THE ENZYMES OF NITRATE ASSIMILATION IN *SCLEROTINIA SCLEROTIORUM*

by

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A thesis submitted in fulfilment of the requirements
for the degree of

Master of Agricultural Science

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February, 1986

PREFACE

Part of the work described in this thesis has been presented at the Australian Biochemical Society Conference (Canberra, 1985) and published in the following journals:

1. Some properties of glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*.
   M.A. Rachim and D.J.D. Nicholas (1985)
   Proc. Aust. Biochem. Soc. 17, 21

2. Glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*.
   M.A. Rachim and D.J.D. Nicholas (1985)
   Phytochemistry 24, 2541-2548

3. Some properties of nitrate reductase from *Sclerotinia sclerotiorum*.
   M.A. Rachim and D.J.D. Nicholas (1986)
   Phytochemistry 25 (in press)

ACKNOWLEDGEMENTS

I wish to express my deepest and sincerest thanks to my supervisor, Prof. D.J.D. Nicholas, Chairman of the Department of Agricultural Biochemistry, Waite Agricultural Research Institute, The University of Adelaide for his constant encouragement, guidance and constructive criticism throughout the progress of the present investigation and in the preparation of manuscript.

I would also like to thank Mr. D. Hein for his assistance with the ^{15}N experiments, Mr. B.A. Palk for preparing the photographic prints, Mrs. M. Brock for the skilful typing of the thesis and to all others who helped me from time to time.

I am grateful to my wife Erna for her patience and unfailing encouragement during the course of this investigation.

The postgraduate scholarship from International Development Programme of Australian Universities and Colleges is gratefully acknowledged.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief no material described herein has been previously published or written by another person except when due reference is made in the text.

If accepted for the award of a M.Ag.Sc. degree, this thesis will be available for loan and photocopying.

M.A. RACHIM

ABBREVIATIONS

The abbreviations for chemicals and symbols in general follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature (*Biochem. J.* (1966) 101: 1-7) or the Instruction to Authors for the Phytochemistry (*Phytochemistry* (1983) 22: 1-7).

CHEMICALS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
BV	Benzyl viologen
BVH	Benzyl viologen (reduced)
CDP	Cytidine 5'-diphosphate
CMP	Cytidine 5'-monophosphate
CMP	Cytidine 5'-triphosphate
Cyt b_{557}	Cytochrome b_{557}
DCPIP	2,6-Dichlorophenolindophenol
DEAE-cellulose	Diethylaminoethyl cellulose
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide (reduced)
FMN	Flavin mononucleotide
$FMNH_2$	Flavin mononucleotide (reduced)
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
IDP	Inosine 5'-diphosphate
IMP	Inosine 5'-monophosphate
ITP	Inosine 5'-triphosphate
MSX	L-methionine-DL-Sulphoximine
MV	Methyl viologen
MVH	Methyl viologen (reduced)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NEM	N-ethylmaleimide
p-CMB	p-chloromercuribenzoate
SDS	Sodium dodecyl sulphate
-SH	Sulphydryl group
SVD	Snake venom phosphodiesterase
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
UDP	Uridine 5'-diphosphate
UMP	Uridine 5'-monophosphate
UTP	Uridine 5'-triphosphate

Symbols and Units

A	absorbance
°C	degree Celcius (centigrade)
cm	centimeter
g	gram
g	unit of gravitational field
hr	hour(s)
kDa	kilo dalton(s)
K_i	inhibitor constant
K_m	Michaelis-Menten constant
L	litre
M	molar
mA	milliampere
mg	milligram
min	minute(s)
ml	millilitre
mm	millimeter
mM	millimolar
mmole	millimole(s)
MW	molecular weight
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
µmole	micromole(s)
N	normal
nm	nanometer
nmole	nanomole(s)
%	percent
P_i	inorganic phosphate
p.s.i.	pound per square inch
U.V.	ultra violet
v	volume
w	weight

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SUMMARY

1. This thesis embodies results of an investigation on some biochemical aspects of enzymes involved in nitrate assimilation in a plant-pathogenic fungus, *Sclerotinia sclerotiorum*. The enzymes are nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthase.

2. Nitrate reductase, purified 118-fold, had a molecular weight of 210 kDa and was composed of 2 dissimilar subunits of 123 and 107 kDa. In addition to using NADPH as an electron donor, the enzyme also utilized reduced viologen dyes and reduced flavin nucleotides as reductants.

3. FAD was isolated from the purified enzyme; however, exogenous FAD was required for maximal activity of NADPH-dependent nitrate reductase *in vitro*. FMN did not substitute for FAD.

4. The enzyme activity was inhibited by metal chelating agents and by flavin and sulphydryl group inhibitors. Azide markedly restricted both NADPH- and MVH-dependent reactions, but inhibition by p-CMB, NEM and amytal was more marked when NADPH was the reductant.

5. Nitrite inhibited nitrate competitively in NADPH-dependent nitrate reduction *in vitro*; however, it is unlikely to inhibit nitrate reductase activity under physiological conditions because the K_i for nitrite was three-fold greater than the K_m for nitrate.

6. Nitrite reductase, the second enzyme in the nitrate assimilation pathway utilized NAD(P)H, reduced viologen dyes and reduced flavin nucleotides as electron donors. NADPH was the most effective electron donor. Maximal activity of the NADPH-dependent nitrite reductase

was achieved by adding FAD to the assay mixture; FMN however, was less effective.

7. Associated with the nitrite reductase enzyme was a hydroxylamine reductase activity as well as diaphorase type activities utilizing either ferricyanide, DCPIP or cytochrome *c* as an electron acceptor.

8. The product of both nitrite and hydroxylamine reductases was ammonia but hydroxylamine was not an intermediate product of nitrite reductase. The stoichiometry of NADPH oxidized to nitrite utilized and ammonia produced in NADPH-dependent nitrite reduction was 3:1:1 and the ratio of NADPH oxidized to ammonia formed in the hydroxylamine reductase-mediated reaction was 1:1.

9. In the presence of FAD, nitrite reductase was inactivated by preincubation with NADPH, but $NADP^+$ was without effect. This inactivation was offset by the substrates (nitrite and hydroxylamine).

10. Nitrite reductase was sensitive to metal binding agents as well as flavin- and sulphydryl group-inihibitors. The inhibition by p-CMB and mepacrine respectively, were reversed by adding cysteine and FAD.

11. Washed felts readily incorporated $^{15}NH_4^+$ into cell-nitrogen but this effect was inhibited by both MSX and azaserine, inhibitors of glutamine synthetase and glutamate synthase respectively. Since glutamate dehydrogenase was not detected in cell-free preparations, this is further evidence that the glutamine synthetase/glutamate synthase pathway is the main route for the assimilation of ammonia into amino acids.

12. Glutamine synthetase, purified by ion exchange and affinity chromatography, had a molecular weight of 490 kDa. The enzyme was composed of 8 identical subunits of 60 kDa. The transferase activity of the

enzyme required Mn^{2+} and ADP for maximal activity whereas the biosynthetic activity required Mg^{2+} and ATP.

13. The enzyme was regulated by feedback inhibition involving amino acids and organic acids and by a adenylylation/deadenylylation mechanism. The transferase activity of the enzyme was also inhibited competitively by the substrates of the biosynthetic reaction, namely glutamate and NH_4Cl, with respect to glutamine.

14. Glutamate synthase had a molecular weight of 220 kDa and was composed of 4 subunits of 53.7 kDa. The enzyme had a specific requirement for NADPH, α-ketoglutarate and glutamine as the electron donor, amino acceptor and amino donor, respectively.

15. Glutamate synthase was regulated by feedback inhibitors including amino acids, organic acids and nucleotides. The enzyme was markedly inhibited by p-CMB (this effect was reversed by cysteine), O-phenanthroline, α,α'-dipyridyl and azaserine.

1. INTRODUCTION

Nitrate is an important nitrogen source for the growth of micro-organisms and plants since it is a predominant form of combined nitrogen present in the environment. The capacity to assimilate nitrate is a feature of many bacteria, fungi and virtually all algae and higher plants, but it is absent from the animal kingdom.

1.1 The biology of *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum along with *S. trifoliorum* and *S. minor* belong to the family of *Sclerotiniaceae* of the class Ascomycotina (Whetzel, 1945). This fungus is widely distributed but it is most common in temperate regions (Reichert, 1958). The fungus has been recognized for many years as a serious pathogen of various plants including about 360 species in 225 genera and 64 families such as alfalfa, bean, celery, lettuce, peanut, potato , spearmint, sunflower, tomato, apricot, cabbage and eggplant (see Purdy, 1979).

Plants may be attacked by this fungus at the seedling stage as well as during maturation and subsequent storage. Under favourable conditions (cool and humid) the fungus invades the tissues of the host and then a light brown watery rot develops and white mycelia grow over the infected tissues (Purdy, 1979). After several days, small and compact bodies (1-8 mm in diameter) develop from the mycelia either on the surface of the host or in cavities within it (Jones, 1974; Abawi *et al.*, 1975). The compact bodies, called sclerotia, are young resting vegetative structures. Active growth of sclerotia takes place only during the 72 hr period subsequent to the appearance of primordia (Cooke, 1970)

and then colour of the mature sclerotia changes from white to black. In soil or plant debris the sclerotia can remain dormant for long periods or alternatively they germinate after a short resting period. Approximately 90% of the life cycle of this fungus is spent in the soil as sclerotia (Adams and Ayers, 1979). Germination of sclerotia gives infective hyphae and in the field the sclerotia usually germinate in spring to form apothecia. The apothecial stalks are 2-3 cm long and 1-2 mm thick whereas the discs are concave, yellowish brown usually 3-8 mm in diameter (Jones, 1974). Cylindrical asci formed in the apothecia contain ascospores (9-15 x 4-7 µm), the sexual spores of the fungus which are produced in large numbers. Later ascospores are liberated from mature asci and when some of them land on a susceptible host, under favourable conditions they germinate and a new cycle of infection commences (Eddins 1937; Dana and Vaughan, 1949; Western, 1971; Jones, 1974; Abawi *et al.*, 1975; Adams and Ayers, 1979; Purdy, 1979; Willetts and Wong, 1980).

Growth of mycelia, production of sclerotia and survival of *S. sclerotiorum* are affected by environmental factors. This fungus grows on agar medium over a range of temperatures (0-35 °C) with an optimum at 20°C (Van den Berg and Leitz, 1968). It can tolerate a wide range of pH values from 2-10 (Tanrikut and Vaughan, 1951) but growth is optimal between pH 4-5.5 (Rai and Agnihotri, 1971). When the fungus is grown in a liquid medium, the pH of the culture filtrate drops to pH 3-4 and then it increases slightly. The low pH coincides with maximum acid production when the specific activities of the Krebs cycle enzymes are high (Le Tourneau, 1979). Oxalic acid is produced by this fungus both in the host tissue as well as in cultures of the fungus (Maxwell and Lumsden, 1970) and fumaric, succinic and glycolic acids are produced during the late exponential stage of growth (Vega *et al.*, 1970).

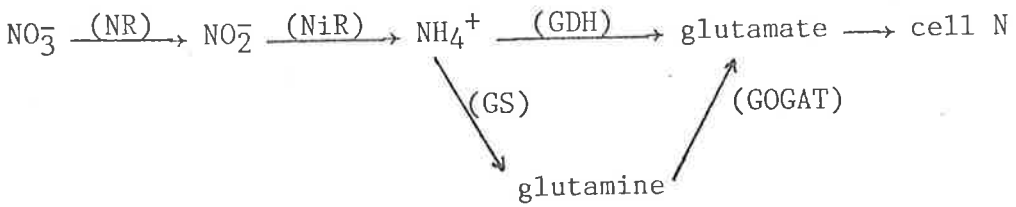
Studies on the nutrition of this fungus indicate that it grows readily on basal salts and a simple carbon source. Some carbon and nitrogen sources are more readily utilized than others. Glucose among monosaccharides and maltose, followed by sucrose among disaccharides are good sources of carbon for growth of the fungus (Rai and Agnihotri, 1971) whereas glycerol and mannitol are poor sources. Of the inorganic nitrogen sources, nitrite supported very little growth of the fungus. On the other hand, nitrate and ammonium supported good growth, and there was no difference between these compounds as a nitrogen source (Tanrikut and Vaughan, 1951; Willis, 1968). Among amino acids, glutamic acid, proline and aspartic acid were the most suitable nitrogen sources whereas lysine, valine and cysteine were not readily utilized (Willis, 1968). In liquid cultures growth was also depressed by deficiencies of phosphate, magnesium and trace elements (Purdy and Grogan, 1954).

