STUDIES ON NITRITE REDUCTASE IN THE HIGHLY PROTEINACEOUS BLUE GREEN ALGA, *Spirulina platensis*

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CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full, for any other Degree or Diploma of any University.

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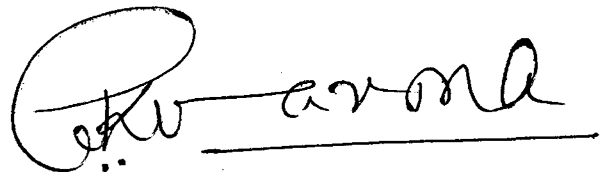

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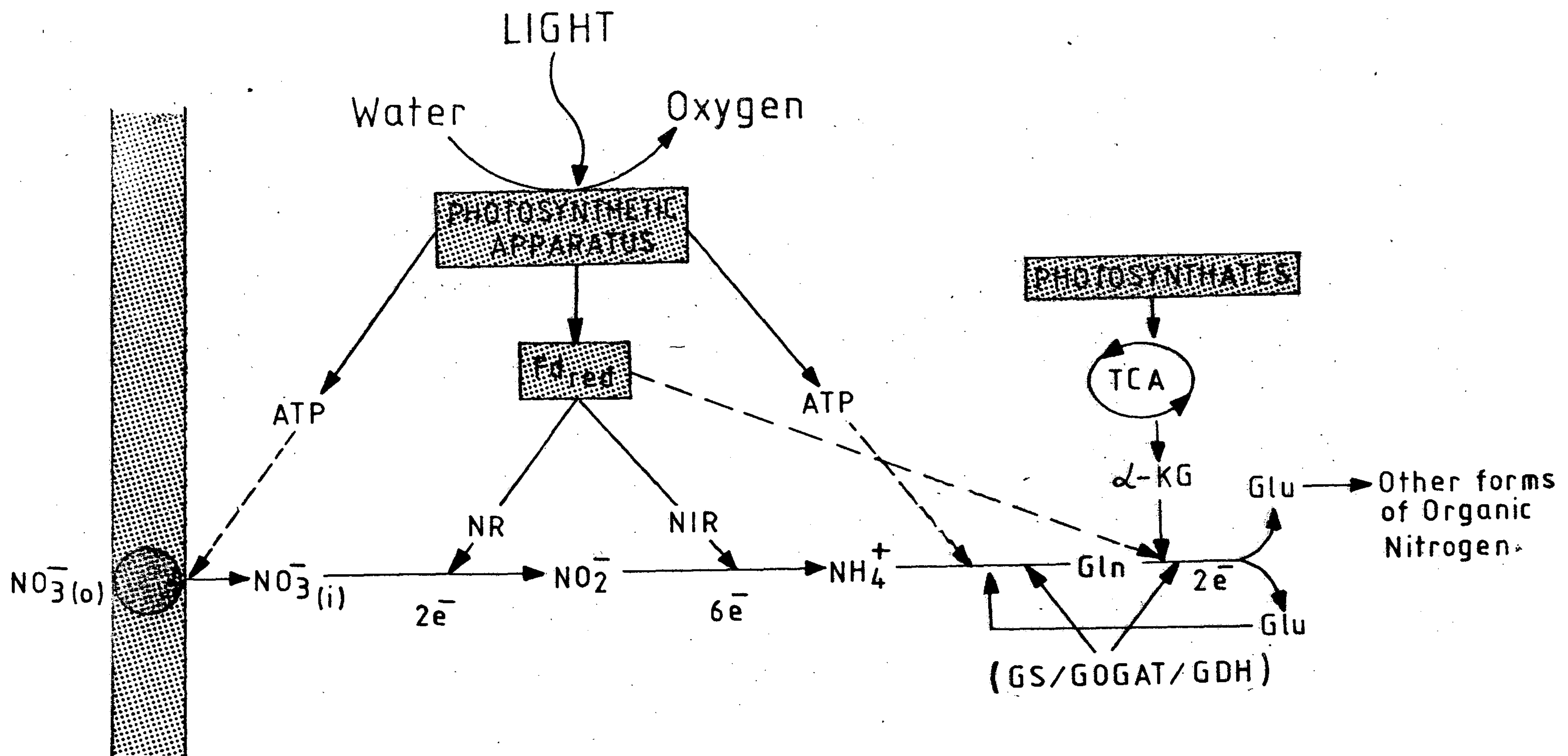
ABBREVIATIONS

App	:	Apparent
BV	:	Benzyl Viologen
^{o}C	:	degree centigrade
e^{-}	:	electron
EDTA	:	Ethylene diamine tetra-acetic acid
FMN	:	Flavin mononucleotide
Fd	:	Ferredoxin
gms	:	Grams
GDH	:	Glutamate dehydrogenase
GS	:	Glutamine synthetase
GOGAT	:	Glutamate Synthase
h	:	Hours
Kd	:	Kelodalton
α-KG	:	α-Ketoglutarate
l	:	Litre
ml	:	millilitre
mM	:	Millimolar
min	:	Minute
nmole	:	nanomole
NR	:	Nitrate reductase
NiR	:	Nitrite reductase
NADH	:	Reduced Nicotinamide adenine dinucleotide
NADPH	:	Reduced Nicotinamide Dinucleotide Phosphate

NED	:	N-(I-Napthyl) ethylenediamine dihydrochloride
p-HMB	:	para hydroxy mercuribenzoate
P	:	Pellet
Sec	:	Second
S	:	Supernatant
W/V	:	Weight/Volume



A SCHEMATIC VIEW OF NITRATE ASSIMILATION IN CYANOBACTERIA

INTRODUCTION

One important property of many blue-green algae is the ability to fix elemental nitrogen. Thus, they make a contribution to the fertility of some crop plants, especially in paddy fields. Another interesting property of blue-green algae is the ability of some species to produce hydrogen gas. Among the many new concepts now being explored in solar energy bioconversions, is their use for the production of hydrogen for fuel purposes. A third important aspect of cyanobacteria is their use as food and feed. *Spirulina platensis* grows as a helicoidal filament with a length of 200-300 μm and a diameter of about 5-10 μm, when developed includes 100-250 cells. Reproduction is accomplished by binary fission in the longitudinal plane. Necrosis of older cells fragment the filaments. *Spirulina* is a non-heterocystous form and does not fix elemental nitrogen. Because of gas vacuoles, characterestic of this species, filaments are buoyant and float on the surface of the water where they may form clumps. If photosynthesis is very intense, turgor pressure becomes too high and the gas vesicles collapse. The cells then lose their buoyance and sink.

The nutritional requirements of Spirulina include nitrogen, phosphorus, sulfur, potassium, sodium, magnesium, calcium and iron; trace amounts of other elements are also required. Chemical composition studies on Spirulina reveal a high protein content. On the average the available lysine is about 85 per cent of the total lysine content. α-Linolenic acid represents 20 per cent of the total fatty acids. A moderate amount of carbohydrates with low calorific value is present. Among the carbohydrate is a phosphorylated cyclitol which is of nutritional interest because it is an iron chelating agent. The protein efficiency ratio (PER) is between 2.2 and 2.6 (74 to 87 per cent of casein). Net protein utilization (NPU) is between 53 and 61 per cent (85 to 92 per cent of casein). Digestibility is between 83 and 84 per cent. The main use of Spirulina is for feeding animals - from crustaceans larvae to human beings.

Although atmospheric air consists of 80 per cent nitrogen (dinitrogen) by volume but this nitrogen can be used only by a few procaryotes. For most of the biological systems including cyanobacteria, nitrate nitrogen serve as the main source of nitrogen for the synthesis of amino acids and other nitrogenous com-

pounds. For this to happen nitrate is reduced by many steps, the first of which represents a two electron transition resulting in the conversion of nitrate to nitrite. Further reduction is catalyzed by nitrite reductase, by which nitrite is reduced to ammonia, a 6 electron transition. These two enzymes are characteristically metalloenzymes.

Studies conducted in this laboratory and elsewhere have clearly documented that ammonia is cytotoxic for cyanobacteria, when used as a nitrogen source. Nitrate is the preferred source of nitrogen for Spirulina. Extensive work is already in progress in this laboratory to elucidate the enzymes in the nitrogen assimilatory pathway in this system. The present dissertation embodies the preliminary ground work preparation towards understanding the mechanism of reduction of nitrite to ammonia by metallo-protein nitrite reductase.

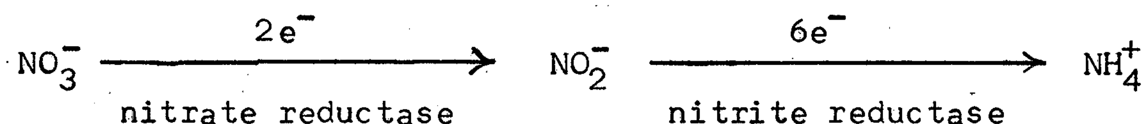
LITERATURE REVIEW

Cyanobacteria like all other living organisms require certain nutrients for growth. These nutrients must contain those chemical elements that are constituents of the cellular materials and that are necessary for the activity of enzyme and transport systems. A great diversity of compounds utilized by microorganisms is only observed with respect to the five elements: nitrogen, sulfur, oxygen, hydrogen and carbon.

Nitrogen is required in large quantities because it accounts for approximately 10 per cent of the dry weight of algal cells. It occurs in the form of ammonia, nitrate, nitrite, nitrogen containing organic compounds and molecular nitrogen. The preferred source of nitrogen is ammonia, which can be utilized practically by all organisms. Nitrate is also taken up and used by many organisms, but not by all. Nitrite is the product of nitrate-nitrite respiration.

Experimental documents in the last one decade have firmly established that the assimilatory nitrate reducing system consists of two metallo-proteins,

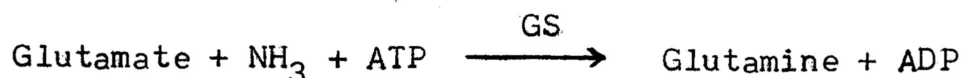
namely nitrate reductase and nitrite reductase which catalyze the step-wise reduction of nitrate to nitrite and ammonia (Beevers and Hageman, 1972; Garrett and Amy, 1978; Losada and Guerrero, 1979, and Lea and Miflin, 1979).



The end product of nitrate reduction, ammonia is incorporated into glutamine.

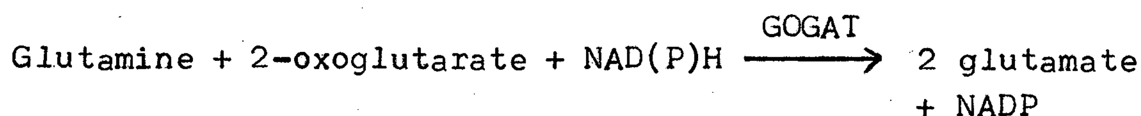
An organism like <u>Klebsiella aerogens</u> (a non diazotrophic strain) assimilates exogenous ammonia <u>via</u> the enzyme glutamate dehydrogenase (GDH), building it into glutamic acid from 2-oxoglutaric acid and thence into protein and other nitrogen containing compounds of biomass.

GDH has only a moderate affinity for ammonia. If the exogenous ammonia concentration is low an alternate two step assimilatory system develops (Brown, Macdonald-Brown and Meers, 1974). First ammonia reacts with pre-existing glutamate to form glutamine, a reaction requiring ATP and mediated by glutamine synthetase (GS):



$$\text{Glutamate} + NH_3 + \text{ATP} \xrightarrow{\text{GS}} \text{Glutamine} + \text{ADP}$$

Secondly, the glutamine reacts with 2-oxoglutarate to form two molecules of glutamate, a reaction mediated by the enzyme glutamate synthase (abbreviated to an acronym GOGAT from its systematic name; Meers, Tempest and Brown, 1970).



$$\text{Glutamine} + \text{2-oxoglutarate} + \text{NAD(P)H} \xrightarrow{\text{GOGAT}} \text{2 glutamate} + \text{NADP}$$

The GS-GOGAT assimilatory pathway has a higher affinity for ammonia. Since it consumes ATP, it is less aeconomic than the GDH pathway. Diazotrophic _Klebsiella_ assimilate the ammonia formed by nitrogenase by GS-GOGAT pathway and so do most other diazotrophic heterotrophs. In the cyanobacteria the assimilation pathway is not clearly understood (Stewart, 1974).

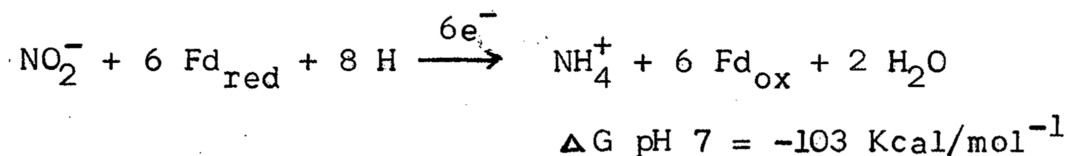
Presence of GS, Glutamate synthase, and GDH in _Rhizobium japonicum_ grown in cultures and in bacteroids has recently been studied by Nicholas and his associates (Varianhos, Bhandari and Nicholas, 1983; Bhandari, Varianhos and Nicholas, 1983).

With this brief introduction to the importance of nitrogen, nitrate reduction and ammonia assimilation,

we shall now review the work so far reported for the last two decades on NiR, in various biological systems, which is our present interest.

NITRITE REDUCTASE:

Nitrite reductase (NiR) is one of the two metallo-enzymes involved in the assimilatory pathway of nitrogen metabolism. It catalyzes the reduction of nitrite to ammonia:



$$NO_2^- + 6\ Fd_{red} + 8\ H \xrightarrow{6e^-} NH_4^+ + 6\ Fd_{ox} + 2\ H_2O$$

$$\Delta G\ pH\ 7 = -103\ Kcal/mol^{-1}$$

The reduction of nitrate can occur by two distinct physiological mechanisms both resulting in the formation of ammonia (Painter, 1970). One involves the conversion of nitrate to nitrite followed by the conversion of nitrite to ammonia. Nitrate reductase and nitrite reductase catalyze the conversion of nitrate to nitrite to ammonia, e.g. in spinach, Chlorella, Anabaena and Anacystis. The second pathway is one in which nitrate is reduced by acting as an electron acceptor to oxygen (Anaerobic respiration) into ammonia, e.g. Pseudomonas aeruginosa, Paracoccus denitrificans, Thiobacillus denitrificans, Rhodopseudomonas palustris (Preuss and Klemme, 1983).

