reduction of $Na^{15}NO_2$ was carried out in the presence of hydrazine, which has been shown by Hollocher et al. (1981) to inhibit utilization of hydroxylamine, and cyclohexanone. The cyclohexanone was added either during the reaction or after reaction was over, to convert hydroxylamine to oxime. The hydroxylamine oxime was then extracted with ether and estimated by using GC/mass spectrometer. We could obtain no evidence for the production of $^{15}NH_2OH$. The data confirm our previous suggestion that at least free hydroxylamine is not an intermediate in the reduction of nitrite to ammonia. If a bound form of hydroxylamine is produced, it must be below 0.01 nmol which is the limit of its detection by GC/MS method employed here.

A. fischeri nitrite reductase utilizes various electron donors: BVH, MVH, $FMNH_2$, $FADH_2$. The K_m values for flavins are much higher as compared with that of BVH; flavins are, therefore, unlikely to be the physiological electron donor

for Achromobacter nitrite reductase. Ferredoxin from either spinach or C. pasteurianum are inactive as electron donor for A. fischeri nitrite reductase. Flavodoxin from C. crispus, on the other hand, acted as an efficient electron donor. Its K_m value is approximately one order of magnitude lower even than that of benzyl viologen. The physiological electron donor for A. fischeri nitrite reductase thus appears to be flavodoxin.

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