1.2 Nitrate assimilation in microorganisms

Nitrate is assimilated *via* a reduction to ammonia followed by an incorporation into amino and amido compounds. The reduction of nitrate to ammonia is catalyzed by two distinct enzymes, namely nitrate reductase (NR) and nitrite reductase (NiR) (Nicholas and Nason, 1954a; Garrett and Amy, 1978). The nitrate assimilation requires a substantial energy expenditure compared with ammonia utilization since eight reducing equivalents are consumed in the reduction of nitrate to ammonia (Nicholas, 1963). Cells assimilating nitrate appear to regulate this reaction at the level of nitrate reductase (Beevers and Hageman, 1969).

The assimilation of ammonia proceeds either *via* glutamate dehydrogenase (GDH) or the glutamine synthetase (GS)/glutamate synthase (GOGAT)

pathway. Glutamate dehydrogenase has a low affinity for ammonia, whereas the glutamine synthetase/glutamate synthase pathway assimilates low concentration of ammonia in microorganisms(Tempest *et al.*, 1970; 1973) and plants (Miflin and Lea, 1976). A summary of the enzymes involved in the nitrate assimilation pathway is as follows:



1.2.1 Nitrate reductase

Nitrate reductase (NAD(P)H-nitrate oxido-reductase, E.C.1.6.6.1-3) catalyzes the reduction of nitrate to nitrite, the first step in the nitrate assimilation pathway, according to the reaction (Nason and Evans 1953; Nicholas and Nason, 1955):

$$NO_3^- + NAD(P)H + H^+ \longrightarrow NO_2^- + NADP^+ + H_2O \qquad [1]$$

The enzyme is nitrate inducible and is rapidly repressed by ammonia (Kinsky, 1961; Lewis and Fincham, 1970). The nitrate reductases from fungi have been characterized and shown to be soluble enzymes with a molecular weight varied between 160 kDa for the enzyme of *Neurospora crassa* and 520 kDa for *Candida utilis* enzyme (Sims *et al.*, 1968; Garrett and Nason, 1969; Antoine, 1974; McDonald and Coddington, 1974; Guerrero and Gutierrez, 1977; Renosto *et al.*, 1981; Minagawa and Yoshimoto, 1982) and the numbers and sizes of subunits differ quite widely. Nitrate reductase from *N. crassa* has two unequal subunits with molecular weights of 115 and 130 kDa (Pan and Nason, 1978). The subunit molecular weights of the enzyme

from *Penicillium chrysogenum* were 97 and 98 kDa (Renosto *et al.*, 1981), and the enzyme from *Aspergillus nidulans* consisted of two types of subunits of 59 and 38 kDa (Minagawa and Yoshimoto, 1982). The nitrate reductase from yeast *Rhodotorula glutinis* has a molecular weight of 230 kDa composed of 2 equal subunits of 118 kDa (Guerrero and Gutierrez, 1977). On the other hand the enzyme from *Torulopsis nitratophila* has a molecular weight of 500 kDa (Rivas *et al.*, 1973) and that for the enzyme from *C. utilis* is 520 kDa with subunits of 260, 130 and 65 kDa (Sims *et al.*, 1968).

Nitrate reductase from *N. crassa* was partially purified and characterized by Nason and Evans (1953) who identified FAD as a prosthetic group in the enzyme. Similarly the purified enzyme from *Asp. nidulans* showed absorption at 450-475 nm indicating the presence of a flavin component (Downey, 1971; McDonald and Coddington, 1974; Minagawa and Yoshimoto, 1982). Two moles of FAD were found in the *Chlorella* enzyme (Solomonson *et al.*, 1975). The FAD was easily dissociated from the enzyme of *N. crassa* (Garrett and Nason, 1967), but not from the *Escherichia coli* enzyme (Nicholas and Nason, 1955).

Nicholas *et al.* (1954) showed that molybdenum was required for the synthesis of nitrate reductase in *N. crassa*. Subsequently they demonstrated that molybdenum was a functional constituent of the enzyme (Nicholas and Nason, 1954b; Nicholas, 1963), one atom molybdenum per mole enzyme of *Asp. nidulans* (Downey, 1973) and *N. crassa* (Jacob and Orme-Johnson, 1980). Studies on valency changes of molybdenum during the enzymatic reduction of nitrate in *N. crassa*, Nicholas and Stevens (1955) concluded that Mo^{5+} was as effective as NADPH as an electron donor for nitrate reductase action whereas Mo^{6+} was without effect. However, Mo^{3+} reduced nitrate non-enzymatically.

Investigations with a purified nitrate reductase (Garrett and Nason, 1969; Guerrero and Gutierrez, 1977; Minagawa and Yoshimoto, 1982) revealed another component of the enzyme namely a haem moiety designated as cytochrome b_{557}. There were two haem components per mole enzyme in *N. crassa* (Garrett and Nason, 1969; Pan and Nason, 1978) and *Chlorella vulgaris* (Solomonson *et al.*, 1975).

The enzyme components (FAD, cytochrome b_{557}, and molybdenum) functioned as electron carriers between the physically separated pyridine nucleotide oxidation site and nitrate reduction site (Campbell and Smarelli, 1978). Amy *et al.* (1977) described an important sulphydryl group which apparently mediated electron transfer between NADPH and FAD. They postulated the sequence of electron transfer mediated by nitrate reductase as follows:

$$\text{NADPH} \longrightarrow \text{-SH} \longrightarrow \text{FAD} \longrightarrow \text{Cyt.}b_{557} \longrightarrow \text{Mo} \longrightarrow NO_3^-$$

Assimilatory nitrate reductase in fungi is mediated by reduced pyridine nucleotides with NADPH as the preferred electron donor (Garrett and Nason, 1969; Rivas *et al.*, 1973; McDonald and Coddington, 1974; Renosto *et al.*, 1982). However, in some yeasts, NADH could also be utilized by the enzyme (Rivas *et al.*, 1973; Guerrero and Gutierrez, 1977). In addition to NAD(P)H, the enzyme from *N. crassa* (Garrett and Nason, 1969; Amy *et al.*, 1977; Pan and Nason, 1978), *P. chrysogenum* (Renosto *et al.*, 1981), *T. nitratophila* (Rivas *et al.*, 1973) and *R. glutinis* (Guerrero and Gutierrez, 1977) utilized dithionite-reduced viologen dyes as an electron donor. Nicholas and Nason (1955), Amy *et al.* (1977), Pan and Nason (1978), Renosto *et al.* (1982) and Minagawa and Yoshimoto (1982) showed that the enzyme could also utilize dithionite-reduced flavin nucleotides as an

electron donor. Excess of dithionite used to reduce benzyl viologen and FMN inactivated nitrate reductase from *Cyanidium caldarium* (Rigano and Aliotta, 1975); however, FMN and nitrate added before the dithionite protected the enzyme against this inactivation. NAD(P)H-linked nitrate reductase activity was stimulated by adding FAD (Downey, 1971; Rivas *et al.*, 1973; McDonald and Coddington, 1974; Guerrero and Gutierrez, 1977; Renosto *et al.*, 1981; 1982).

The K_m values for nitrate and NADPH of nitrate reductase in fungi range from 60-200 µM and 10-62 µM, respectively (Garrett and Nason, 1969; McDonald and Coddington, 1974; Rigano and Aliotta, 1975; Guerrero and Gutierrez, 1977; Renosto *et al.*, 1981; 1982). Varying concentrations of NADPH had no effect on the K_m for nitrate and *vice versa* (McDonald and Coddington, 1974) and they suggested that there was no interaction between sites for nitrate and NADPH.

The NAD(P)H-dependent nitrate reductase activity in purified preparations was inhibited by p-hydroxymercuribenzoate (Nicholas and Nason, 1955; Garrett and Nason, 1969; McDonald and Coddington, 1974; Guerrero and Gutierrez, 1977; Pan and Nason, 1978); this inhibition could be overcome with cysteine or dithiothreitol. Much larger concentrations of the mercury compound (1 mM) did, however, restrict the $FADH_2$-dependent or MVH-linked nitrate reductase activities (Garrett and Nason, 1969) whereas intermediate concentrations (20-200 µM) resulted in a stimulation of the MVH-nitrate reductase from *N. crassa*. Azide and cyanide also inhibited both NADPH- and MVH-dependent nitrate reduction (Minagawa and Yoshimoto, 1982). Solomonson and Vennesland (1972) showed that azide, cyanate, thiocyanate and nitrite reacted rapidly with the enzyme from *Chlorella* and these compounds inhibited NADH-nitrate reductase competitively with respect

to nitrate, whereas cyanide and hydroxylamine reacted slowly with the reduced form of the enzyme to give an inactive product which can be slowly reactivated in the presence of nitrate.

1.2.2 Nitrite reductase

Nitrite reductase (NAD(P)H : nitrite oxidoreductase, E.C.1.6.6.4), the second enzyme in the nitrate assimilatory pathway catalyzes the reduction of nitrite, the product of nitrate reductase action, to ammonia in a six-electron transfer reaction. The reduction proceeds apparently with no release of intermediate compounds (Dunn-Coleman *et al.*, 1984) in the reaction:

$$NO_2^- + 6e^- + 8H^+ \longrightarrow NH_4^+ + 2\,H_2O \qquad [2]$$

The enzyme from fungi and other microorganisms is induced in cells grown in the presence of nitrate or nitrite (Pateman *et al.*, 1967; Garrett, 1972). The purified enzyme has a molecular weight of 290 kDa (Lafferty and Garrett, 1974) and is composed of 2 subunits of 140 kDa each (Garrett and Amy, 1978; Prodouz and Garrett, 1981).

Nitrite reductase purified from *N. crassa* was shown to be flavoprotein containing iron and copper (Nicholas *et al.*, 1960); the two metals were concentrated in purified enzyme preparations, and extracts from mycelia grown in the absence of copper or iron exhibited low nitrite reductase activity which was restored when the omitted metal was added to the extract. It was suggested that copper is required for the terminal step, coupling the electron transfer sequence to nitrite (Nicholas, 1963). Vega *et al.* (1975) demonstrated that nitrite reductase from *N. crassa*

contains siroheme as a prosthetic group and that the siroheme component functions in the enzymatic catalysis of nitrite reduction.

The assimilatory nitrite reductase from fungi and other non-photosynthetic microorganisms differs from the assimilatory nitrite reductase from photosynthetic organisms. Thus in the non-photosynthetic organisms, reduced pyridine nucleotides are utilized for the reduction of nitrite to ammonia. In *Escherichia coli* (Kemp and Atkinson, 1966) and *Azotobacter chroococcum* (Vega *et al*, 1973) nitrite reductase utilizes NADH only as an electron donor and in *T. nitratophila* (Rivas *et al*., 1973) the enzyme is NADPH specific, whereas in *Asp. nidulans* (Pateman *et al*., 1967) and *N. crassa* (Lafferty and Garrett, 1974; Vega *et al*., 1975; Vega, 1976; Greenbaum *et al*., 1978; Prodouz and Garrett, 1981) either NADPH or NADH is utilized. *In vitro* the reduced pyridine nucleotide-dependent activities required exogenous FAD for maximal activity (Lafferty and Garrett, 1974; Garrett and Amy, 1977). The enzyme also utilized dithionite-reduced viologen dyes as electron donors but under these conditions FAD was not required (Cook and Sorger, 1968; Lafferty and Garrett, 1974).