The assimilatory NiR is of two types marked by a well defined electron donor specificity:

(a) Ferredoxin Nitrite reductase: which is often present in photosynthetic organisms.

(b) NAD(P)H Nitrite reductase: which is present in non-photosynthetic organisms. This second type has been further classified into three types (Guerrero, Vega and Losadà, 1981) based on their specificity for different pyridine nucleotides:

(i) NAD(P)H-NiR: It could use either NADPH or NADH as electron donor. This type of NiR occur in fungi e.g. *Neurospora crassa* (Garrett and Amy, 1978).

(ii) NiR having marked specificity for NADPH as an electron donor as in yeast, *Torulopsis nitrophila* (Rivas, Guerrero, Paneque and Losada, 1973).

(iii) NADH-NiR: This type of enzyme is specific for NADH as electron donor and has been reported in *Azotobacter* and *Escherichia coli* (Vega, Guerrero, Leadbetter and Losada, 1981).

All these enzymes catalyse the stoichiometric reduction of nitrite to ammonia which implies the unusual transfer of 6 electrons. No free nitrogen compounds of an intermediate redox state were detected (Guerrero and Amy, 1978). The quantitative details revealed that NiR levels in different cells and

tissues are usually much higher than that of NR and accordingly, the accumulation of nitrite is seldom observed (Guerrero, Vega and Losada, 1981). *In vitro* experiments revealed that nitrite is inhibitory at concentrations which were tolerated in other experiments (Hewitt, Hucklesby and Betts, 1967; Sanderson and Cocking, 1964).

OCCURRENCE

Nitrite reductase has been studied in all groups of the plant kingdom. Amongst higher plants, it has been reported in *Spinacea oleratia* (Vega, Cardenas and Losada, 1980; Lancaster, Batie, Kamin and Knaff, 1982), barley (Borne and Miflin, 1973; Ida, Mori and Morita, 1974), *Cucurbita pepo* (Aparicio, 1975; Hucklesby, Banwell and Hewitt, 1975; Hucklesby, 1972).

In algae, NiR has been extensively studied in *Chlorella fusca* (Zumft, 1972), a green alga. Ho, Ikawa and Nisizawa (1976) have also reported the presence of NiR in a red alga *Porphyra yezoensis*.

Amongst fungal species, NiR has been studied in *Torulopsis nitrophila* (Rivas, Guerrero, Paneque and Losada, 1973), *Neurospora crassa* (Lafferty and Garrett, 1974). NiR in bacteria has been reported from *Azotobacter chroococcum* (Vega, Guerrero, Leadbetter and Losada, 1973), *Achromobacter fischeri* (Prakash

and Sadana, 1972), Micrococcus denitrificans (Lam and Nicholas, 1969), Escherichia coli (Siegel and Kamin, 1971), Salmonella typhimurium (Siegel, Kamin and Reuger, 1969) and Clostridium perfringenes (Sekiguchi, Seki and Ishomoto, 1983).

In cyanobacteria, Hattori and Uesugi (1968) reported for the first time, a 40 fold purified NiR from a filamentous Anabaena cylindrica. Later in 1977, Manzano reported a 30 fold purified NiR dependent on ferredoxin as an electron donor in single celled Anacystis nidulans. Recently, Mendez and Vega (1981) reported a 736 fold purified Fd-NiR with preliminary kinetic investigations from Anabaena sp. 7119 (previously named Nostoc muscorum).

LOCALIZATION:

The intracellular localization of Fd-NiR in chloroplasts from different C_3 and C_4 plants was well established (Dalling, Tolbert and Hageman, 1972 a,b; Grant, 1970; Wallsgrove, Lea and Miflin, 1979). However, Lips and Avissar (1972) claimed that it is localized within the microbodies. Emes and Fowler (1979) have established that NiR and GS are plastid located enzymes and that the induction of nitrate assimilation is connected with the plastid localized pentose-phosphate-pathway in pea roots. This adds support to the view of nitrate assimilation being

coupled to carbohydrate oxidation (Emes and Fowler, 1983).

In fungi, both, NR and NiR are dependant on reduced pyridine nucleotides and are apparently found in the cytoplasm, where NAD(P)H is generated in dissimilatory reactions (Guerrero, Vega and Losada, 1981).

No direct demonstration seems to be available showing the localization of NR and NiR among the eukaryotic algae. Nevertheless, Guerrero, Vega and Losada (1981) inferred from various experiments with chlorophyll fluorescence done by Kessler and Zumft (1973), in *Chlorella* and *Ankistodesmus*, that NiR and NR is located in the chloroplasts. They also found that nitrite reduction is more directly coupled to light reaction of photosynthesis than nitrate reduction.

This is particularly evident in cyanobacteria, where both NR and NiR are tightly bound to chlorophyll containing fractions and are able to utilize photosynthetically generated reducing power *via* ferredoxin (Guerrero, Vega and Losada, 1981). Although, these enzymes were associated with particles containing photosynthetic activity (Ortega, Castilla

and Carenus, 1976; Candau, Manzano and Losada, 1976). The Fd-NiR is usually a soluble protein, with the exception of the Fd-NiR isolated from *Anacystis nidulans*. NiR from *Anacystis nidulans* was associated with the particulate fraction and prolonged ultra-sonication was required to get the enzyme in the soluble fraction (Menzano, Candau, Gomez-mereno, Ratimpo and Losada, 1976).

REDUCING POWER:

The reducing power required for the activity of NiR is supplied either through the electron transport chain of the non-cyclic phosphorylation by the photolysis of water or in the dark, by molecular hydrogen or by reduced carbon compounds. Here the reducing power generated, through any one of the pathways mentioned above is transferred to the actual site of nitrite reduction by redox coenzymes, redox metabolites or reduced substrates. However, the supply of reducing power in chlorophyllous organisms is different from that of non-photosynthetic organisms (Garrett and Amy, 1978).

The physiological reductant for NiR in non-chlorophyllous organisms remains unknown (Beevers and Hageman, 1972; Dalling, Hucklesby and Hageman,

Hewitt, 1975). However, it has been widely accepted that NiR in such organisms is able to utilize reduced carbon compounds for nitrite reduction (Losada, Guerreo and Vega, 1981). In fungi, both NR and NiR utilize reduced pyridine nucleotides, generated through dissimilatory processes (Garrett and Amy, 1978).

In support of the above view, several authors have demonstrated that the growth of the fungi, Aspergillus nidulans, on nitrate, affected several enzymes in the pentose phosphate pathway, which is the major source of NAD(P)H generation in this organism. This was also proved by the two classes of mutants, defective in their pentose phosphate metabolism, designated pppA and pppB, which were characterized by poor levels of nitrate and nitrite reductases (Hankinson, 1974).

Studies on chlorophyllous organisms indicated that they utilize water as the reductant in light (Losada, Guerrero and Vega, 1981). However, the photosynthetically generated reduced coenzymes and their utilization in nitrate assimilation was found to be directly coupled to photosynthesis, which gave rise to the concept of 'photosynthetic assimilation of nitrate' (Emes and Fowler, 1983).

Studies on green algae, Ankistodesmus sp. and Scenedesmus sp. showed that they were capable of using molecular hydrogen, for the reduction of nitrite to ammonia after an adaptation time of several hours (Kessler, 1957, 1964) under anaerobic conditions. In such algae and certain strains of Chlorella, the reduction of nitrite proceeded much faster than the reduction of nitrate. Stephenson and Stickland (1931) found that the bacteria containing the enzyme hydrogenase, was able to utilize molecular hydrogen. Woods (1938) found that 4 molecules of hydrogen were needed for the reduction of one nitrate molecule in Clostridium welchii and two strains of E. coli.

Among cyanobacteria, particularly in Anacystis nidulans, Anabaena cylindrica and Anabaena sp. 7119, NR and NiR were found to be associated with chlorophyll containing membrane fractions and utilize photosynthetically generated reducing power via ferredoxin (Manzano, 1976, 1977, 1978). Unsupplemented lamellar preparations of Anacystis nidulans can carry out stoichiometric photochemical reduction of nitrate with water as electron donor (Candau, Manzano and Losada, 1976). It is also observed that on an average and net basis the proportion of electrons

coming from water photolysis and required for nitrate assimilation is as much as about half of that required for the assimilation of carbon dioxide (Flowes, Ramos, Herrero and Guerrero, 1983). Hence, nitrate metabolism can be considered a 'genuine photosynthesis process' (Losada and Guerrero, 1979).

Ferredoxin is an electron donor for both enzymes of nitrate reducing systems in prokaryotic photosynthetic systems. But in eukaryotic photosynthetic systems, NiR is the only one which is Fd-dependant, as NR is a NAD(P)H dependant enzyme (Mendez, Guerrero and Vega, 1981).

Though the studies with Anacystis and Anabaena revealed that they could not use the molecular hydrogen in dark, Candau (1976) reported that the active stable particles from Anacystis could use water as electron donor for nitrite reduction both, in light and anaerobic conditions. It can be concluded finally, based on the experiments conducted with photosynthetic organisms, that the following electron donors are found to be effective as sources of reducing power for NiR activity (i) illuminated chlorophyllous particles, (ii) NAD(P)H, (iii) H_2-hydrogenase.

PURIFICATION AND CHARACTERIZATION:

Nitrite reductase from higher plants (Hucklesby, 1976; Vega, Kamin, Cardenas and Losada, 1977, 1980), green alga (Zumft, 1972), red algae (Ikawa and Nissizawa, 1976) have been purified to electrophoretic homogeneity by using conventional techniques which provided NiR preparations with specific activities of 50-110 U/mg protein. Ida (1977) purified spinach NiR by using an affinity chromatographic column, Fd-Sepharose.

NiR from Pseudomonas aeruginosa was reported to be purified upto 500 folds by a fractionation procedure using ammonium sulfate precipitation and $CuSO_4$ gel treatment by Nicholas (1960), NAD(P)H-NiR from Neurospora crassa (Greenbaum, Prodouz and Garrett, 1978); NADH-NiR from E. coli (Coleman, 1978) have been purified to homogeneity using various chromatographic techniques. Affinity chromatography on Blue-Dextran Sepharose appears to be a decisive step in purification. However, Greenbaum, Prodouz and Garrett (1978) got homogenous NiR preparation having a specific activity of 26.9 U/mg protein in Neurospora crassa. The highest specific activity reported from E. coli has been 5.3 U/mg protein. Higher specific

activities could not be achieved because of the instability of the enzyme (Coleman, Carnish, Bouden and Cole, 1979).

Fungal NiR was also found to be markedly unstable in vitro, probably because of its sensitivity to oxygen. An excellent protection to the enzyme was obtained by the inclusion of dithionite and FAD in all buffers used during purification procedures (Vega, Guerrero Leadbetter and Losada, 1973).

Reports on Fd-NiR from cyanobacteria are very scarce. In 1968 Hattori and Uesugi reported a 40 fold purified Fd-NiR from Anabaena cylindrica using acetone precipitation and chromatography on DEAE cellulose columns. In 1977, Manzano purified Fd-NiR upto 30 folds from Anacystis nidulans. Recently, Mendez and Vega (1981) reported a 763 fold purified NiR using ion exchange chromatography in Anabaena sp. 7119. However, the high content of phycobiliprotein which remains tightly bound to the enzyme during purification prevented further purification.

PROPERTIES:

The properties of NiR of photosynthetic organisms and non-photosynthetic organisms are discussed separately in view of their striking differences.

(i) Photosynthetic organisms: Ida and Morita (1973) showed that spinach NiR had a molecular weight of 72,000 daltons, and consisted of two equal subunits. However, Lancaster, Vega, Kamin and Orme-Johnson, Orme-Johnson, Kruger and Siegel (1979) reported that spinach NiR had a molecular weight of 66,000 daltons. It was found to be a monomeric protein, consisting of a single polypeptide chain made up of 600 amino acids. These results are in agreement with other reports on NiR molecular weight determination which were reported to vary between 61,000 daltons and 70,000 daltons as in *Chlorella* (Zumft, 1972); *Duneliella terticola* (Grant, 1970), *Anabaena* (Hattori and Uesugi, 1968). Mendez, Guerrero and Vega (1981), in *Anabaena* sp. 7119 reported NiR having a molecular weight of 52,000 daltons with a Stokes radius of 3.09 nm and a sedimentation coefficient of 4.07 S, which was similar to NiR from Anacystis (MW 54,000 daltons, Stokes radius 2.97 nm and a sedimentation coefficient of 4.6 S) (Manzano, 1977). The NiR from *Anabaena* sp. 7119 could be stored at -20°C for several months without significant loss in activity. Enzyme suspended in 50 mM Tris HCl, pH 7.5, containing 150 mM NaCl were stable at 2°C for a week. At room tempera-

ture, the half life of NiR was found to be approximately 40 hours. Repetitive freezing and thawing of enzyme or prolonged dialysis against low ionic strength buffer caused a significant loss in activity (Mendez and Vega, 1981).

The Km values differed from species to species. Ida and Morita (1973) found that K_m of leaf NiR in spinach varied from 100 to 300 µM of nitrite (Ramirez, Delcampo, Paneque and Losada, 1966). Bourne and Miflin (1973) reported an exceptional K_m of 2 mM with respect to nitrite in roots and leaves of barley. Hattori and Uesugi (1968) found that K_m values of NiR for nitrite, MV and Fd were 5×10^{-5} M, 2×10^{-4} M and 5×10^{-5} M respectively in Anabaena cylindrica. Other details regarding pH, subunits and K_m are given in Table 1.

Plant and algal NiRs were found to be specific for ferredoxin or its artificial substitute, methyl or benzyl viologen and could not utilize NAD(P)H directly (Creswell, Hageman, Hewitt and Hucklesby, 1965; Grant and Amy, 1970; Hattori and Uesugi, 1968; Hewitt, Hucklesby and Natton, 1976; Garrett and Amy, 1978; and Guerrero, Vega and Losada, 1981).