The nitrite reductases from higher plants and algae utilize reduced ferredoxin as the primary electron donor (Zumft, 1972). The enzymes from *Anabaena cylindrica* can also use NADPH as an electron donor but NADH was inactive (Hattori and Myers, 1966). This reaction required ferredoxin which mediated the transfer of electrons from NADPH to nitrite. Ferredoxin could be replaced by either methyl viologen or benzyl viologen but not by FAD or FMN. Ferredoxin reduced chemically with dithionite or enzymatically with NADPH as well as chemically-reduced viologen dyes were effective electron donors whereas dithionite-reduced FMN and FAD were inactive (Hattori and Uesugi, 1968). Although chemically-reduced viologen

dyes and flavins served as electron donor for the enzyme from a green alga *Dunaliella tertiolecta*, neither NADPH nor NADH functioned as a reductant even when exogenous FAD was added (Grant, 1970).

The NAD(P)H-dependent nitrite reductase from *N. crassa* also catalyzes other electron-transfer reactions *in vitro* (Vega, 1976). These activities included NAD(P)H-hydroxylamine reductase, NAD(P)H-diaphorase activity using either mammalian cytochrome *c*, 2,6-dichlorophenolindophenol, ferricyanide or menadione as an electron acceptor. The NAD(P)H-hydroxylamine reductase activity displayed by nitrite reductase had some similar properties to the NAD(P)H-nitrite reductase activity. The FAD-dependent enzyme was inactivated upon preincubation with NAD(P)H and FAD and the inactivation was protected by substrates, inhibited by cyanide and was sensitive to sulphydryl reagents (Lafferty and Garrett, 1974; Vega *et al* ., 1975; Vega, 1976). Kinetic observations with the enzyme from *E. coli* (Kemp and Atkinson, 1966) indicated that hydroxylamine and nitrite were both reduced at the same site. The K_m values for nitrite and hydroxylamine respectively were 0.01 and 3 mM for the *N. crassa* enzyme (Lafferty and Garrett, 1974), 0.01 and 1.5 mM for *E. coli* nitrite reductase (Kemp and Atkinson, 1966), and 0.05-0.08 and 5-8 mM for the enzyme from *Bacillus fischeri* (Prakash and Sadana, 1972). They all suggested that nitrite is the physiological substrate since hydroxylamine did not appear to be an intermediate product in nitrite reduction. Moreover, McElroy and Spencer (1956) showed that hydroxylamine at 0.1 mM was toxic to the growth of *N. crassa*.

The reduced pyridine nucleotide-dependent nitrite and hydroxylamine reductases which required FAD for maximal activity, were more heat-labile and more sensitive to inhibition by p-hydroxymercuribenzoate than the

dithionite-reduced viologen dependent activity (Lafferty and Garrett, 1974). The inhibition by p-hydroxymercuribenzoate could be reversed by either cysteine or glutathione. Nitrite reductase was also inhibited by cyanide, sulphite and O-phenanthroline.

Nitrite reductase has some similarities with sulphite reductase since both enzymes catalyze a 6-electron reduction, utilize similar electron donors and prosthetic groups and are affected by the same inhibitors (Prabhakararao and Nicholas, 1969; 1970; Murphy *et al.*, 1974; Greenbaum *et al.*, 1978; Siegel *et al.*, 1982; Janick *et al.*, 1983). The sulphite reductase was originally classified as nitrite reductase since this enzyme from *E. coli* also reduced nitrite and hydroxylamine to ammonia (Kemp *et al.*, 1963; Siegel *et al.*, 1973). Similarly the sulphite reductase from yeast could also catalyze these reactions (Yoshimoto and Sato, 1968; Prabhakararao and Nicholas, 1970). However, the K_m values of sulphite reductase for nitrite and hydroxylamine were too high for nitrite to be a substrate under physiological conditions (Siegel, 1975; Krueger and Siegel, 1982). Moreover, it is noteworthy that nitrite reductase from *E. coli* (Kemp and Atkinson, 1966) and *N. crassa* (Greenbaum *et al.*, 1978) did not reduce sulphite, although Lafferty and Garrett (1974) found that sulphite was a competitive inhibitor of nitrite reductase in *N. crassa*.

1.2.3 Metabolism of ammonia into amino acids

The main pathway of ammonia assimilation in microorganisms and plants was earlier accepted to be the reductive amination of α-ketoglutarate (Davies, 1968; Sims *et al.*, 1968) which is catalyzed by the enzyme glutamate dehydrogenase, E.C.1.4.1.2 (Shapiro and Stadtman, 1970) as follows:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NAD(P)H \rightleftharpoons \text{glutamate} + NAD(P)^+ + H_2O \quad [3]$$

In *Aerobacter aerogenes* it was found that glutamate dehydrogenase operated only when ammonia was in excess. Indeed in cells grown with limited amounts of ammonia, glutamate dehydrogenase was repressed (Meers *et al.*, 1970). Kinetic studies have demonstrated that glutamate dehydrogenase has a low affinity for ammonia (Sakamoto *et al.*, 1975; Tyler, 1978). It is now thought that the assimilation of ammonia into amino acids at low ammonia concentrations occurs *via* the glutamine synthetase/glutamate synthase pathway (Tempest *et al.*, 1970; 1973; Miflin and Lea, 1976; 1980; Hummelt and Mora, 1980a):

$$\text{glutamate} + NH_4^+ + \text{ATP} \xrightarrow{Me^{2+}} \text{glutamine} + \text{ADP} + \text{Pi} \qquad [4]$$

$$\alpha\text{-ketoglutarate} + \text{glutamine} + \text{NAD(P)H} \longrightarrow 2\ \text{glutamate} + \text{NAD(P)} \qquad [5]$$

The coupling of glutamine synthetase and glutamate synthase reactions results in an irreversible route for the production of glutamate. Because of this irreversibility and high affinity for ammonia both enzymes of this pathway can function in a very efficient manner when the cellular levels of free ammonia are low (Sawhney and Nicholas, 1978b; Murrell and Dalton, 1983; Florencio and Ramos, 1985).

1.2.3.1 Glutamine synthetase

Glutamine synthetase (L-glutamate : ammonia ligase (ADP forming), E.C.6.3.1.2) catalyzes the ATP-dependent production of glutamine from ammonia and glutamate as shown in reaction [4]. In addition to the transamination of the amino group of glutamine to an α-ketoacid, the amide-nitrogen of glutamine formed may serve as a nitrogen donor for the synthesis of histidine (Neidle and

Waelsch, 1959), tryptophan and p-aminobenzoic acid (Weiss and Srinivasan, 1959; Gibson and Gibson, 1962), purines (Buchanan, 1960), pyridine nucleotides (Preiss and Handler, 1958), cytidine nucleotides (Hurlbert and Chakraborty, 1961), citrulline (Levenberg, 1961), glucosamine (Leloir and Cardini, 1953) and carbamyl phosphate (Pierard and Wiame, 1964). The glutamine synthetase has been shown to play an important role in nitrogen metabolism in bacteria (Nagatani *et al.*, 1971; Brown *et al.*, 1974), fungi (Sims *et al.*, 1974; Quinto *et al.*, 1977), algae (Wolk *et al.*, 1976; Thomas *et al.*, 1977) and in higher plants (Lea and Miflin, 1974; Stewart and Rhodes, 1976). The enzyme from bacteria has a molecular weight in the range 617-720 kDa and contains twelve identical subunits (Kleinschmidt and Kleiner, 1978; Alef *et al.*, 1981; Bhandari *et al.*, 1983; Murrell and Dalton, 1983; Kimura *et al.*, 1984; Kumar and Nicholas, 1984). The molecular weight of the enzyme from eukaryotic organisms, namely between 360 and 470 kDa, is relatively smaller than that from prokaryotes (Sims *et al.*, 1974; Palacios, 1976; Lin and Kapoor, 1978; Mitchell and Magasanik, 1983; Beudeker and Tabita, 1985). The enzymes from *N. crassa* and other fungi are composed of 8 subunits except that glutamine synthetase from *Saccharomyces cerevisiae* consists of 10-12 subunits (Mitchell and Magasanik, 1983).

The most extensive biochemical studies on glutamine synthetase have been carried out in *E. coli* (Ginsburg and Stadtman, 1973; Tempest *et al.*, 1973). They established that glutamine synthetase in *E. coli* could respond to changes in extracellular concentrations of ammonia. The enzyme was rapidly inactivated (biosynthetic

activity, reaction 4) when the ammonia concentration was high, but it still retained γ-glutamyltransferase activity [reaction 6]:

$$\text{glutamine} + NH_2OH \xrightarrow[\text{arsenate}]{ADP, Me^{2+}} \gamma\text{-glutamylhydroxamate} + NH_3 \qquad [6]$$

The glutamine synthetase from microorganisms is regulated by three different but related mechanisms namely covalent modification by adenylylation, activation by divalent cations and feedback inhibition by the end-products of glutamine metabolism. The native enzyme from *E. coli* has 12 identical subunits arranged in two hexagons, the whole aggregate behaving as a spherical particle which is stabilized by divalent cations (Brown, 1980). Each subunit contains a specific tyrosyl residue which can be adenylylated enzymatically (Kingdon *et al.*, 1967) to form a 5'-adenylyl-*o*-tyrosyl derivative. A fully adenylylated enzyme is biosynthetically inactive and removal of these adenylyl groups resulted in restoration of the biosynthetic activity (Shapiro *et al.*, 1967; Tyler, 1978). The extent of adenylylation, measured as an adenylylation number, is usually estimated by the transferase reaction [6] where the adenylylated and deadenylylated forms show different cation specificities (Shapiro and Stadtman, 1970; Brown, 1980).

The biosynthetic activity of a fully adenylylated enzyme was dependent on Mn^{2+} whereas the biosynthetic activity of the unadenylylated enzyme required Mg^{2+} (Shapiro and Stadtman, 1970). In contrast to the biosynthetic activity, Mn^{2+} was required for transferase activity of both adenylylated and deadenylylated forms of the enzyme. Furthermore, the addition of Mg^{2+} to the Mn^{2+}-dependent transferase assay inhibited the activity of the adenylylated

subunits only (Stadtman *et al.*, 1970) and so the relative state of adenylylation can be measured by comparing the activity in the presence of Mn^{2+} only with that of both Mn^{2+} and Mg^{2+}. The adenyl group can be removed from adenylylated enzyme *in vitro* by treating the enzyme with snake venom phosphodiesterase (Vairinhos *et al.*, 1983; Murrell and Dalton, 1983; Kumar and Nicholas, 1984; Kimura *et al.*, 1984; Masters and Madigan, 1985).

Feedback inhibition of glutamine synthetase by a number of possible end products of glutamine metabolism was first observed in *E. coli* (Woolfolk and Stadtman, 1964) and subsequently found to occur in several other organisms including *Az. vinelandii* (Kleinschmidt and Kleiner, 1978), *Chlorobium vibrioforme* f. *thiosulphatophilum* (Khanna and Nicholas, 1983b), *Anabaena* L-31 (Tuli and Thomas, 1981) and *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Clostridium pasteurianum* (Hubbard and Stadtman, 1967). The enzyme from *B.subtilis* (Deuel and Stadtman 1970), *B. stearothermophilus* (Wedler *et al.*, 1976) and *Anabaena* (Stacey *et al.*, 1979) was not regulated by adenylylation but feedback inhibition appeared to be the main mechanism for controlling glutamine production. A partially adenylylated enzyme from *E. coli* was more sensitive to feedback inhibition than the unadenylylated enzyme (Brown, 1980). Each subunit had specific binding sites for the feedback inhibitors such as CTP, glucosamine-6-phosphate, carbamyl phosphate, tryptophan, histidine, AMP and a single site for amino acids including serine, glycine and alanine (Brown, 1980).

1.2.3.2 Glutamate synthase

The reductive transfer of the amino group from glutamine to α-ketoglutarate to produce two molecules of glutamate [reaction 5] is catalyzed by glutamate synthase (glutamine (amide) : 2-oxo-glutarate amino transferase oxidoreductase, E.C.1.4.1. 13-14). This enzymewas first detected in *A. aerogenes* grown at low concentrations of ammonia (Tempest *et al.*, 1970). Similar results were observed with *A. aerogenes*, *Erwinia coratovora*, *Ps. fluorescens*, *B. subtilis* var. *niger*, *B. subtilis* W23; *B. megaterium* (Meers *et al.*, 1970). Since then the enzyme has been purified from *E. coli* (Miller and Stadtman, 1972; Miller, 1973), *A. aerogenes* (Trotta *et al.*, 1974), *Thiobacillus thioparus* (Adachi and Suzuki, 1977), *B. megaterium* (Hemmila and Mantsala, 1978), *Cb. vibrioforme* f. *thiosulphatophilum* (Khanna and Nicholas, 1983b), and *Derxia gummosa* (Wang and Nicholas, 1985) and its properties studied. Tempest *et al.* (1973) and Brown *et al.* (1973) claimed that eukaryotic organisms including *Sac. cerevisiae*, *C. utilis*, *Asp. nidulans* and *N. crassa* do not contain glutamate synthase. Subsequently, however, the enzyme was detected in some species of *Schizosaccharomyces* and *Saccharomycodes ludwiggi* (Brown *et al.*, 1973, Johnson and Brown, 1974) and in *Sac. cerevisiae* (Roon *et al.*, 1974). More recently glutamate synthase has been purified from *Sac. cerevisiae* (Masters and Rowe, 1979), *N. crassa* (Hummelt and Mora, 1980b), *Chlamydomonas reinhardii* (Galvan *et al.*, 1984) and also from higher plants (Boland and Benny, 1977; Wallsgrove *et al.*, 1977; Hirasawa and Tamura, 1984).

The enzyme from microorganisms has a molecular weight in the range 146-840 kDa and the number of subunits varies from 2-8 subunits

(Miller and Stadtman, 1972; Adachi and Suzuki, 1977; Hemilla and Mantsala, 1978; Masters and Rowe, 1979; Yelton and Yoch, 1981; Galvan *et al.*, 1984). The purified glutamate synthase from *E. coli* was found to be an iron-sulphur protein (Miller and Stadtman, 1972; Miller, 1973) made up of eight subunits, *viz.* four large (molecular weight 135 kDa) and four small (53 kDa). The *A. aerogenes* and *Sac. cerevisiae* enzyme was also composed of 2 unequal subunits (Trotta *et al.*, 1974; Masters and Rowe, 1979) but the enzyme from *N. crassa* was composed of a single type of monomer with a molecular weight more than 200 kDa. Both iron and sulphur were present in the heavy subunit which also bound glutamine (Trotta *et al.*, 1974) whereas the light subunit did not bind the substrate. The enzyme contained flavin (Trotta *et al.*, 1974; Adachi and Suzuki, 1977; Masters and Rowe, 1979); and in *B. subtilis* there were eight molecules per mole of enzyme (Miller, 1973).

In bacteria and fungi, reduced pyridine nucleotides were electron donors for the enzyme (Adachi and Suzuki, 1974; Roon *et al.*, 1974; Masters and Rowe, 1979; Hummelt and Mora, 1980a; 1980b; Vairinhos *et al.*, 1983; Wang and Nicholas, 1985) whereas reduced ferredoxin functioned in algae and higher plants (Wallsgrove *et al.*, 1977; Galvan *et al.*, 1984; Hirasawa and Tamura, 1984). The partially purified glutamate synthase from *Tb. thioparus* had a specific requirement for NADPH, glutamine and α-ketoglutarate (Adachi and Suzuki, 1977). The K_m values for NADPH, α-ketoglutarate and glutamine respectively, were in the range 3-15 μM, 7-300 μM and 130-1100 μM (Miller and Stadtman, 1972; Trotta *et al.*, 1974; Adachi and Suzuki, 1977; Hemmila and Mantsala, 1978; Yelton and Yoch, 1981).

1.3 Aims of the study

This thesis is concerned with biochemical aspects of the assimilation of nitrate into glutamate in a plant pathogenic fungus *Sclerotinia sclerotiorum*. These studies include the following topics:

(i) Purification and characterization of nitrate and nitrite reductases as well as glutamine synthetase and glutamate synthase.

(ii) Regulation mechanisms for these assimilatory enzymes.

(iii) Use of ^{15}N-labelled $(NH_4)_2SO_4$ and appropriate inhibitors, namely MSX for glutamine synthetase and azaserine for glutamate synthase to determine the pathway of ammonia incorporation into glutamine and glutamate in washed mycelia.

2. MATERIALS AND METHODS

2.1 Culturing *Sclerotinia sclerotiorum*

2.1.1 Growth conditions

Culture of *S. sclerotiorum* was kindly supplied by Dr. M. Carter, Dept. of Plant Pathology of Waite Agricultural Research Institute. The fungus was maintained on Czapek-Dox agar plates containing per litre: glucose, 15g; $NaNO_3$, 2g; KH_2PO_4, 1g; KCl, 0.5g; $MgSO_4.7H_2O$, 0.5g; $FeSO_4.7H_2O$, 10 mg; $ZnSO_4.7H_2O$, 10 mg; $CuSO_4.5H_2O$, 5 mg; $MnSO_4.H_2O$, 0.42 mg; $NaMoO_4.2H_2O$, 0.40 mg; and agar (for solid medium), 20g. Mycelial discs (1 cm^2) from the outermost growth zone of 4-5 day old cultures grown on agar plates at 25°C were used as inocula. Into each 100 ml sterile culture medium in 250 ml conical flasks, stoppered with cotton wool, was added aseptically 3 discs of inocula. After incubating at 30°C on a gyratory shaker for 4 days the contents of each flask were transferred into 1L conical flasks containing 300 ml of sterile culture medium and incubation continued for 2 days. These felts were used for the extraction and assays of nitrate reductase, glutamine synthetase and glutamate synthase. The felts used for nitrite reductase preparations were incubated for 4 days.

2.1.2 Harvesting

The felts harvested by centrifugation at 9,000 *g* for 5 min at 4°C in a Sorvall RC-2B refrigerated centrifuge, were washed three times with cold buffers which vary with the enzyme preparation as indicated later. The felts were then blotted between towel paper to remove water. Except for nitrite reductase preparations, the blotted mycelial pads could be stored

at -15°C for 1 month without loss of enzyme activity. Fresh mycelial felts were always used for nitrite reductase preparations.

2.2 Enzyme methods

2.2.1 Nitrate reductase assay

Nitrate reductase activity was determined by either measuring the production of nitrite colorimetrically or by following the nitrate-dependent oxidation of NADPH spectrophotometrically at 340 nm in a 1 cm quartz cuvette in a Varian Techtron model 635.

2.2.1.1 Colorimetric

The enzyme activity was determined at 30°C using either NAD(P)H, reduced viologen dyes or reduced flavin nucleotides as an electron donor.

When NAD(P)H was used as an electron donor the enzyme was assayed in open test tubes (1.3 x 10 cm). The reaction mixture contained (in 1 ml final volume): 50 mM phosphate buffer (pH 7.1); 1 mM KNO_3; 0.2 mM NAD(P)H; 10 µM FAD and an appropriate aliquot of the enzyme. The reaction was started by the addition of NAD(P)H and stopped after 15 min by adding 0.1 ml M Zn-acetate followed by 1.9 ml 95% (v/v) ethanol. Test tubes containing the same reaction mixture but the reaction terminated at zero time were included as controls. The reaction mixture was centrifuged at 3,000 *g* for 10 min in a bench MSE centrifuge. Nitrite produced in 1 ml aliquots was determined as described in Section 2.4.1.

The reaction mixture for the nitrate reductase assay with either MVH, BVH, $FADH_2$ or $FMNH_2$ as an electron donor was 50 mM

phosphate buffer (pH 7.5); 10 mM KNO_3; 0.2 mM MV, BV, FAD or FMN; 1 mM $Na_2S_2O_4$, in 1% (w/v) $NaHCO_3$ and an appropriate aliquot of the enzyme. The assay was carried out in test tubes (1 x 10 cm) fitted with subaseals. The tubes were evacuated by a water suction pump *via* needles inserted into the rubber caps and then flushed with argon for 2 min. The reaction was started by adding $Na_2S_2O_4$ (freshly prepared in 1% w/v $NaHCO_3$ under argon) and stopped after 15 min by rapid oxidation of the electron donor system by stirring the reaction mixture in a Vortex mixer. Zero time controls were always included and the nitrite produced was determined by the procedure of Hewitt and Nicholas (1964) as described in Section 2.4.1.

2.2.1.2 Spectrophotometric

The NADPH-linked nitrate reductase activity was also measured by following the rate of nitrate-dependent NADPH oxidation in a 1 cm quartz cuvette at 340 nm using a recording spectrophotometer (Varian Techtron model 635). The reaction mixture (in a 3 ml final volume) contained: 50 mM phosphate buffer (pH 7.1); 1 mM KNO_3; 0.2 mM NADPH; 10 µM FAD and an appropriate aliquot of the enzyme. The reaction was started by adding nitrate.

2.2.2 Nitrite reductase and hydroxylamine reductase assays

Nitrite reductase activity was assayed by measuring the utilization of nitrite, the production of ammonia and by following the oxidation of NAD(P)H.

2.2.2.1 Colorimetric

The enzyme activity was determined at 30°C using either NAD(P)H, reduced viologen dyes or reduced flavin nucleotides as the reductant.

The NAD(P)H-dependent nitrite utilization was measured in open test tubes at 30°C. The reaction mixture in a total volume of 1 ml contained: 50 mM phosphate buffer (pH 7.0); 0.1 mM KNO_2; 0.2 mM NAD(P)H; 10 µM FAD and an appropriate aliquot of the enzyme. The reaction was started by adding NAD(P)H and terminated after 10 min by adding 0.1 ml M Zn-acetate following by 1.9 ml 95% (v/v) ethanol. The reaction mixture was centrifuged at 3,000 *g* for 10 min in a bench centrifuge and nitrite determined in aliquots of the supernatant as described in Section 2.4.1. The reaction terminated at zero time was always included as control.

The NADPH-dependent ammonia production was measured as for the NADPH-dependent nitrite utilization described earlier, except that the total volume was 2 ml. The reaction was terminated by adding 25 µl 40 mM KCN. Ammonia formed was determined by the phenol-hypochlorite reaction (Russell, 1944) following a microdiffusion procedure in Conway units, as described in Section 2.4.2. Zero time controls were always included in these experiments.

For determining ammonia production in the hydroxylamine reductase assay, hydroxylamine-hydrochloride (12 mM) was used instead of nitrite as an electron acceptor. The composition and concentration of other components of the reaction mixture was as for the nitrite reductase assay described earlier.

For determination of nitrite reductase activity with either MVH, BVH, $FADH_2$ or $FMNH_2$ as an electron donor, the reaction mixture (in 1 ml final volume) contained: 50 mM phosphate buffer (pH 7.3); 0.1 mM KNO_2; 0.2 mM MV, BV, FAD or FMN; 1 mM $Na_2S_2O_4$ and an appropriate aliquot of the enzyme. The assay was carried out under

anaerobic conditions as described for the MVH-nitrate reductase assay (Section 2.2.1.1). The reaction was stopped after 10 min by vigorous agitation in a Vortex mixer. Nitrite was determined in 0.25 ml aliquots as described in Section 2.4.1. Zero time controls were always included in these experiments.