Table 1: Summary of the properties of Nitrite Reductases in biological systems

Source	Molecular weight (Kd)	Sub-units	Electron donor	Specific activity µ/mg	Purification (folds)	Nitrite Km µM	Prosthetic Groups Siroheme	4Fe-4S	pH optima	References
Spinacea oleratia	61	I	Fd	108	EH	360	I	I	7.5	Lancaster et al., 1979, Vega et al., 1980
Chlorella fusca	63	I	Fd	92	EH	NR	NR	R	7.5	Zumft, 1972
Porphyra yezoensis	63	NR	Fd	9	R	810	R	NR	7.5	Ho et al., 1976
Anabaena cylindrica	68	NR	Fd	10.7	40	50	NR	R	7.5	Vega and Garrett, 1975
Anabaena sp 7119	57	NR	Fd	21.5	763	NR	NR	R	7.4	Uesugi and Hattori, 1968
Anacystis nidulans	54	NR	Fd	R	30	R	NR	R	8.0	Mendez and Vega, 1981 Flores et al., 1983
Neurospora crassa	290	2	NAD(P)H	27	EH	10	R	NR	7.5	Menzano, 1977; Menzano et al., 1976.
Escherichia coli K 12	190	2	NADH	16.7	EH	5	R	R	8.0	Coleman et al., 1978

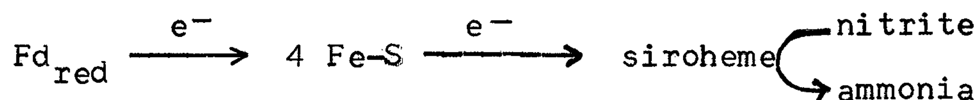
R - Reported, data not available; NR - Not reported; EH - Electrophoretic Homogeneity.

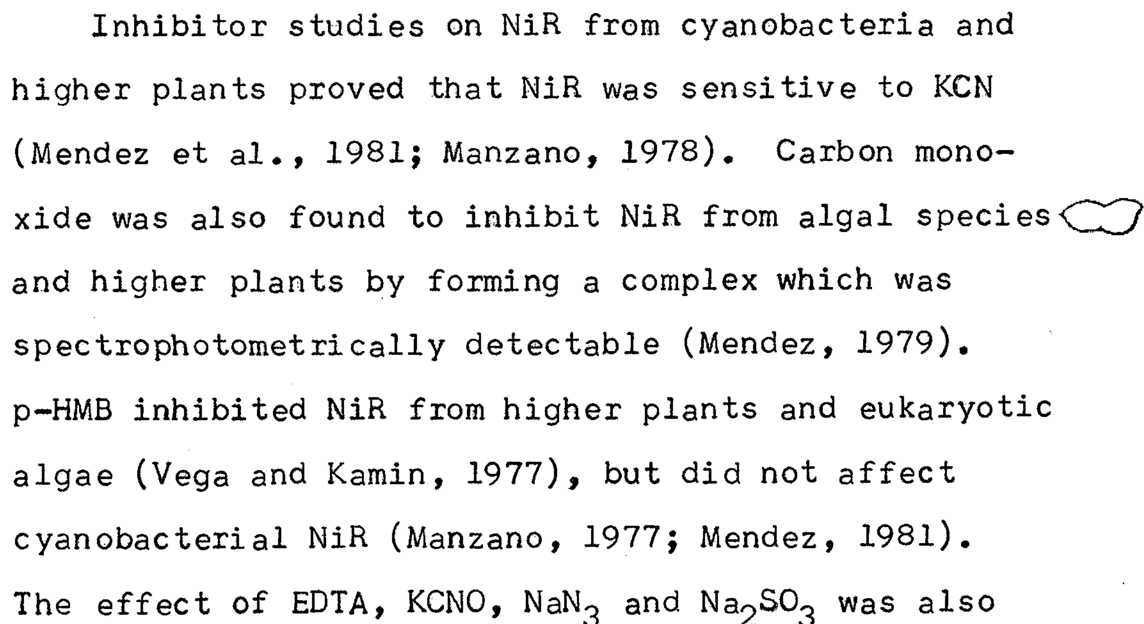
NiRs from photosynthetic systems have generally been reported to contain a heme prosthetic group which is of the siroheme type. It has an iron-tetrahydroporphyrin group of the isobacterochlorin type with eight carboxylic acid side chains (Murphy Siegel, Tove and Kamin, 1974). A similar prosthetic group is characteristic of assimilatory and dissimilatory sulfite reductases.

Iron is an essential element for NiR synthesis. The purified enzyme shows characteristic absorption peaks at 380-390 nm and 570-580 nm and contains 3 atoms of iron/molecule of enzyme. The iron content has been estimated to be 2 Fe atoms/molecule in Cucurbita pepo (Hucklesby, James, Banwell and Hewitt, 1976) and Chlorella (Zumft, 1972). Vega and Kamin (1977) showed the presence of 3 Fe atoms/ molecule of enzyme in spinach. However, ESR studies indicated that there were 5 Fe atoms in spinach, where one was in the siroheme, and 4 were present in the form of Fe-S centres (Lancaster et al., 1979; Guerrero, Vega and Losada, 1981). In higher plants in addition to siroheme, there is an Fe-S centre acting as a prosthetic group (Aparacio, Knaff and Malkin, 1975; Commack, Hucklesby and Hewitt, 1978; Mendez Herrero and Vega, 1981).

Experiments with <u>Anabaena</u> sp. 7119 suggested the association of a heme chromatophore with the enzyme. The presence of Fe-S centres was also suggested by observing the close association of acid-labile sulfide in NiR activity (Mendez and Vega, 1981). The cyanobacterial NiR resembles that of higher plants in many respects including the nature of prosthetic group (Flores, Ramos, Herrero and Guerrero, 1983).

Kinetic studies have suggested that nitrite was bound to siroheme and was reduced to ammonia. The pathway of electron flow to nitrite via NR was represented as follows:





Inhibitor studies on NiR from cyanobacteria and higher plants proved that NiR was sensitive to KCN (Mendez et al., 1981; Manzano, 1978). Carbon monoxide was also found to inhibit NiR from algal species and higher plants by forming a complex which was spectrophotometrically detectable (Mendez, 1979). p-HMB inhibited NiR from higher plants and eukaryotic algae (Vega and Kamin, 1977), but did not affect cyanobacterial NiR (Manzano, 1977; Mendez, 1981). The effect of EDTA, KCNO, NaN_3 and Na_2SO_3 was also

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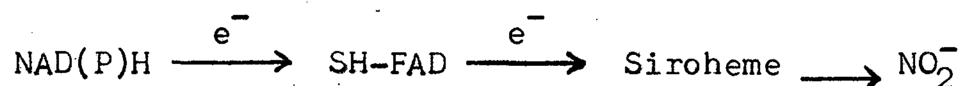
insignificant in Anabaena (Mendez, 1981). However, hydroxylamine and sulfite to a lesser extent, were competitive inhibitors of Anabaena NiR with respect to nitrite.

(ii) Non-photosynthetic organisms: NiRs of non-photosynthetic organisms differ from that of photosynthetic organisms in having higher molecular weight, FAD as cofactor and dependence on pyridine nucleotides for reducing power. Prakash and Sadana (1972) and Siegel, Murphy and Kamin (1973) studied the NiRs from Achromobacter fischeri and Escherichia coli and determined the molecular weight as 95,000 d and 190,000 d respectively. E. coli NiR had 12-20 Fe atoms and 12-15 acid labile sulfate groups (Siegel, 1971). NiR from Azotobacter had a single polypeptide (67,000 d) and a disassociable FAD and utilized NADH (Guerrero, Losada and Leadbetter, 1973). Cresswell, Hageman and Hewitt (1962) found that K_m of E. coli NiR was lower than 40 μM. Experiments with Achromobacter and Azotobacter showed that NiR in these systems had K_ms of 50 μM and 5 μM towards nitrite, respectively (Vega, Guerrero, Leadbetter and Losada, 1973). Neurospora crassa NiR had a K_m of 10 μM for nitrite (Garrett and Amy, 1978; Greenbaum, Prodoux and Garrett, 1978).

In yeast and N. crassa NiR was found to have molecular weights of 350 and 290 Kd respectively. The nNiR from N. crassa had two similar subunits of 140 Kd and contained FAD, siroheme and probably Fe-S centres as prosthetic groups. Lafferty and Garrett (1974) reported that NiR required FAD to utilize NADPH or NADH as electron donor. The siroheme was the centre of interaction of nitrite on the enzyme, where the conversion of nitrite to ammonium took place. No free intermediates were observed (Garrett, 1978).



$$NAD(P)H \xrightarrow{e^-} SH\text{-}FAD \xrightarrow{e^-} Siroheme \longrightarrow NO_2^-$$

Inhibitor studies on NADPH-NiR revealed that sulfite, arsenate and cyanide inhibited NAD(P)H-NiR competitively (Coleman, 1978). CO and pHMB were also found to inhibit NAD(P)H dependant NiR (Vega, 1970, 1978 and 1981).

The interconversion between an active and inactive form of NR has been confirmed, but needs further conformation for NiR. However, this process seems to be of general metabolic significance and is apparently related to a redox change of the enzyme protein. Losada (1973) showed that inactivation of NiR in Azotobacter could be prevented by nitrite, but not by nitrate or ammonium.

MATERIALS AND METHODS

Chemicals:

Tris (pH 7-9), Methyl viologen, NADH, NADPH, FMN and Tetra zolium salt were obtained from the Sigma Chemical Company, St. Louis, M.O., USA. Sephadex G-25 was purchased from Pharmacia Fine Chemicals. Folin's reagent was purchased from Chemicals Division, Patel Chest Institute, Delhi. N-(1-Naphtyl) ethlenediammonium dichloride (NED) and sulfanilamide were obtained from Loba-Chemie-Indoaustranal Co., India. All other chemicals used were of analytical grade. Culture of blue-green alga *Spirulina platensis* was taken from the stock maintained at the Microbiology Laboratory, School of Life Sciences, Jawaharlal Nehru University.

Growth:

Spirulina platensis was grown in prescribed medium (Menon and Varma, 1979). The medium contained in gm/l: $NaHCO_3$, 18; K_2HPO_4 0.5; $NaNO_3$, 2.5; K_2SO_4, 1.0; NaCl, 0.1; $MgSO_4 \cdot 6\ H_2O$, 0.2; $CaCl_2$, 0.4; $FeSO_4 \cdot 7\ H_2O$, 0.01; EDTA, 0.08; and A_5 solution 1 ml.

A_5 solution contained in gm/l: Boric acid, 2.9; $MnCl_2$, 1.81; $ZnCl_2$, 0.11; $CuSO_4$, 0.01; ammonium molybdate, 0.18. pH of the medium was maintained between 9-10. Cultures were grown in 4 litres Hafkins flask with constant bubbling of sterile air at a temperature of 28 $\pm$ 1°C and under constant illumination.

Harvesting of Cells:

Cultures in the mid-log phase of growth (10 to 12 day old) were harvested by vacuum filteration through a Buchner funnel using a Whatman No.1 filter paper. Cells filtered from one litre of medium were washed with 200 ml of phosphate buffer (pH 7.4, 10 mM).

Preparation of cell-free extract:

All preparative procedures henceforth were carried out at 0-4°C, unless otherwise mentioned. Harvested cells were suspended in phosphate buffer (pH 7.4, 10 mM) at a ratio of 1:10 (W/V). Cell suspensions were disrupted at 12 Kcs/sec, 20 KH_z using a MSE 150 W ultrasonic disintegrator fitted with a titanium probe. The sonicated cell suspension was centrifuged at 33,000 g for one hour using a Sorvall RC-5 centrifuge. The supernatant, S_{33}

was then ultracentrifuged at 140,000 g for 1.5 h in a Beckman ultracentrifuge. The supernatant from this step was designated as S_{140}.

Desalting of the enzyme preparation:

5 ml batches of S_{140} samples were loaded on to a Sephadex G-25 column (2 x 12.5 cm) pre-equilibrated with phosphate buffer (pH 7.4, 10 mM) containing 1 µM Methyl Viologen. The samples were eluted with the same buffer and 2 ml fractions were collected and assayed for enzyme activity. The enzyme activity was found to be stable for 8 weeks at $0^{o}C$. The desalted samples were used for all further experiments.

Assay procedure for Nitrite reductase:

NiR activity was assayed by the modified method described by Ramirez, del-Campo, Paneque and Losada (1966). Assay mixture contained in a final volume of 1 ml: 0.7 ml phosphate buffer (10 mM, pH 7.4); 0.1 ml desalted enzyme containing 100-130 µg protein; 0.05 ml MV (15 mM); 0.05 ml $NaNO_2$, 7.5 mM; $Na_2S_2O_4$ (25 mg/ml neutralized with 0.29 M $NaHCO_3$). Control consisted of reaction mixtures in which MV was deleted and substituted with equivalent quantity of double glass distilled water. The reaction was initiated by adding $Na_2S_2O_4$ (0.1 ml). $Na_2S_2O_4$ and MV

were prepared freshly each day for experiments. The enzyme assay was carried out at 40°C for 30 minutes in a rotatory water bath. The reaction was stopped by vigorous stirring using a cyclomixer, after which 0.1 ml of this mixture was transferred to test tubes containing 1.9 ml of distilled water. The tubes were shaken over a cyclomixer to oxidise the excess $Na_2S_2O_4$ and left for 20 minutes. 0.5 ml of 1 per cent sulfanilamide (prepared in 1.5 N HCl) and 0.5 ml of 0.02 per cent NED were added and thoroughly mixed. Color development was carried out for 15 minutes after which the optical density was read at 540 nm using a Shmadzu spectrophotometer (UV 240).

Protein assay:

Protein was assayed by the method of Lowry, Rosenbrough, Farr and Randall (1951) using Bovine Serum Albumin as standard (Fig 2).