2.2.2.2 Spectrophotometric

The rate of nitrite-dependent NAD(P)H oxidation was followed in a 1 cm quartz cuvette at 340 nm using a Varian Techtron Spectrophotometer. The 3 ml total reaction mixture contained: 50 mM phosphate buffer (pH 7.0); 0.1 mM KNO_2; 0.2 mM NAD(P)H; 10 µM FAD and an appropriate aliquot of the enzyme. The solution was mixed thoroughly by inverting the cuvette several times before the reaction was started by adding nitrite.

For determination of hydroxylamine reductase, the electron acceptor was hydroxylamine-hydrochloride (12 mM). Diaphorase type activities were measured with either ferricyanide (0.2 mM), DCPIP (0.5 mM) or mammalian cytochrome c (0.05 mM) as electron acceptors.

2.2.3 Glutamine synthetase assay

Glutamine synthetase activity was determined by the transferase and biosynthetic reactions according to the procedure of Shapiro and Stadtman (1970).

2.2.3.1 Transferase reaction

The enzyme activity was determined in a reaction mixture (1 ml) in test tubes (1.3 x 10 cm) containing: Imidazole-HCl buffer

(pH 7.0), 40 mM; glutamine, 30 mM; hydroxylamine-hydrochloride (neutralized with 2M NaOH), 30 mM; $MnCl_2.4H_2O$, 0.3 mM; potassium arsenate, 20 mM; ADP, 0.4 mM and an appropriate aliquot of the enzyme. The reaction was started by adding the enzyme and the reaction mixture was incubated for 15 min at 37°C. The reaction was terminated by adding 2 ml of the following reagent mixture: 3.3% (w/v) $FeCl_3$ and 2% (w/v) TCA in 0.25N HCl. The colour was read at 540 nm in a 1 cm glass cuvette using a Shimadzu (QV-50) spectrophotometer. The amount of γ-glutamylhydroxamate produced was determined from a standard curve. Zero time controls were always included in these experiments.

2.2.3.2 Biosynthetic reaction

The biosynthetic activity was determined in reaction mixture, in a final volume of 0.5 ml, containing: Imadazole-HCl buffer (pH 7.0), 50 mM; glutamate, 100 mM; NH_4Cl, 50 mM; either $MgCl_2$, 50 mM or $MnCl_2.4H_2O$, 5 mM; ATP, 7.5 mM and an appropriate aliquot of the enzyme. The reaction started by adding the enzyme, was carried out in test tubes (1.3 x 10 cm) at 37°C. After 15 min the reaction was terminated by adding 3 ml of 1% (w/v) $FeSO_4.7H_2O$ in 0.015N H_2SO_4 followed by 0.3 ml of 6.6% (w/v) ammonium molybdate in 7.5N H_2SO_4. The colour developed after 1 min was read at 660 nm in a 1 cm glass cuvette in a Shimadzu (QV-50) spectrophotometer. The amount of P_i formed was calculated from a standard curve using KH_2PO_4 as the standard. Zero time controls were always included in these experiments.

2.2.4 Glutamate synthase assay

The activity of the enzyme was determined at 30°C by measuring the initial rate of oxidation of NADPH in a 1 cm quartz cuvette at 340 nm using a Varian Techtron spectrophotometer. The reaction mixture, in 3 ml final volume, contained: 100 mM phosphate buffer (pH 8.0) ; 6 mM glutamine; 3 mM α-ketoglutarate; 0.1 mM NADPH and an appropriate aliquot of the enzyme.

2.2.5 Determination of Michaelis-Menten constant (K_m)

In order to determine the K_m values, the enzyme activity was measured at various concentrations of one substrate, in the presence of saturating concentrations of the other substrate. The K_m value was then estimated from a double reciprocal plot of the velocity of the reaction against concentration of substrate as described by Lineweaver and Burk (1934).

2.2.6 Determination of inhibitor constant (K_i)

For determining the K_i values, the enzyme activity was measured at various concentrations of substrate in the presence of various concentrations of inhibitor. The K_i values were determined from reciprocal plots of the rate of reaction against inhibitor concentration as described by Dixon and Webb (1979).

2.2.7 Determination of molecular weights

2.2.7.1 Gel filtration

The molecular weight of the purified enzyme was determined by gel filtration through a Sepharose 6B column (78 x 2 cm) according

to the method of Andrews (1970). The column prepared and equilibrated as described in Section 2.3.2.3, was calibrated with thyroglobulin (MW 669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and albumin (68 kDa) as protein markers. Void volume of the column was determined from the elution of blue-dextran. The molecular weight was estimated from plot of log (MW) against distribution coefficient (K_{av}). The K_{av} values were calculated according to the following equation (Andrews, 1970):

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$

where V_e = the elution volume

V_o = the void volume

V_t = the bed volume

2.2.7.2 Electrophoresis

The molecular weights of the enzyme subunits were determined by polyacrylamide (12.5% w/v) slab gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulphate. The enzyme solution was boiled in a waterbath for 3 min in the presence of 1% (w/v) SDS and 5% (v/v) β-mercaptoethanol. To facilitate layering, glycerol (5%, v/v) was added to each sample and bromophenol blue was used as a tracking dye. The running buffer used was 0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS. Electrophoresis was performed at a constant current of 20 mA through the stacking gel and then increased to 30 mA in the resolving gel. The tracking dye was allowed to travel approximately 0.5 cm from the bottom of the gel before termination of electrophoresis. The proteins used as markers were phosphorylase *b* (subunit MW 94 kDa), albumin (67 kDa), ovalbumin

(43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

2.2.8 Separation and identification of flavin from nitrate reductase

Tubes containing the purified enzyme were placed in a boiling water bath for 15 min. After cooling the precipitated protein was removed by centrifugation at 100,000 *g* for 30 min. The supernatant was loaded onto a DEAE-cellulose column (DE-32, 3 x 12 cm) equilibrated with 50 mM phosphate buffer (pH 7.5). After washing with the same buffer, the column was eluted with a 200 ml linear gradient of NaCl (0-0.3M) in the buffer and 2 ml fractions were collected using a LKB Ultrafrac fraction collector. All yellow coloured fractions were pooled, desalted by passing through a Sephadex G-10 column (1.5 x 15 cm) and then concentrated in a Speed Vac Concentrator, model SVC 100H (Savant Instrument, N.Y.).

Aliquots of the isolated flavin as well as the appropriate standards namely FAD and FMN (2 mg/ml) were spotted 7.5 cm apart on a Whatman 3MM paper (23 x 57 cm). The chromatograms were developed for 30 hr in a descending sequence using t-butanol : water (60:40) as the solvent system. After drying, the flavins were located on the chromatograms by viewing under U.V. light.

2.2.9 Adenylylation and deadenylylation of glutamine synthetase

The extent of adenylylation of glutamine synthetase was determined by measuring the transferase activity (as described in Section 2.2.3.1) with and without 60 mM $MgCl_2$ (Shapiro and Stadtman, 1970; Michalski *et al.*, 1983; Vairinhos *et al.*, 1983). The values obtained were then used to determine the adenylylation state (n) of the enzyme in the following formula:

$$n = 8\text{-}8\left(\frac{\text{activity with 0.3 mM } Mn^{2+} + \text{60 mM } Mg^{2+}}{\text{activity with 0.3 mM } Mn^{2+}}\right).$$

Percent of adenylylation was determined by dividing n by 8 then multiplying by 100.

Since the adenylylated enzyme can be deadenylylated by treatment with snake venom phosphodiesterase (SVD), 500 µg of SVD was added to 10 ml of a purified preparation of glutamine synthetase. After incubating for 1 hr at 37°C the mixture was passed through G-10 column (1.5 x 10 cm) to separate AMP which was cleaved from the adenylylated enzyme. Fractions collected from the column (G-10 fractions) were then assayed for its transferase activity with or without 60 mM $MgCl_2$. The values obtained were then used to determine the adenylylated state as described above for the untreated enzyme.

To establish that AMP was cleaved from the adenylylated enzyme, the AMP released was detected by polyacrylamide gel electrophoresis. A sample for electrophoresis was prepared as follows: 500 µg SVD was added to 10 ml purified enzyme preparation. After incubating at 37°C for 1 hr, the mixture was concentrated to 250 µl using a Speed Vac Concentrator. The sample (100 µl) and 10 µl (0.2 mg/ml) AMP standard were loaded separately onto the polyacrylamide gel (12.5%, w/v). To facilitate layering, glycerol was added to the sample to a final concentration of 5% (v/v). Prior to loading the samples, the gel was equilibrated for 15 min at 20 mA. The running buffer used was 0.09M Tris, 0.08M boric acid and 0.93g/1 Na_2-EDTA (pH 8.4). The electrophoresis was performed for 15 min at 20 mA then at 30 mA for 2.5 hr at room temperature. The AMP was detected in the gels by U.V. light by reference to an appropriate standard.

2.3 General techniques

2.3.1 Incorporation of ^{15}N into washed felts

Washed felts (approximately 1g fresh weight) were suspended, with gentle shaking, into 20 ml of N-free culture medium. The suspensions were incubated with and without L-methionine-DL-sulphoximine (2 mM) or azaserine (1 mM) for 2 hr at 30°C. Then into each sample was added $(^{15}NH_4)_2SO_4$ (5 mM final concentration) and incubation continued for a further 1 hr. The felts, filtered through a Whatman paper No. 541 in a glass funnel connected to a vacuum flask, were washed thoroughly with cold glass double distilled water. The felts were then dispensed into micro-Kjeldahl flasks containing 4 ml of 36N H_2SO_4 and 24g of Kjeldahl catalyst (7g HgO + 93g K_2SO_4) for a 3 hr digestion. The ammonia produced by NaOH treatment of digested samples was distilled in a Markham apparatus into 1% (w/v) boric acid which was then titrated with 0.01N sulphuric acid. After adding 0.1 ml N H_2SO_4, the solution was concentrated to 2 ml by heating on a hot plate. The ammonium N was converted into N_2 by adding alkaline hypobromite in rigorously evacuated Rittenberg tubes (Brownell and Nicholas, 1967). Measurements of ^{15}N enrichment were carried out in a 602E Mass spectrometer (ISOMASS , Middlewich, Cheshire, U.K.).

2.3.2 Preparations of columns

The columns were prepared according to the instructions given by the manufacturers; Amicon Corporation for Matrex Gel Blue A, Pharmacia Fine Chemicals for Blue Sepharose CL-6B and Sepharose 6B, and Whatman Ltd. for DEAE-cellulose.

2.3.2.1 Matrex Gel Blue A

The column packed by pouring the preswollen gel into a glass column in one operation was generated with four bed volumes of 8M Urea in 0.5N NaOH and then equilibrated with 0.1M phosphate buffer (pH 7.5). Before reuse, the column was always regenerated as described above. When not in use the column was stored at 4°C at pH 7.5 in the presence of 0.2% (w/v) sodium azide.

2.3.2.2 Blue Sepharose CL-6B

The freeze-dried powder of Blue Sepharose CL-6B was swollen for 15 min and washed with glass distilled water on a sintered glass filter. About 200 ml of distilled water was used for each gram of dry powder, giving a final volume of approximately 3.5 ml gel. The gel was packed into a glass column and then equilibrated with either 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM $MnCl_2$, for purifying glutamine synthetase or 50 mM Tris-HCl buffer (pH 7.5) for glutamate synthase purification. For reuse, the gel was regenerated with 4-5 washing cycle of alternate 10 column volumes of 0.1M Tris-HCl buffer containing 0.5M NaCl (pH 8.5) and 10 column volumes of 0.1M Na-acetate buffer containing 0.5M NaCl (pH 4.5), and then washed with the equilibration buffer. When not in use the gel was stored at 2°C in the appropriate buffer in the presence of 0.1% (w/v) sodium azide.