PROCEDURES FOR VARIOUS EXPERIMENTS

(1) Localization of Nitrite Reductase:

For localizing NiR in *Spirulina*, cells were harvested, sonicated for 2.5 min and the homogenate was centrifuged at 33,000 g for one hour. The resultant supernatant (S_{33}) and pellet (P_{33}) were assayed for enzyme activity. Enzyme was localized in the

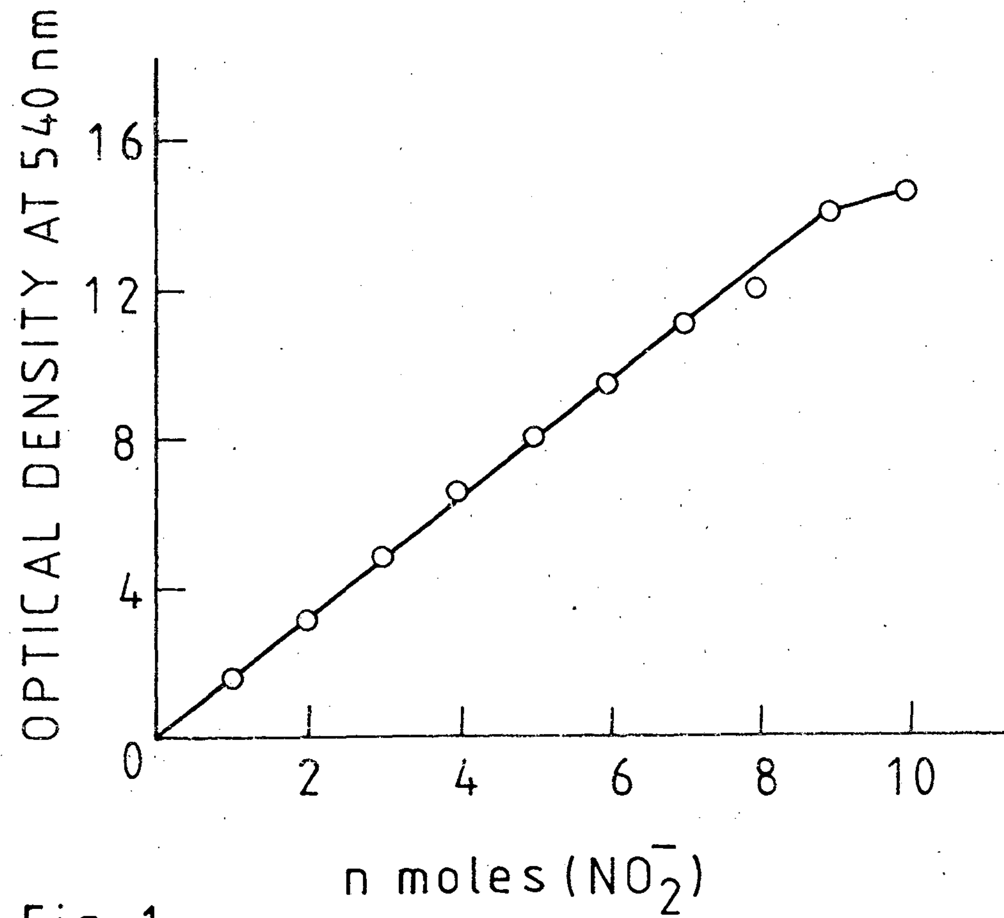


Fig. 1.

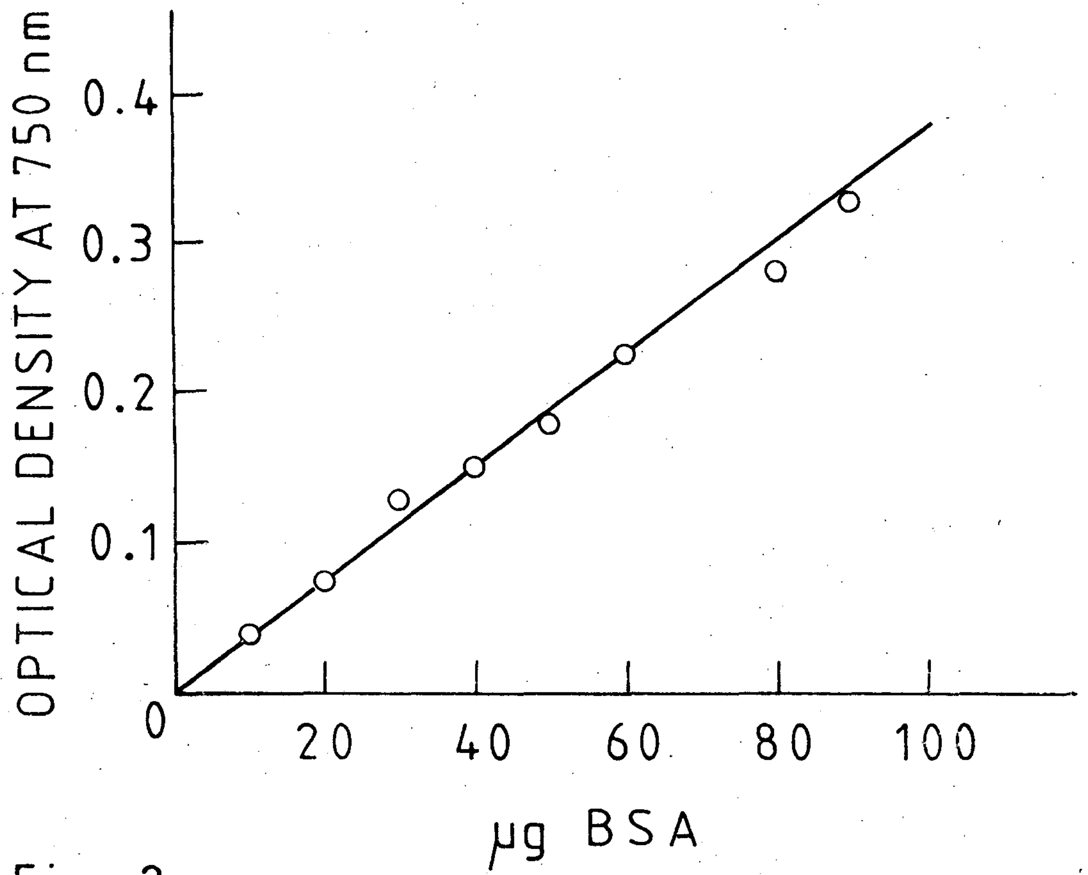


Fig. 2

supernatant fraction. The S_{33} fraction was then ultracentrifuged at 140,000 g for 1.5 h and the resultant pellet (P_{140}) and supernatant (S_{140}) were assayed for NiR activity.

(2) Effect of varying sonication periods:

40 ml cell suspension (maintained at 0–4°C) was sonicated 12 Kcs and 5 ml samples were withdrawn after 30, 60, 90, 150, 210, 270 and 330 seconds interval. Samples were centrifuged at 33,000 g and the NiR activity was assayed both, in supernatant and pellet fractions.

(3) Effect of buffers:

To determine suitable buffer for extraction procedures as well as enzyme assay, two batches of cells were sonicated in phosphate buffer (10 mM, pH 7.4) and Tris-HCl (pH 7.4, 10 mM) respectively. The cell homogenate was centrifuged as described before and the supernatants were used for NiR assay. The enzyme assay was carried out in two different types of reaction mixtures, one containing Tris-HCl buffer (pH 7.4, 10 mM) and the other containing phosphate buffer (pH 7.4, 10 mM) and both crude extracts were tested for NiR activity.

(4) Effect of varying incubation periods:

To determine the incubation period which gave enzyme activity in its linear phase of reaction, enzyme reactions were carried out for varying incubation periods, 5, 10, 20, 25, 30, 40, 50, 60, 120 and 240 minutes. Enzyme activity after these incubation periods were assayed as described before.

(5) Effect of temperature on NiR activity:

For the determination of optimal temperature for NiR activity, enzyme reaction was carried out at 15, 20, 30, 35, 40, 45, 50 and 55°C using reaction mixtures pre-incubated at the specified temperatures. NiR activity was assayed after 30 min. Results were expressed in terms of nmoles NO_2^- reduced/mg protein/ 30 min.

(6) Effect of pH on enzyme activity:

Reaction mixtures of varying pH were prepared by using phosphate buffers of pH 6, 6.4, 6.8, 7, 7.2, 7.4, 7.6, 7.8, 8 and 9 in the reaction mixtures. Enzyme activity was assayed after 30 minutes of incubation at 40°C.

(7) Protein concentration:

To determine optimal protein concentration of enzyme extract, which contained suitable amount of

enzyme, and responded linearly to the amount of substrates in the normal reaction mixture; the enzyme samples with protein concentration of 0.21 mg/ml were diluted in such a way that the extracts had 20, 42, 84, 133, 150, 170, 190 and 210 µg of protein/0.1 ml. 0.1 ml of these extracts were used for NiR assay.

(8) Effect of different protectors during desalting:

To determine the effect of different protectors during desalting, 5 ml aliquots of S_{140} samples were loaded onto a Sephadex G-25 column (prepared as described before) and eluted with phosphate buffer containing (i) methyl viologen 1 mM, (ii) cysteine, 1 mM, (iii) EDTA, 0.2 mM and (iv) EDTA plus cysteine and (v) buffer with no additive. The column was pre-equilibrated each time with buffer containing different protectants, before eluting the enzyme sample with the buffer containing the protectant. Two ml samples were collected and assayed for NiR activity.

(9) Determination of suitable electron donor:

For studying the effect of different electron donors on NiR activity, reaction mixtures were prepared containing 0.05 ml, NADH (0.3 mM); 0.05 ml NADPH, (0.3 mM); 0.1 ml $S_2O_4^-$ (14 mM) plus 0.05 ml

methyl viologen (15 mM) and 0.1 ml $S_2O_4^-$ plus FMN 0.05 ml (0.3 mM) as electron donors. Enzyme activity was assayed by adding equal quantities of desalted S_{140} samples.

(10) Optimal concentration of methyl viologen for NiR reaction:

To determine optimal concentration of MV, different initial concentrations of MV, 1, 2.5, 5, 7.5, 10, 12.5, 15, 20 and 30 mM were prepared from a stock of 30 mM and added to reaction mixtures. Enzyme activity was assayed using equal volumes of desalted S_{144} samples, at 40°C.

(11) Effect of varying nitrite concentration:

Reaction mixtures containing the following initial concentrations of $NaNO_2$ were prepared from a 20 mM stock, 1.5, 2, 2.5, 3, 4, 5, 7.5 and 10 mM. Using these substrates concentrations, enzyme reaction was carried out as described before. From the data obtained, a v (nmoles of nitrite reduced/mg protein/hr) vs s (concentration of NO_2) was plotted. With the same results a double reciprocal plot (I/v vs I/s) was drawn to find the K_m^{app} of NiR for nitrite.

(12) Dependence of NiR Synthesis on Iron:

Iron free medium (usually containing 2 x 10^{-2} µM Fe as contamination) was prepared. Fresh *Spirulina*

cells (0.17 g) washed thoroughly with iron free medium was transferred to one litre of iron free medium containing 8 mM NH_4Cl as sole nitrogen source and allowed to grow under continuous illumination and bubbling of air. The growth curve was measured by checking the optical density of cells at 420 nm, after every 24 h interval. Cells in the mid-log phase (8 days) were harvested and were transferred to media (30 mM $NaNO_3$ as nitrogen source) containing iron at varying concentrations, 5, 20, 36, 50, 100 μM, and the culture was grown under conditions described before. NiR activity was assayed in these cultures after 2, 4 and 8 days. NiR was also assayed in iron free medium grown cells and which served as control.

RESULTS

(1) Localization of enzyme: Nitrite reductase activity was primarily recovered from the supernatant fractions (S_{33} and S_{140}) of the cell homogenate. Very little activity was detected in sedimentable fractions (Table 2). Nitrite reductase thus seems to be a soluble protein. The supernatant, S_{140} was desalted using a G-25 column and approximately 2.5 fold purification was achieved (Table 2).

(2) Effect of varying sonication periods: A sonication period of 2.5 min yielded NiR with highest specific activity (Fig 3). Beyond 2.5 min a rapid drop in specific activity was observed. For further extraction procedures, cells were sonicated for 2.5 min.

(3) Effect of varying incubation periods: Enzyme assay was carried out for time periods varying from 5 min to 40 min (Fig 4). The assay was linear upto 30 min after which it reached a plateau. Consequently, all reactions were run for 30 min in a reciprocating water bath, which was in the linear phase of the reaction rate.

(4) Effect of temperature: Highest enzyme activity was recorded at 40°C, above this incubation temperature, a

decline in activity was recorded (Fig 5). All experiments were carried out at 40°C in a preadjusted water bath shaker.

(5) Effect of varying pH: Optimal enzyme activity was obtained at a pH of 7.4 (Fig 6). Reaction mixture for all future experiments were made using phosphate buffer (K^+/K^{++}) at a pH of 7.4. A rapid decline in activity was noticed beyond a pH of 8.0.

(6) Effect of varying protein concentrations: The reaction was found to be linear with increasing concentrations of protein upto 130 µg/ml of reaction mixture. Beyond this concentration, saturation was achieved (Fig 7). Rest of the work was done using enzyme sample in the range of 100-130 µg/ml protein per reaction mixture.

(7) Selection of buffer during extraction procedure and enzyme assay: The enzyme preparations isolated and assayed in phosphate buffer invariably yielded higher (100 per cent) enzyme activity (Table 6). The same enzyme extract when incubated with Tris-HCl in the assay mixture showed a sharp decline (about 50 per cent) in activity as compared to the former set. Enzyme sample prepared in Tris-HCl buffer also showed almost 40-50 per cent decrease in NiR activity

irrespective of the buffers used in the reaction mixture. Our results have clearly indicated that phosphate buffer was suitable for both, enzyme preparation as well as its activity.

(8) Effect of different protectors during desalting: Table 4 shows the effect of various enzyme protectors used during elution procedures of the enzyme extract. Methyl viologen (1 µM) was found to provide maximum protection to the enzyme, as revealed by highest recovery. There was almost a 76 per cent increase in the activity as compared to the sample eluted in phosphate buffer containing no protectant. Cysteine alone, or in a combination with EDTA yielded a marginal increase in activity, whereas, EDTA alone was found to decrease the recovery. Hence, during desalting procedures on G-25 Sephadex, the buffer was invariably supplemented with 1 µM Methyl viologen.

(9) Determination of electron donor: NiR in *Spirulina* sp. was found to be a MV dependant enzyme. No activity was obtained when NADH or NAD(P)H was used as the electron donor. FMN in combination with sodium dithionite did not yield any enzyme activity (Table 5). For experiments, a combination of 14 mM $Na_2S_2O_4$ and 15 mM MV was used as the electron donor.

(10) Effect of varying concentrations of MV: A V vs S plot of increasing methyl viologen final concentrations vs nitrite reduced/mg protein/hr showed a linear trend upto a MV concentration of 1.5 mM. Michaelis Menten plot (I/v vs I/s) showed a K_m^{app} of 4×10^{-4} M for MV (Fig 8).

(11) Effect of varying substrate concentrations: Fig 9 shows a 1/v vs 1/s between concentration of nitrite added as substrate and enzyme activity. From this a plot a K_m^{app} of 3×10^{-4} M was calculated. A v vs s plot showed a v max of 300 nmoles/mg protein/hr.