2.3.2.3 Sepharose 6B

The preswollen gel of Sepharose 6B was diluted with glass distilled water and stirred gently with a glass rod. After standing for 30 min, fine particles were removed by decantation and the gel

was degassed under reduced pressure. The homogenous slurry of the gel was poured in one operation into a glass column until the desired column height was attained. The column was equilibrated with the appropriate buffer. When not in use, the column was stored at 2°C in the presence of 1% (w/v) sodium azide.

2.3.2.4 DEAE-cellulose

Cellulose ion exchanger, DE-32, was stirred with a glass rod in 15 volumes of 0.5N HCl. After leaving for 30 min, the supernatant was decanted and the gel was washed in a funnel with double distilled water until the pH of the filtrate was 4. It was then stirred in 15 volumes of 0.5N NaOH and left for a further 30 min. This second treatment was repeated and the gel was again washed in a funnel with double distilled water until the pH of the filtrate was 8. The gel was then equilibrated with either 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM $MnCl_2$ or 50 mM Tris-HCl buffer (pH 7.5). The column was packed by pouring the homogenous slurry into a glass column in one operation. After the gel had settled, the appropriate buffer was passed through the column until the pH of the effluent was the same as that of the equilibration buffer. For each use, DE-32 was regenerated afresh as described above. When not in use the gel was stored at 2°C in aqueous solution of 0.1% (w/v) sodium azide.

2.4 Other determinations

2.4.1 Nitrite

Nitrite was determined by the procedure of Hewitt and Nicholas (1964). The red azo dye in 1 ml of the aliquot was developed by adding 1 ml of 1%

(w/v) sulphanilamide in 1.5N HCl followed by 1 ml of 0.02% (w/v) aqueous solution of N-1-naphthylethylenediamine dihydrochloride. After standing for 15 min the absorbance at 540 nm was read in a 1 cm glass cuvette using a Hitachi Perkin Elmer spectrophotometer. The concentration of nitrite was determined from a standard curve.

2.4.2 Ammonia

The micro-diffusion technique of Conway (1962) was used and ammonia was determined by the phenol-hypochlorite reaction according to Russell (1944). Into test tubes (1.3 x 10 cm) kept in an ice bath, 1.5 ml of sample was added followed by 50 µl 3 mM $MnCl_2$, 1 ml of cold 25% (w/v) phenol in 2.7N NaOH and 1 ml of cold hypochlorite reagent. The contents were mixed by gentle rotation of the tubes which were then inserted quickly into a boiling water bath for about 5 min. After cooling the colour was read at 625 nm using a Hitachi Perkin Elmer spectrophotometer. The concentration of ammonia was calculated from a standard curve.

The hypochlorite reagent was prepared as follows: 25g of calcium hypochlorite was thoroughly ground and then dissolved in 300 ml of hot double distilled water. Into the hypochlorite solution 135 ml of ammonia-free 20% (w/v) K_2CO_3 was added with stirring and after mixing thoroughly, the mixture was heated briefly to about 90°C. After cooling, the volume was made to 500 ml. Then the mixture was filtered and the filtrate was stored in a brown bottle in the dark at 2°C.

2.4.3 Protein

Protein was determined by the dye binding method of Bradford (1976). To 1 ml sample containing 10 to 100 µg protein in test tubes (1.3 x 10 cm)

was added 3 ml Bradford reagent (0.01%, w/v, Coomassie Brilliant Blue G-250; 4.7%, v/v, ethanol; and 8.5%, w/v, phosphoric acid). The contents were mixed in a Vortex unit. After stranding for 5 min, the colour was read at 595 nm using a Hitachi Perkin Elmer spectrophotometer. The protein content was determined from a standard curve using bovine serum albumin as the protein standard.

2.5 Biochemicals, chemicals and other materials

2.5.1 Biochemicals and chemicals

All nucleotides, amino acids and organic acids, bovine serum albumin, cytochrome *c* (horse heart), hydroxylamine-hydrochloride, γ-glutamylhydroxamate, L-methionine-DL-sulphoximine, azaserine, Tris (hydroxymethyl) aminomethane, imidazole, dithiothreitol, p-chloromercuribenzoate, rotenone, amytal, acrylamide, N,N'-methylene-bis-acrylamide, methyl viologen and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Snake venom phosphodiesterase was obtained from Boehringer Mannheim (West Germany). The following protein markers: α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin, phosphorylase *b*, aldolase, catalase, ferritin and thyroglobulin were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Sodium diethyldithiocarbamate was from Merck (Darmstadt, West Germany), 2,6-dichlorophenolindophenol was from Ajax Chemicals Ltd. (Sydney, Australia) and benzylviologen was from B.D.H. Ltd. (Poole, England). $(^{15}NH_4)_2SO_4$ (5.25% atom excess) was purchased from L'Office National Industrial de l'Azote (ONIA), Marseille, France.

All other chemicals were of the highest purity available.

2.5.2 Other materials

Matrex Gel Blue A was purchased from Amicon Corporation (Lexington, Mass, USA). Blue Sepharose CL-6B and Sepharose 6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE-cellulose (DE-32) and Whatman 3MM paper were from Whatman Ltd. (Kent, England).

3. RESULTS

3.1 Nitrate reductase

3.1.1 Purification

Nitrate reductase was purified 108-fold with a recovery of 39% by ammonium sulphate fractionation and affinity chromatography. All operations during the purification were carried out at 4°C. The preparation of the mycelial felts from cultures is described in Section 2.1.2.

In a typical preparation 20g of frozen mycelial felts were thawed and homogenized in 60 ml of cold 0.1M phosphate buffer (pH 7.5) containing 5 mM EDTA, 5 mM cysteine, 10 µM FAD and 10% (w/v) glycerol. The homogenate was passed twice through a French pressure cell at 20,000 p.s.i. and then centrifuged at 27,000 *g* for 20 min. Nitrate reductase was recovered in the supernatant fraction (S_{27}) which was used as crude extract.

Solid ammonium sulphate was slowly added to the crude extract with constant stirring under argon to a 40% ammonium sulphate saturation. During the addition of ammonium sulphate, the pH of solution was maintained at 7.5 by adding cold 0.1M NaOH. After standing in ice for 30 min the mixture was centrifuged at 20,000 *g* for 15 min. The pellet taken up in 0.1M phosphate buffer (pH 7.5) was dialyzed for 4 hr against 3L of the same buffer and then loaded onto a Matrex Gel Blue A column (100 x 15 mm). Prior to loading the enzyme, 50 ml of BSA (2 mg/ml in 0.1M phosphate buffer (pH 7.5) containing 0.2 mM EDTA and 0.1 mM DTT) was passed through the column which was then washed with 150 ml of the same buffer. After loading the enzyme the column was again washed with the buffer containing 0.5 mM ATP and 0.5 mM $NADP^+$, and then with 0.1M phosphate buffer (pH 7.5). The enzyme was eluted

with 0.1M phosphate buffer (pH 7.5) containing 1 mM NADPH. Details of the purification of the enzyme are given in Table 1.

3.1.2 Properties

3.1.2.1 Molecular weight

The purified nitrate reductase has a molecular weight of 210 kDa as determined by gel filtration on a Sepharose 6B column (Fig. 1A). Estimation of subunit molecular weight of the enzyme by mean of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate showed two major bands. Thus the enzyme contains two subunits with molecular weights of 107 and 123 kDa (Fig. 1B).

3.1.2.2 Effect of pH

The pH dependence of nitrate reductase activity was determined with either NADPH or MVH as an electron donor in the range of 6.5 to 8.0 (Fig. 2). The pH optima were 7.1 and 7.5 for the NADPH- and the MVH-dependent reactions, respectively.

3.1.2.3 Electron donors

A variety of compounds have been tested as electron donors for the purified enzyme (fraction 3, Table 1). The results in Table 2 indicate that both NADPH and NADH function as reductants but NADPH was more effective. For the NADPH- and NADH-dependent reactions FAD was required for maximal activity; there was no enzyme activity in the absence of added FAD or when FMN substituted for FAD. The

TABLE 1: Purification of nitrate reductase

The enzyme activity from each fraction was measured with either MVH (values without brackets) or NADPH (values in brackets) as an electron donor. The reaction mixture was as described in Section 2.2.1.1.

One unit of enzyme activity corresponds to 1 nmole NO_2^- produced/min.

Fractions	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
1. Crude extract (S27)	3138 (1854)	265.2	11.83 (6.99)	-	100
2. Precipitate from 0-40% $(NH_4)_2 SO_4$ saturation, dialyzed against 0.1M phosphate buffer (pH 7.5) for 4 hr	3121 (1334)	153.6	20.32 (8.68)	2	99
3. Fraction 2 loaded onto a Matrex Gel Blue A column (10x1.5cm) and eluted with 1 mM NADPH in 0.1M phosphate buffer (pH 7.5)	1236 (488)	0.888	1391.89 (549.32)	118	39

FIG. 1A: Molecular weight determination of nitrate reductase by gel filtration.

The enzyme (fraction 3, Table 1) was passed through a Sepharose 6B column as described in Section 2.2.7.1. Elution buffer was 0.1M phosphate (pH 7.5) containing 0.1M NaCl. The K_{av} values were calculated from $K_{av} = (V_e-V_o)/(V_t-V_o)$ where V_e is the elution volume, V_o is the void volume and V_t is the bed volume. The marker proteins of known molecular weight were:

(○) ferritin;
(■) catalase;
(▲) aldolase; and
(●) albumin.

FIG. 1B: Estimation of subunit molecular weight by SDS-polyacrylamide gel electrophoresis.

The molecular weight of the subunit of the purified enzyme (fraction 3, Table 1) was determined by polyacrylamide (12.5%) slab gel electrophoresis in the presence of 0.1% (w/v) SDS, as described in Section 2.2.7.2. Relative mobilities (R_f) of the proteins were calculated by dividing the migration distance of protein with the movement of the tracking dye at the end of the run. The standard proteins used were:

(○) phosphorylase *b*;
(■) albumin;
(▲) ovalbumin;
(●) carbonic anhydrase;
(□) trypsin inhibitor; and
(△) α-lactalbumin.