(12) Dependence of NiR synthesis on the iron in the culture medium: Our data presented in Fig 10 (a,b) indicated that the presence of iron in the culture medium was essential for *in vivo* production of NiR in *Spirulina platensis*. In this experiment, the cells were initially grown on an iron deficient medium, in the presence of 8 mM NH_4Cl as sole nitrogen source, which sharply repressed synthesis of NiR (Fig 10 a). After 8 days, the washed cells with low NiR activity were transferred to a fresh media containing 30 mM nitrate as the sole nitrogen source and specified amounts of iron ($FeCl_3$), as indicated. After 2, 4, (Fig 10 a) and 8 days of growth (Fig 10 b) in iron enriched media, the cells were harvested, washed and

tested for NiR levels. It was observed that cells grown from 36 to 50 μM iron concentrations in the culture media had the highest NiR activity (Fig 10 b). It is interesting to note that at 100 μM Fe^{3+} concentration, enzyme activity showed a sharp decline after the eighth days of growth.

Table 2 : Distribution of NiR activity in cell-free extracts from *Spirulina platensis*

Cell-free extracts	Specific activity (nmoles NO_2 reduced/ mg protein/30 min.)	Relative activity (percent)
Supernatant -33	210	100
Supernatant -140	315	150
Pellet -33	18	9
Pellet -140	15	7

Cells grown with 30 mM of nitrate as sole nitrogen source was harvested and resuspended in 10 mM phosphate buffer (pH 7.4) in a ratio of 1:10 (W/V). They were disrupted by sonication at 4°C. The cell homogenate was clarified at 33,000 g for 60 min. The pellet was resuspended in the same buffer (W/V = 1:10). The supernatant S_{33} was then spun at 140,000 g for 90 min. The pellet was again suspended in phosphate buffer (10 mM, pH 7.4). MV-NiR activity was assayed in all the fractions. In controls, MV was omitted. Specific activity was defined as nmoles of nitrite utilized per mg of protein per 30 min. Pellet suspensions were made in the ratio of 1:10 (W/V).

Table 3: Partial Purification of Nitrite reductase

Fraction	Specific activity (nmoles NO_2 reduced/mg protein/30 min.)	Fold purification
S_{33} (33,000 g)	200	1.0
S_{140} (140,000 g)	304	1.5
Sephadex G-25	466	2.33

Sephadex G-25 column (12.5 x 2 cms) was pre set at 4°C. column was equilibrated and eluted using phosphate buffer (10 mM, pH 7.4) containing 1 µM methyl viologen.

Table 4: Effect of various enzyme protectors on NiR levels

Protectants added	Concen-tration (mM)	Specific activity (nmoles NO_2 reduced/mg protein/30 min)	Relative Activity (percent)
Extraction buffer	10	288	100
+ MV	0.001	436	176
+ EDTA	0.2	272	90-
+ Cysteine	1.0	312	140
+ EDTA + Cysteine	0.2+1.0	328	115

5 ml fractions of S_{140} sample was desalted through a G-25 column (12.5 x 2 cms) using phosphate buffer (10 mM, pH 7.4) containing the protectors at specified concentrations. Buffer without any protectant served as base value (100 per cent).

Table 5: Determination of ideal electron donor for NiR activity

Electron donor	Concentration (mM)	nmoles NO_2 reduced/30 min.	Relative activity (Percent)
NADH	0.3	0.1	5.0
NADPH	0.3	0.01	0.5
MV	15.0	0	0
$S_2O_4^{=}$	14.0	0.3	15
+ MV	15.0	2.2	100
+ FMN	0.3	0.3	16

The reaction mixture included in a final volume of 1 ml: desalted enzyme (100 µg protein); 7 µmoles phosphate buffer (pH 7.4), 7.5 mM NO_2 and the different electron donors and cofactors at indicated concentrations. After running the reaction for 30 min at 40°C, the unconsumed nitrite in the reaction mixture was measured/each reaction mixture. Data expressed are the average values obtained by three independent experiments.

Table 6: Selection of buffer for extraction and enzyme assay

Extraction buffer (pH 7.4)	Buffer used in assay (pH 7.4) 7 µmoles/Reaction mixture	Specific activity	Relative activity (percent)
Potassium phosphate (K^{++}/K^{+})	Potassium phosphate	250	100
	Tris-HCl	130	52
Tris-HCl	Tris-HCl	113	45
	Potassium phosphate	173	69

Cells were harvested, washed and suspended in either potassium phosphate or Tris-HCl buffer (1:10 /W/V). Cell suspension was sonicated and centrifuged at 33,000g. These two sets of enzyme preparations were tested for NiR activity using reaction mixtures containing 7 µmoles of either Tris-HCl or phosphate buffer (pH 7.4). Specific activity was expressed as nitrite utilized/mg protein/ 30 min.

Fig 3: Effect of varying sonication periods: Algal cells were sonicated for various periods and batches of 5 ml aliquots were withdrawn. The suspension was centrifuged at 33,000g for one hour. The S_{33} fractions were assayed for NiR activity.

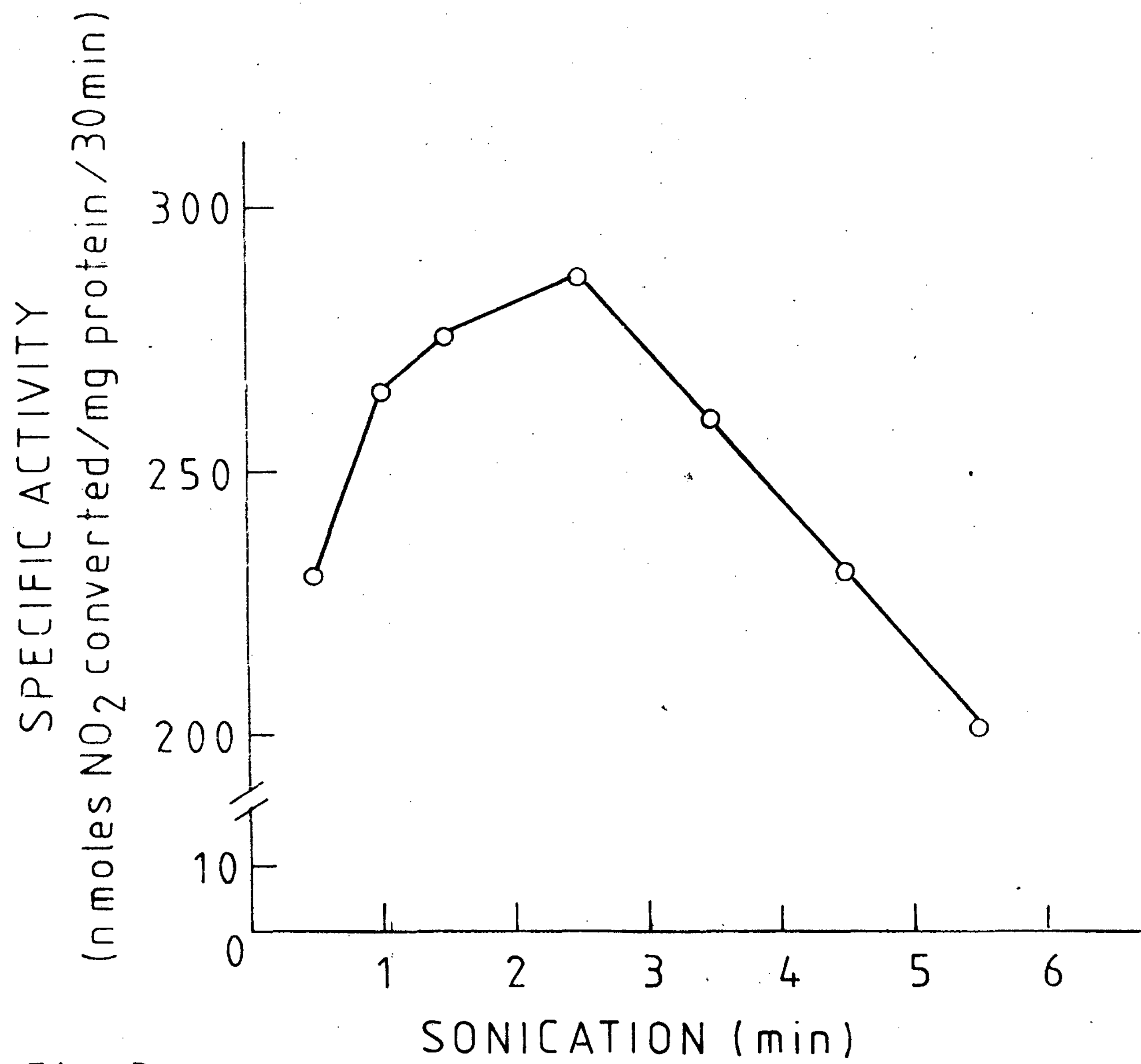


Fig. 3

Fig 4: Effect of varying incubation periods on NiR activity: For incubation experiments, S_{140} fractions were used. The reaction mixtures were incubated for specified time intervals which varied from 5 to 240 min.

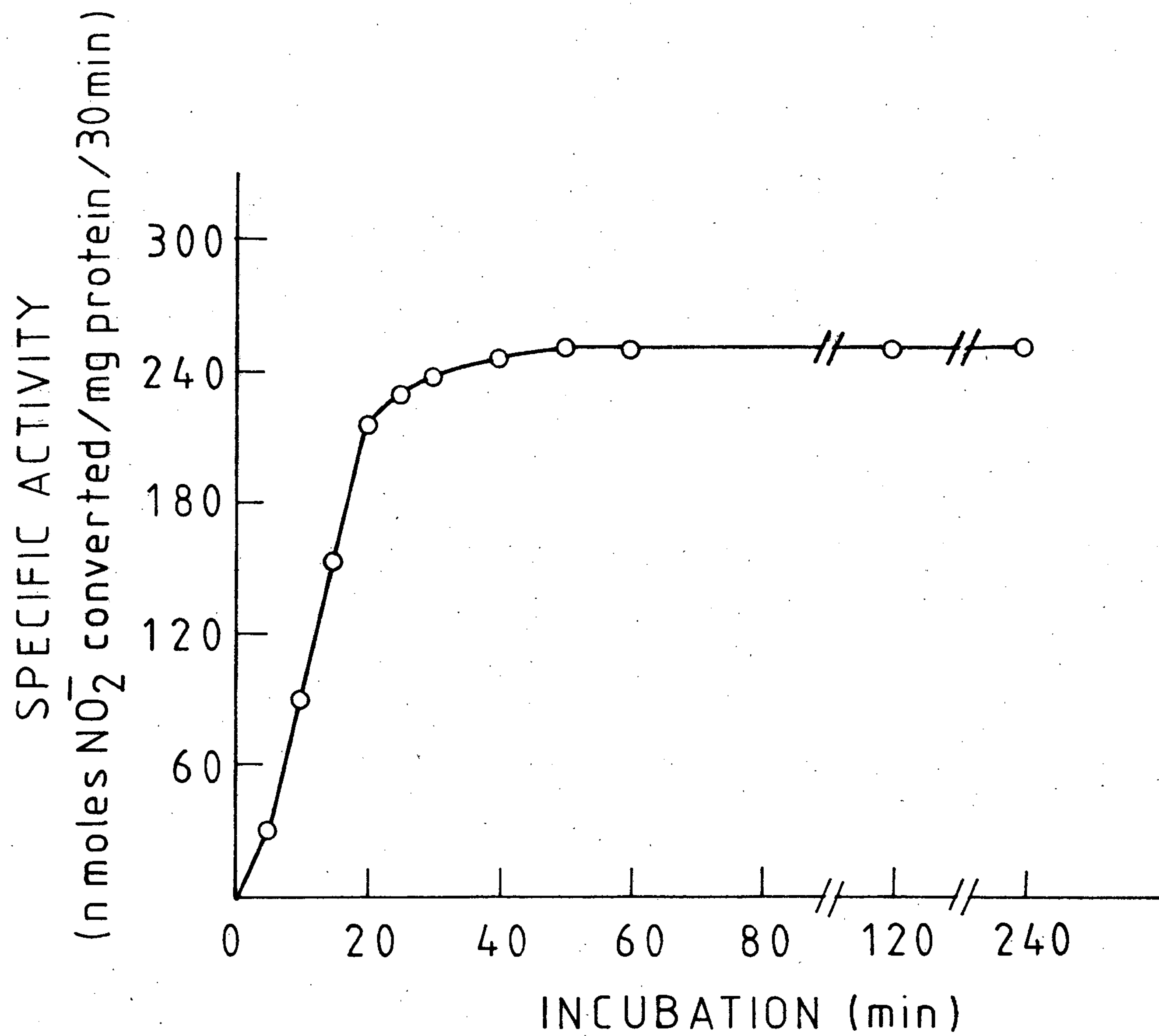


Fig. 4

Fig 5: Effect of Temperature on Enzyme Activity: The specified temperatures which varied from 15 to 60°C were set in reciprocating water bath shaker. After stabilizing the temperatures, the reaction was carried out for 30 minutes. Other details are as in Materials and Methods.

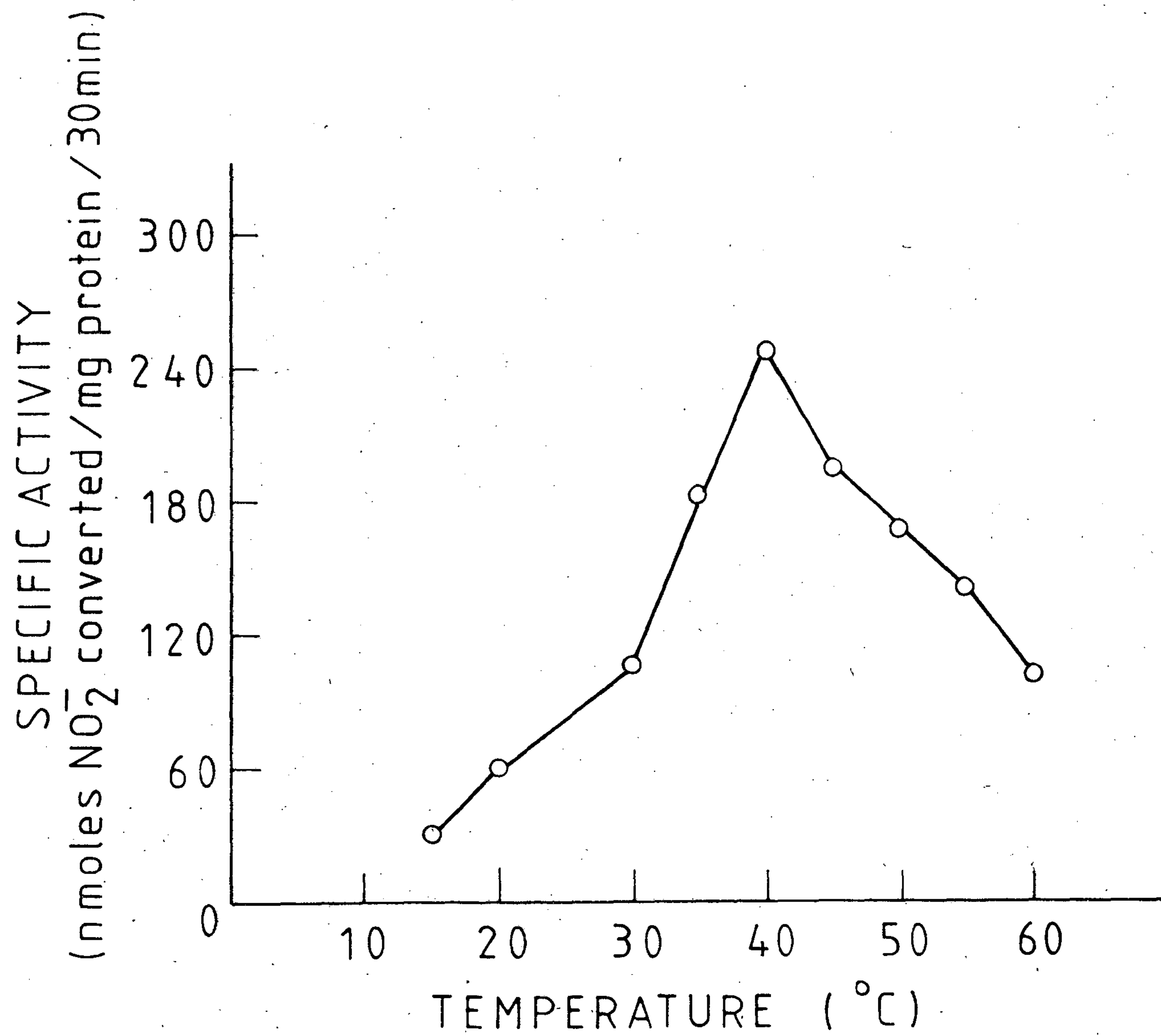


Fig. 5

Fig 6: Effect of pH on NiR activity: Reaction mixture using 10 mM phosphate buffer, adjusted in the pH range of 6-9 were prepared. Incubation mixtures were incubated at 40°C for 10 min. before the reaction was initiated.

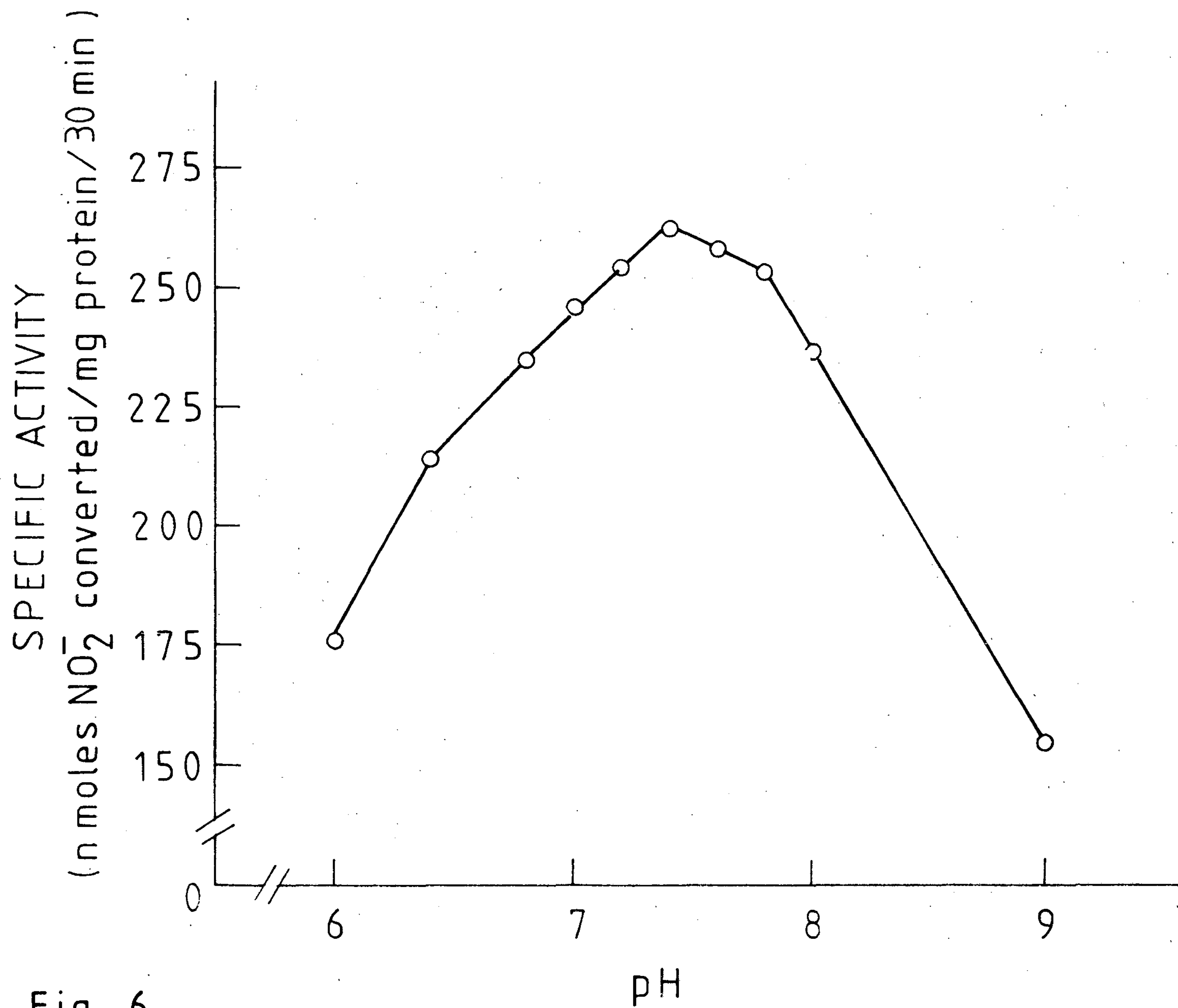


Fig. 6.

Fig 7: Influence of protein concentration on NiR activity: Protein obtained from desalted enzyme preparation was adjusted to specified concentrations by diluting the stock enzyme extract with phosphate buffer. The range of protein concentration tested in our studies varied from 10 µg to 210 µg per reaction mixture.

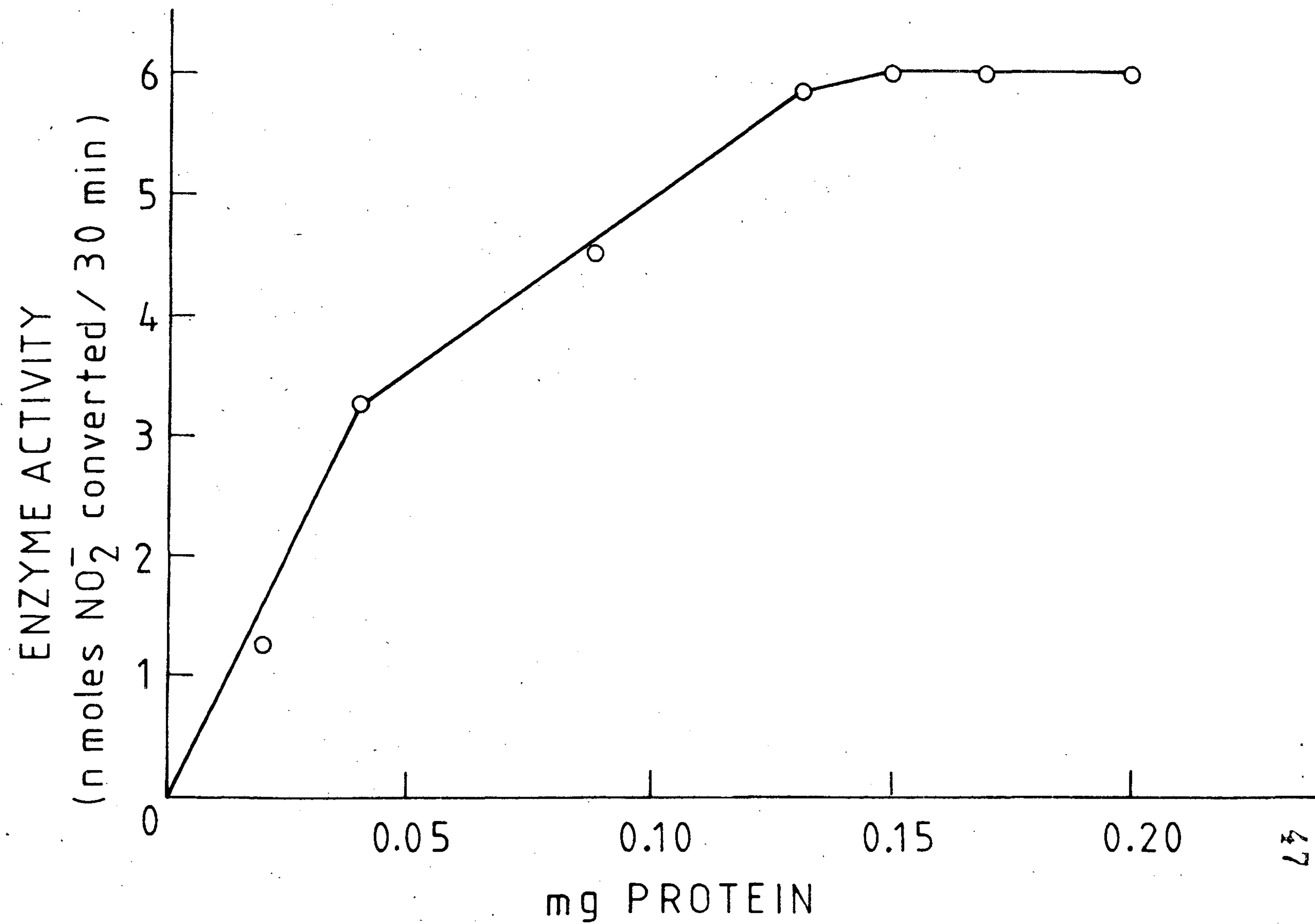


Fig. 7

Fig 8 : MV concentrations-Enzyme action: Reaction mixtures containing (mM) 0.1 to 2.0 of MV (final concentrations) were assayed. Using Michaelis Menten (double reciprocal plot) (1/v vs 1/s) the K_m values were calculated.

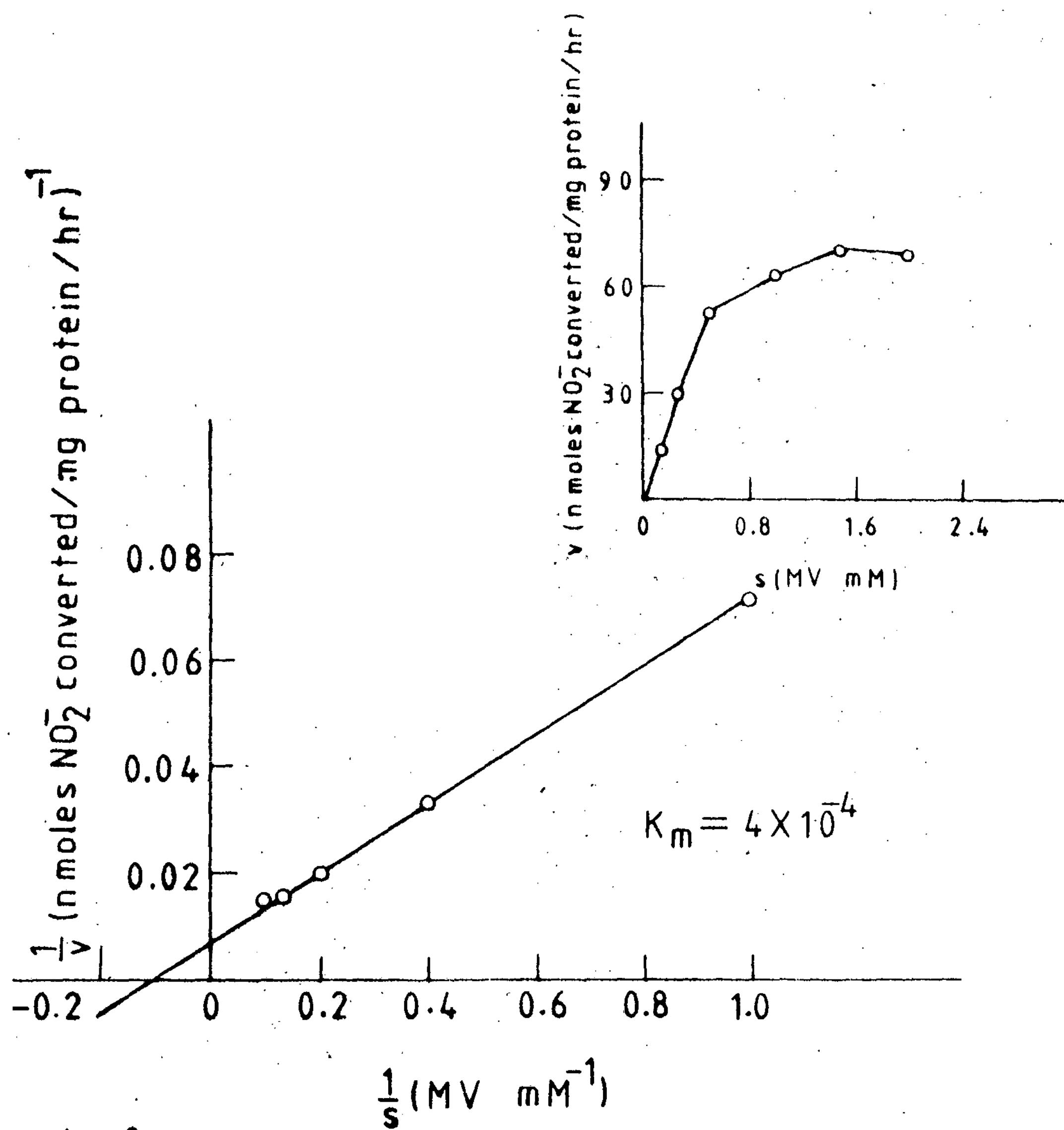


Fig. 8.

Fig 9: Nitrite concentration and its effect on NiR activity: A Michaelis Menten plot was prepared using the values obtained from the assay of reaction mixtures containing nitrite at specified final concentrations ranging from 0.1 to 1.0 mM. The K_m^{app} for nitrite was calculated. The velocity was defined as specific activity (nmoles nitrite reduced/mg protein/h).