FIG 1A

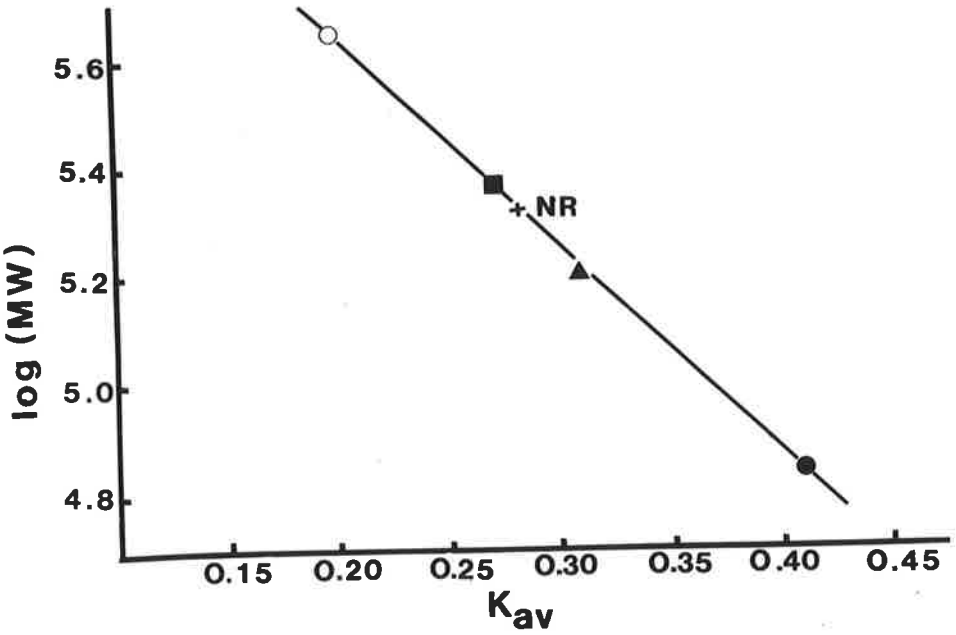

5.6
5.4
5.2
5.0
4.8
log (MW)
NR
0.15
0.20
0.25
0.30
0.35
0.40
0.45
K_{av}

FIG 1B

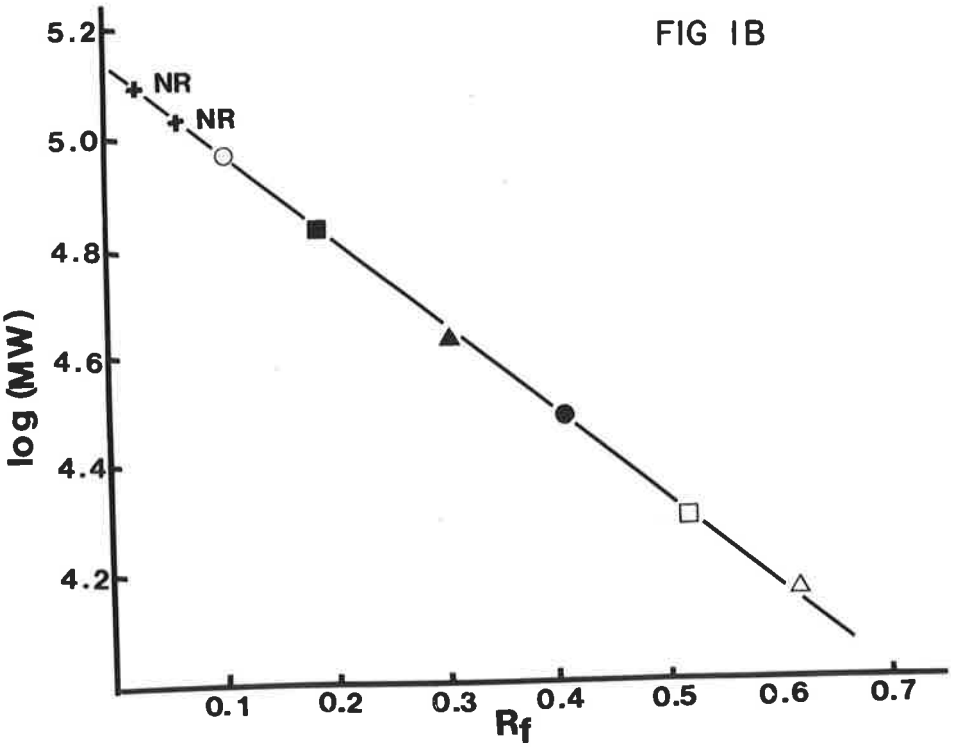

5.2
5.0
4.8
4.6
4.4
4.2
log (MW)
NR
NR
0.1
0.2
0.3
0.4
0.5
0.6
0.7
R_f

FIG. 2: Effect of pH on nitrate reductase activity.

Activity of the enzyme (fraction 3, Table 1) was measured at various pH values with MVH (□) and NADPH (■) respectively as an electron donor. The reaction mixture was as described in Section 2.2.1.1.

FIG 2

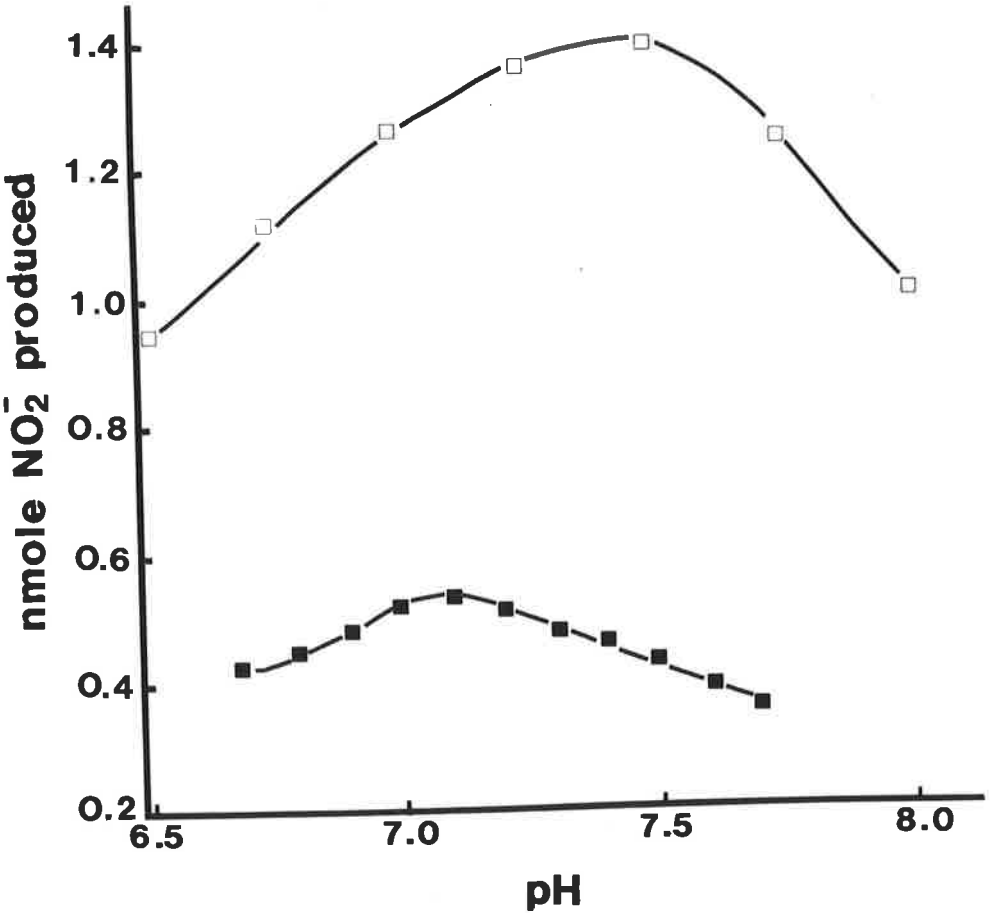


TABLE 2: Electron donors for the purified nitrate reductase activity

The assay conditions were as described in Section 2.2.1.1. When NAD(P)H was used as an electron donor the reaction mixture (in a final volume of 1 ml) was:

50 mM phosphate buffer (pH 7.1); 1 mM KNO_3; 0.2 mM NAD(P)H; 10 µM FAD or FMN and 10 µg enzyme (fraction 3, Table 1).

The reaction mixture with either MVH, BVH, $FMNH_2$ or $FADH_2$ as a reductant was:

50 mM phosphate buffer (pH 7.5); 10 mM KNO_3; 0.2 mM MV, BV, FMN or FAD and 1 mM $Na_2S_2O_4$ (in 1% w/v $NaHCO_3$, freshly prepared) and 10 µg enzyme. The enzyme preparation (fraction 3, Table 1) was used after dialyzing in 50 mM phosphate buffer (pH 7.5) for 4 hr.

The results are expressed as % activity of control (using NADPH as an electron donor). The specific activity of the control was 551 nmole NO_2^- produced/min/mg protein.

Electron donors	Activity (%)
NADPH	0
NADPH, FAD	100
NADPH, FMN	0
NADH	0
NADH, FAD	24
NADH, FMN	0
$Na_2S_2O_4$	0
$Na_2S_2O_4$, MV	242
$Na_2S_2O_4$, BV	149
$Na_2S_2O_4$, FAD	21
$Na_2S_2O_4$, FMN	43

data in Table 2 also show the effects of dithionite reduced-viologen dyes and -flavins on nitrate reduction. At a concentration of 0.2 mM of reductant, the enzyme activity with MVH and BVH as an electron donor was about 2.5- and 1.5-fold respectively, of that with NADPH at a similar concentration. On the other hand dithionite reduced-FMN or -FAD were less effective electron donors.

3.1.2.4 K_m values for substrate, reductant and cofactor

The effects of various concentrations of nitrate on enzyme activity with various electron donors are illustrated in Figs. 3A, 3B, 4A and 4B. The apparent K_m values for nitrate calculated from double reciprocal plots using NADPH, MVH, $FMNH_2$ and $FADH_2$ as an electron donor were (μM): 33, 1700, 150 and 71, respectively.

The K_m values for NADPH and FAD in the NADPH-dependent reaction were 40 and 0.2 μM, respectively (Figs. 5A and 5B), whereas that for MVH was 1.8 μM (Fig. 6).

3.1.2.5 Characterization of flavin isolated from the purified enzyme

The yellow-coloured fraction isolated from the boiled purified nitrate reductase (fraction 3, Table 1) as described in Section 2.2.8, showed an absorption band around 450 nm which disappeared on the addition of sodium dithionite. This spectral change indicated that the enzyme contains a flavin compound.

The isolated flavin compound, along with authentic samples of FMN and FAD were chromatographed on a 3MM Whatman paper. In the dried chromatograms observed under UV light (Fig. 7) the flavin isolated from nitrate reductase was found to have co-migrated with FAD.

FIG. 3: Double reciprocal plots of the effects of various nitrate concentrations on NADPH- and MVH-dependent nitrate reductase activities.

A: NADPH as an electron donor

B: MVH as an electron donor

Nitrate reductase activities were determined as described in Section 2.2.1.1, except that the concentration of nitrate in the assay mixture was varied as indicated. The purified enzyme preparation (fraction 3, Table 1) was used after dialyzing against 3L of 50 mM phosphate buffer (pH 7.5) for 4 hr.

S = substrate (nitrate, mM)

V = activity (μmole NO_2^- produced/min/mg protein).

FIG 3A

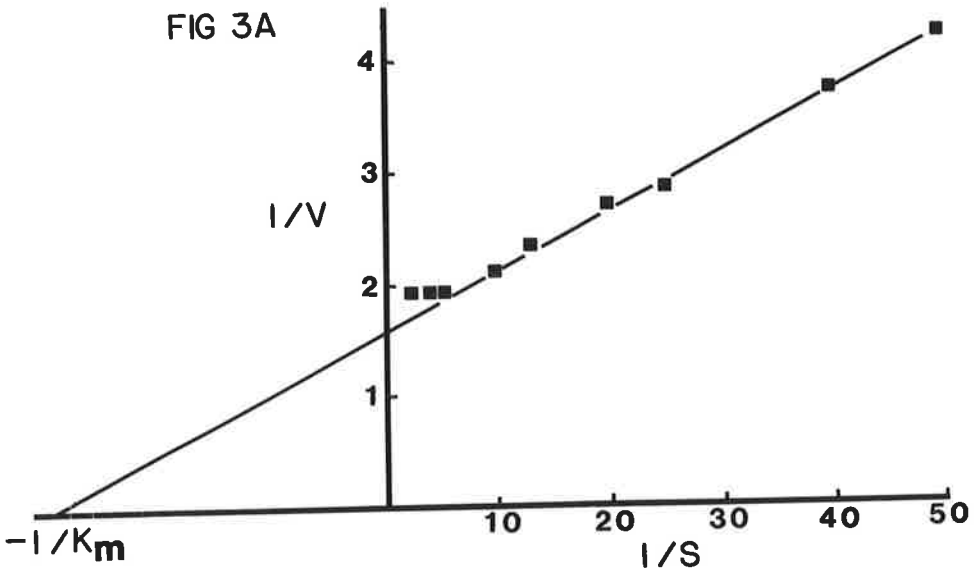


FIG 3B

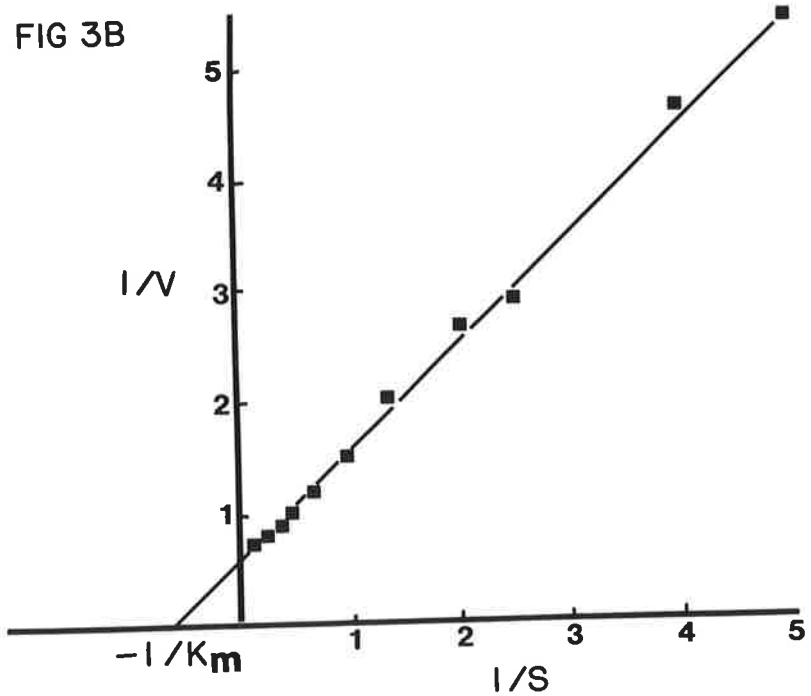


FIG. 4: Double reciprocal plots of the effects of various concentrations of nitrate on $FMNH_2$- and $FADH_2$-linked enzyme activities.