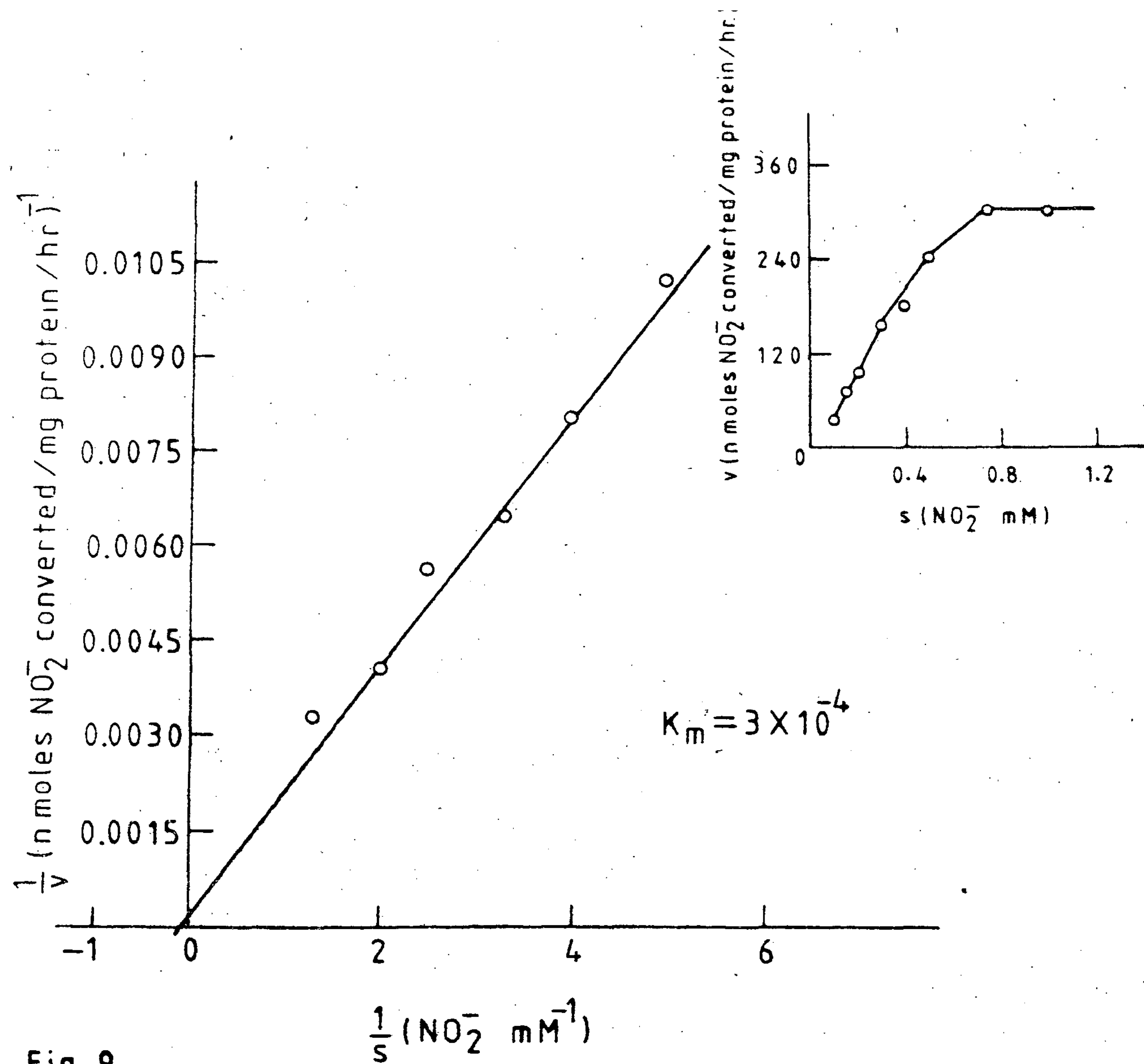


Fig. 9

Fig 10 a,b: Iron dependance for enzyme synthesis: Spirulina cells washed with iron-free medium were inoculated and grown in an iron deficient medium for 8 days with 8 mM NH_4Cl as sole nitrogen source. After this incubation, the culture was harvested, washed with cold phosphate buffer and the cells were transferred to batches of media containing varying iron concentrations i.e. (µM) 5, 20, 36, 50 and 100 and 30 mM $NaNO_3$ as nitrogen source. A portion of the culture was tested for NiR level. The algal cultures were allowed to grow under normal growth conditions as described in Materials and Methods. Cells were used for NiR studies after 2,4 (Fig. 10a) and 8 days (Fig 10b).

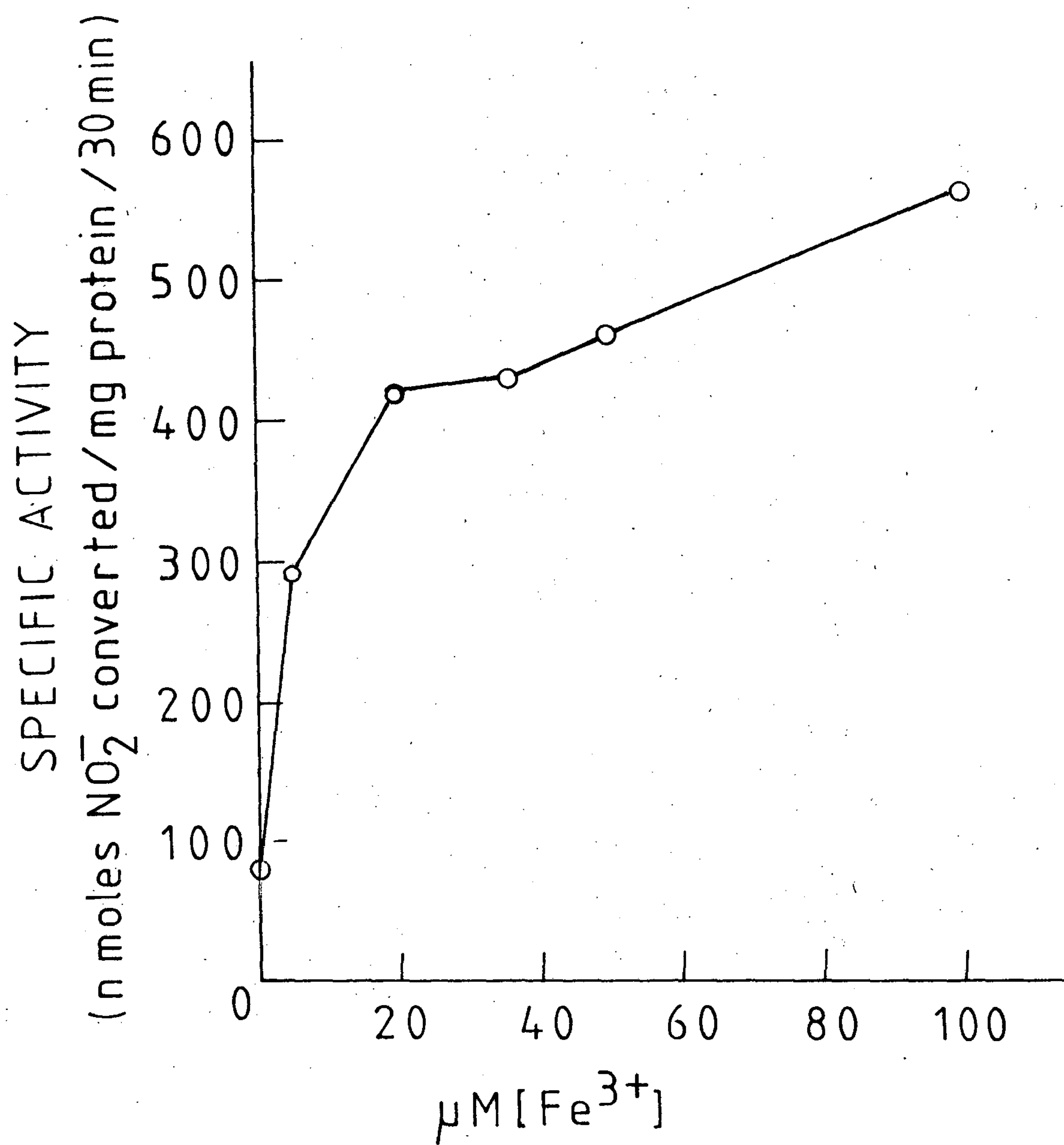


Fig. 10. a

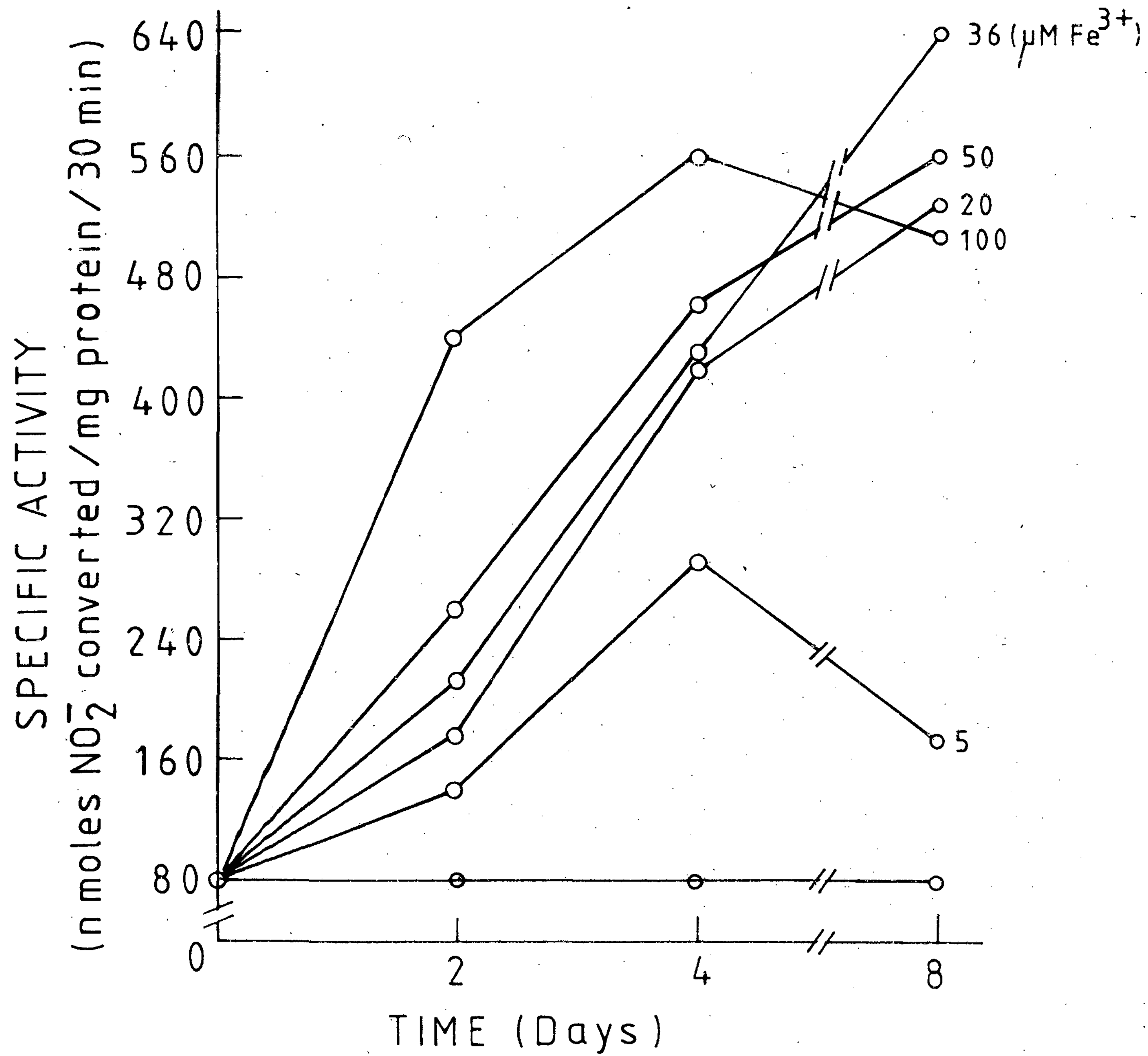
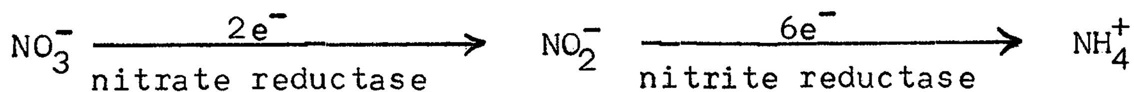


Fig. 10. b

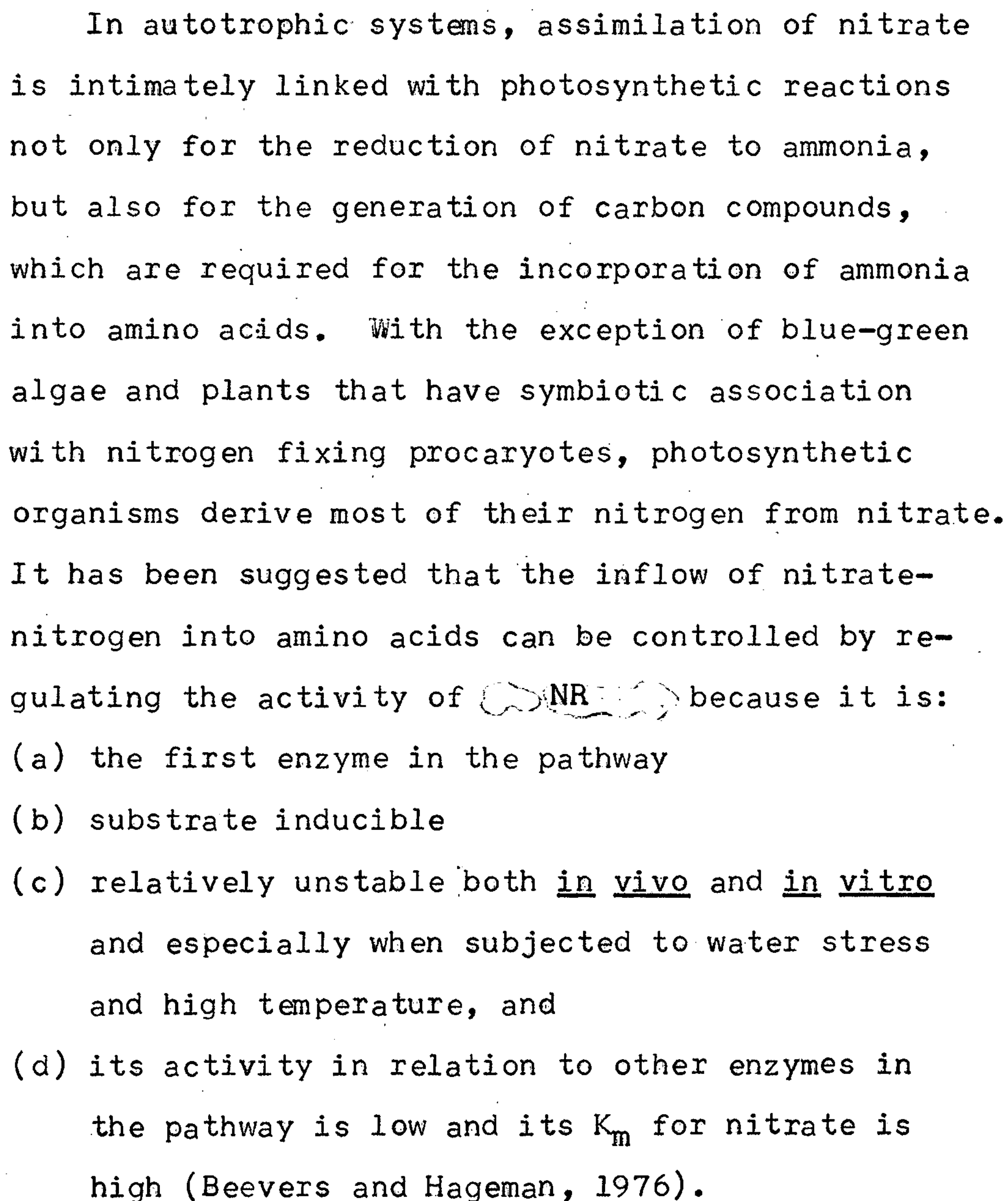
DISCUSSION

Nitrogen plays a key role in high agricultural production. Amelioration of agricultural soils of the world which are predominantly nitrogen-deficient is achieved by the application of nitrogenous fertilizers manufactured by high cost technology. Their utilization is, however, poor (Subba Rao, 1977). This necessitates the development of an understanding of factors which regulate their uptake and assimilation by plants and photosynthetic organisms. Thus, it is apparent that the nitrate reduction by algae and higher plants is one of the most fundamental biological processes by which inorganic oxidised nitrate is reduced to ammonia with water as ultimate reductant at the expense of solar energy (Losada, Guerrero and Vega, 1981), before being assimilated into amino acids.

Reduction of nitrate to ammonia is catalyzed by two independent enzymes, nitrate reductase (NR) and nitrite reductase (NiR).



$$NO_3^- \xrightarrow[\text{nitrate reductase}]{2e^-} NO_2^- \xrightarrow[\text{nitrite reductase}]{6e^-} NH_4^+$$



In autotrophic systems, 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. With the exception of blue-green algae and plants that have symbiotic association with nitrogen fixing procaryotes, photosynthetic organisms derive most of their nitrogen from nitrate. It has been suggested that the inflow of nitrate-nitrogen into amino acids can be controlled by regulating the activity of NR because it is:

(a) the first enzyme in the pathway

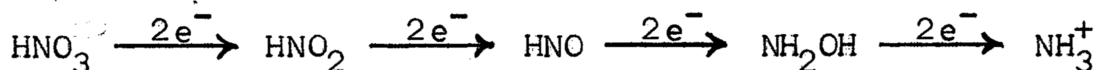
(b) substrate inducible

(c) relatively unstable both *in vivo* and *in vitro* and especially when subjected to water stress and high temperature, and

(d) its activity in relation to other enzymes in the pathway is low and its K_m for nitrate is high (Beevers and Hageman, 1976).

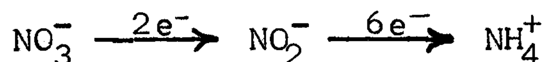
The nitrite produced by the activities of the enzyme nitrate reductase rarely accumulates in living cells under aerobic conditions. Usually

nitrite is converted to ammonia by nitrite reductase which is present in considerably high quantities than nitrate reductase. The first convincing demonstration of the occurrence of nitrite reductase in extracts of photosynthetic tissues was made by Hageman, Cresswell and Hewitt (1962). These workers were able to demonstrate that tissue extracts when fortified with reduced benzyl viologen as electron donor were capable of stoichiometrically converting nitrite to ammonia. The crude enzyme preparations also possessed the capacity for hydroxylamine reduction and superficially it appeared that nitrate reduction could proceed according to the scheme originally proposed by Meyer and Schulze (1884) in which hyponitrite and hydroxylamine functioned as intermediates:



$$HNO_3 \xrightarrow{2e^-} HNO_2 \xrightarrow{2e^-} HNO \xrightarrow{2e^-} NH_2OH \xrightarrow{2e^-} NH_3^+$$

Only in the last few years, extensively purified preparations have been obtained, which are active in nitrite reduction but devoid of hydroxylamine reductases (Naik, Abrol, Nair and Rama Rao, 1982).

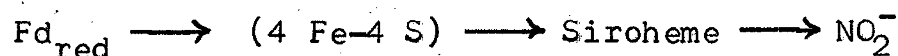


$$NO_3^- \xrightarrow{2e^-} NO_2^- \xrightarrow{6e^-} NH_4^+$$

It is now believed that the reduction of nitrite to ammonia is catalyzed by one protein, nitrite reductase, in which the iron porphyrin prosthetic group, siroheme, of the enzyme functions in the transfer of six electrons. There are no free intermediates.

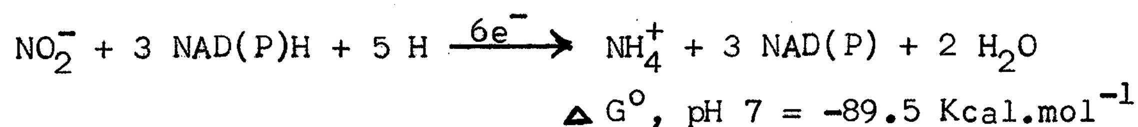
Basically, two types of assimilatory NiRs, marked by well defined electron donor specificity have been illustrated:

(1) Ferredoxin-nitrite reductase, the characteristic of photosynthetic organisms,



$$Fd_{red} \longrightarrow (4\ Fe\text{-}4\ S) \longrightarrow \text{Siroheme} \longrightarrow NO_2^-$$

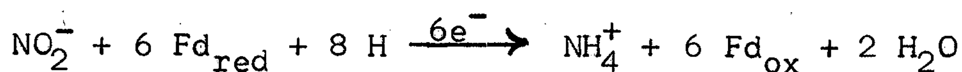
(2) NAD(P)H nitrite reductase, typical of non-photosynthetic organisms,



$$NO_2^- + 3\ NAD(P)H + 5\ H \xrightarrow{6e^-} NH_4^+ + 3\ NAD(P) + 2\ H_2O$$

$$\Delta G^o,\ pH\ 7 = -89.5\ Kcal.mol^{-1}$$

Both the enzymes catalyze the stoichiometric reduction of nitrite to ammonia, which implies the universal transfer of six electrons, also occurring in the reduction of dinitrogen to ammonia catalyzed by nitrogenase and the reduction of sulfite to sulfide, catalyzed by sulfite reductase:



$$NO_2^- + 6\ Fd_{red} + 8\ H \xrightarrow{6e^-} NH_4^+ + 6\ Fd_{ox} + 2\ H_2O$$

$$\Delta G^o,\ pH\ 7 = -103.5\ Kcal.mol^{-1}$$

Flavodoxin can also substitute for ferredoxin as the intermediate electron donor for different nitrite reductases (Bothe, 1977).

Both nitrate and nitrite reductases are tightly bound to chlorophyll containing membrane fractions and are able to utilize photosynthetically generated reducing power by implicating ferredoxin (Manzano, Candau, Gomez-Moreno, Relimpo and Losada, 1976). Characteristically assimilatory ferredoxin nitrite reductase is usually a soluble enzyme in blue-green algae with the exception of *Anacystis nidulans* and *Anabaena cylindrica* which needed unusually long ultrasonication to obtain active soluble fractions. Cells of *Anacystis nidulans* were required to be suspended in 50 mM Tris-HCl, pH 7.5 containing 1 mM EDTA and then sonicated for 10 minutes at 20 Kcs. Cells of *Anabaena cylindrica* were sonicated at 10 Kcs for 5 minutes (Hattori and Uesugi, 1968). Whereas, Mendez and Vega (1981) have succeeded in extracting NiR from cells of *Anabaena* sp. 7119 (formerly *Nostoc muscorum* by sonication at 20 Kcs and 75 W for two periods of 30 secs each.

The results presented for Spirulina platensis (Table 2) suggest that NiR is a soluble protein and is obtainable after 2 to 2.5 min of sonication at 12 Kcs, thereby indicating a close resemblance to Anabaena sp. 7119. Interestingly, in this characteristic, it differs a great deal from a taxonomically related cyanobacterium Anacystis nidulans.

Suitable choice of buffers during extraction procedures and enzyme assays is an important consideration. Earlier workers have successfully used Tris-HCl buffer for NiR preparation from Spinach (Vega and Kamin, 1977), Chlorella (Zumft, 1977), Anabaena sp. 7119 (Mendez and Vega, 1981) and Anacystis nidulans (Menzano, Candau, Gomes-Moreno, Relimpo and Losada, 1976). Contrary to these reports, enzyme extracts of Spirulina made in Tris-HCl buffer showed almost 50 per cent inhibition and the stability of the enzyme was poor. The results recorded in the present investigation is in conformity with Anabaena cylindrica (Hattori and Uesugi, 1968) as phosphate buffer (K^{+}/K^{++}) was a suitable choice for the stability and the activity of the enzyme (Table 6).

Optimal pH for NiR activity reported for most of the blue-green algae was around 7.5, has also been

found in *Spirulina* (Fig. 6). Only exception reported so far is in *Anabaena* sp. 7119 where 8.0 was the optimal pH.

NiR from *Spirulina* seems to favour higher temperature, 40°C for maximal activity (Fig 5) which is in contradiction to earlier reports in cyanobacteria where maximum activity was obtained around 25°C.

Active nitrite reductases in *Anabaena* sp. 7119 and *Anacystis nidulans* did not require any extra enzyme protectant, while Hattori and Uesugi (1968) have recorded a better protein stabilization with cysteine (1×10^{-3} M) when included in the elution buffer. Crude extracts in this investigation also retained more activity and stability when methyl viologen (1 µM) was included in the buffer during preparation procedures (Table 4), than elutate obtained after treating with cysteine or that which did not receive any protectant.

Methyl viologen in the presence of sodium dithionite was the best electron donor for the reduction of nitrite (Table 5). The present observation is in agreement with previous reports with different photosynthetic systems including spinach (Ramirez et al., 1979), *Chlorella* (Zumft, 1972), *Anabaena cylindrica* (Hattori and Uesugi, 1968), *Anabaena* sp. 7119 (Mendez

and Vega, 1981) and _Anacystis nidulans_ (Manzano et al. 1977). The reduced pyridine nucleotides (NADPH/NADH) by themselves failed to serve as electron donor in experiments with _Spirulina_ NiR, which is again in accordance with earlier reports (Guerrero, Vega and Losada, 1981).

Role of iron seems to be essential both _in vivo_ synthesis of NiR and for its catalytic activity _in vitro_. ESR studies indicated that spinach NiR had 5 iron atoms, one localized in siroheme and the rest of the 4 were in the form of (4Fe-4S) tetranuclear centre (Lancaster et al, 1979). Zumft (1972) also concluded similarly, after a study on the metal components of the nitrite reducing system from _Chlorella_. A close association of radioactive ^{59}Fe with NiR was documented thereby, strongly suggesting that iron is an integral constituent of the enzyme. Atomic absorption spectroscopy of NiR shows the presence of two atoms of iron per molecule of enzyme. More recently Mendez and Vega (1981) have also claimed that iron is an essential element for NiR production in _Anabaena_ sp. 7119. This preliminary investigation point out that iron is required for _in vivo_ synthesis of NiR in _Spirulina platensis_ (Fig 10).

SUMMARY

It is now well documented that nitrate-nitrogen is reduced to the ammonium levels before being incorporated into organic compounds. Recent work suggests that only two enzymes catalyze the entire reduction of nitrate to ammonium. The first is nitrate reductase ($NO_3^- \longrightarrow NO_2^-$); the second is nitrite reductase which catalyze conversion of nitrite to ammonia ($NO_2^- \longrightarrow NH_4^+$).

The mass culture of algae, fashionable about three decades ago has re-emerged as a legitimate goal. Cyanobacteria growing in natural situations are used by several human cultures as food and several commercial undertakings successfully export Spirulina from Mexican and Taiwanese lagoons. This has encouraged the belief that Spirulina may have a major role in cattle and human food substitutes. Whether the extra claims for the nutritional giving properties as Single Cell Protein (SCP) of this organism currently being advocated by several Japanese and USA organizations helps or hinders this belief remains to be seen.

The present dissertation primarily pertains to update the literature concerning the nitrite reduction to ammonia *in vivo* and *in vitro* in cyanobacteria. This work also envisages the cultivation of *Spirulina platensis*, a rich source of protein (SCP) in our laboratory conditions. Most of the growth requirements have been standardized and considerable success has been achieved for optimum cell cultures and harvest. Routinely cultures were grown in 4 litre Hafkin's flask with constant bubbling of sterile air at a temperature of 28 $\pm$ 1°C and under constant illumination.

For obtaining active cell-free extracts, cells were suspended in phosphate buffer (pH 7.4, 10 mM) and disrupted at 12 Kcs/sec using a MSE ultrasonicator. Cell homogenate was separated in a Sorvall RC-5 centrifuge. Supernatant fractions were later desalted through a Sephadex G-25 column. Methyl viologen served as a good enzyme stabilizer.

The enzyme Nitrite reductase appears to be a soluble protein with pH and temperature optima of 7.4 and 40°C respectively. Methyl viologen, besides being an effective enzyme protector, was the most suitable electron donor in the combination with dithionite.

An apparent K_m value of NiR for MV and nitrite were 3×10^{-4} and 4×10^{-4} respectively. Iron was an essential component for the *in vivo* synthesis of NiR and in vitro activity of *Spirulina platensis*.

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