A: $FMNH_2$ as a reductant.

B: $FADH_2$ as a reductant.

The activity of the purified nitrate reductase (fraction 3, Table 1) was determined as described for the standard enzyme assay in Section 2.2.1.1, except that the concentration of nitrate was varied as indicated. The enzyme preparation (fraction 3, Table 4) was dialyzed against 3L of 50 mM phosphate buffer (pH 7.5) for 4 hr before it was used in the assay.

S = substrate (nitrate, mM)

V = activity (μmole NO_2^- produced/min/mg protein).

FIG 4A

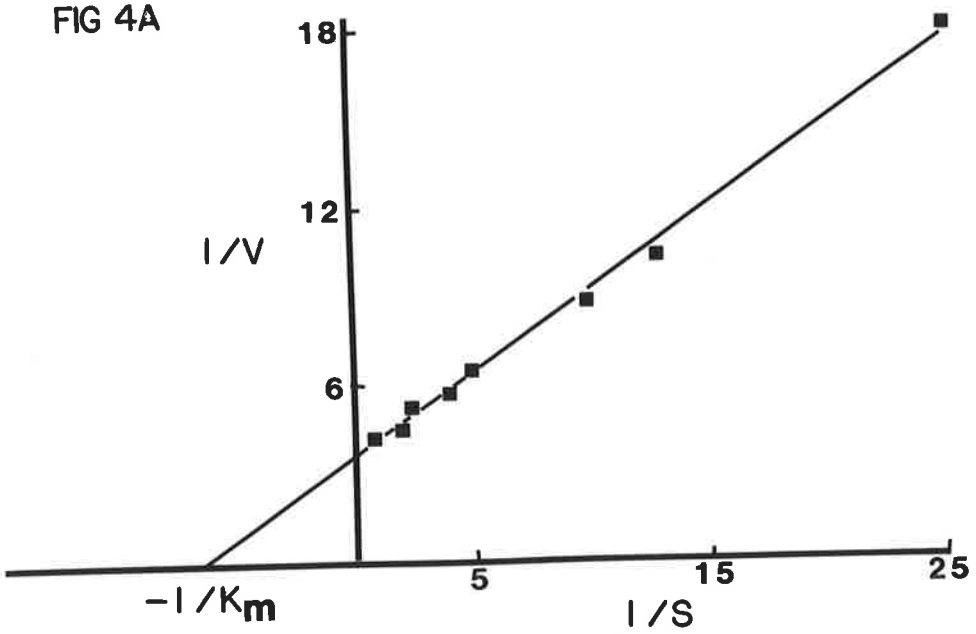


FIG 4B

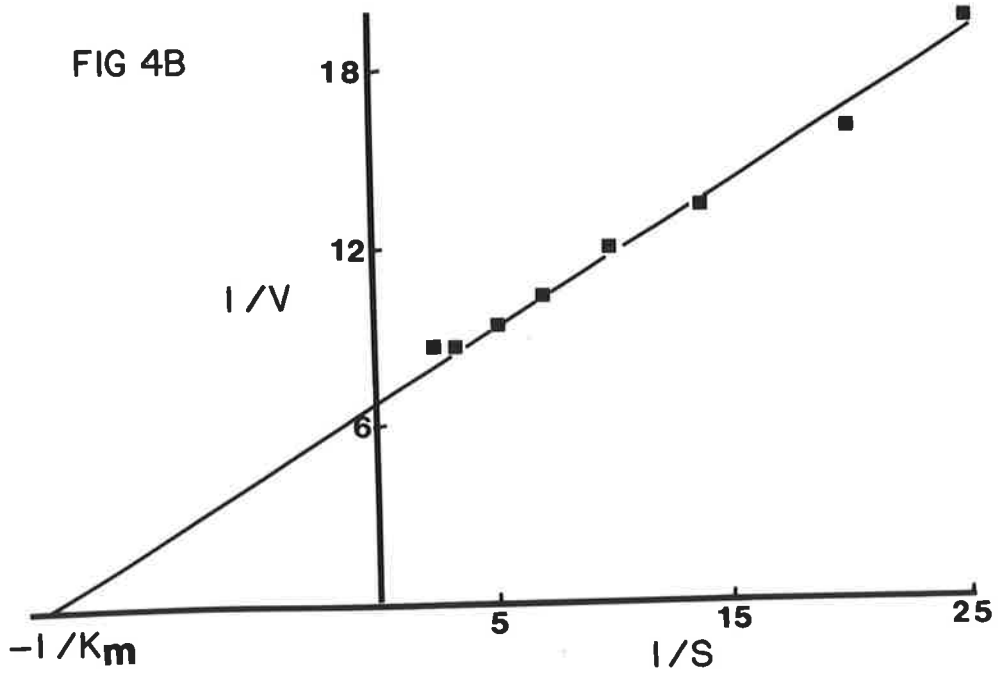


FIG. 5: Double reciprocal plots of the effects of varying NADPH and FAD concentrations respectively on NADPH-dependent nitrate reductase activity.

A: various concentration of NADPH.
B: various concentration of FAD.

Nitrate reductase activity of the purified enzyme (fraction 3, Table 1) was measured as described in Section 2.2.1.1, except that the concentration of either NADPH or FAD was varied as indicated. The purified enzyme (fraction 3, Table 1) was dialyzed against 3L of 50 mM phosphate buffer (pH 7.5) for 4 hr before it was used in the assay.

V = activity of the NADPH-dependent reaction (μmole NO_2^- produced/min/mg protein).

FIG 5A

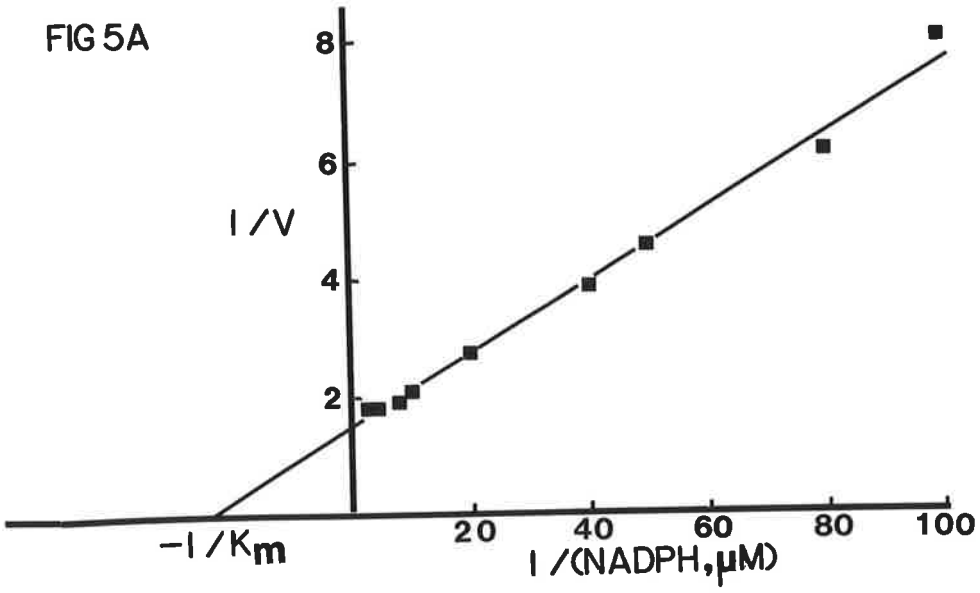

8
6
1/V
4
2
-1/Km
20
40
60
80
100
1/(NADPH,μM)

FIG 5B

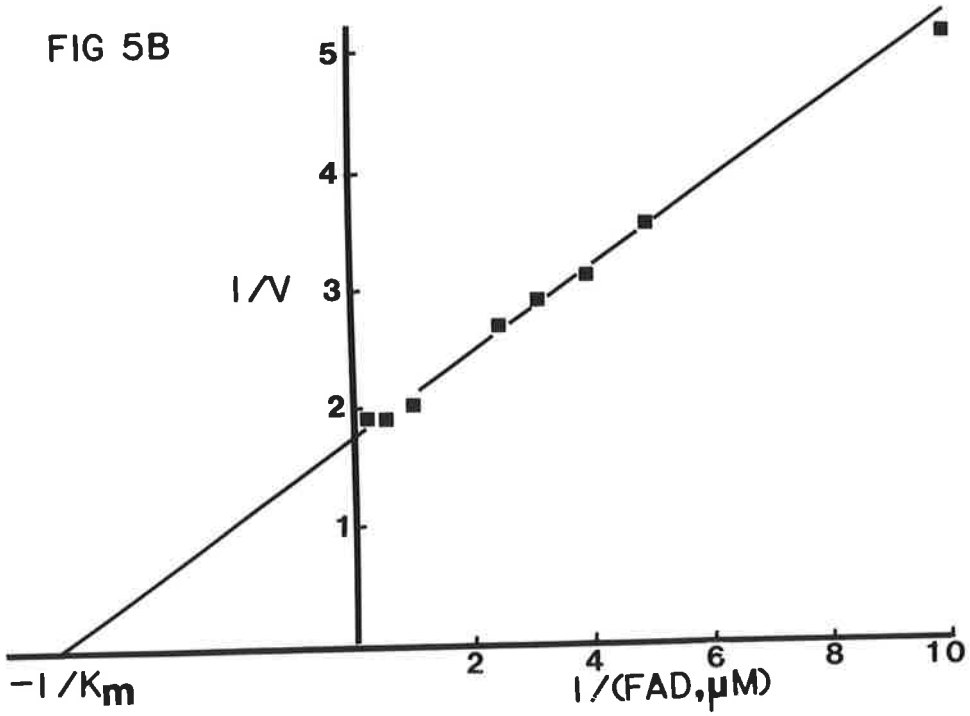

5
4
1/V
3
2
1
-1/Km
2
4
6
8
10
1/(FAD,μM)

FIG. 6: A double reciprocal plot of the effect of various concentrations of reduced methyl viologen on nitrate reductase activity.

The activity of the purified enzyme (fraction 3, Table 1) was determined as described in Section 2.2.1.1, except that the concentration of reduced methyl viologen was varied as indicated. The purified enzyme (fraction 3, Table 1) was used after dialyzing against 3L of 50 mM phosphate buffer (pH 7.5) for 4 hr.

V = activity (μmole NO_2^- produced/min/mg protein).

FIG 6

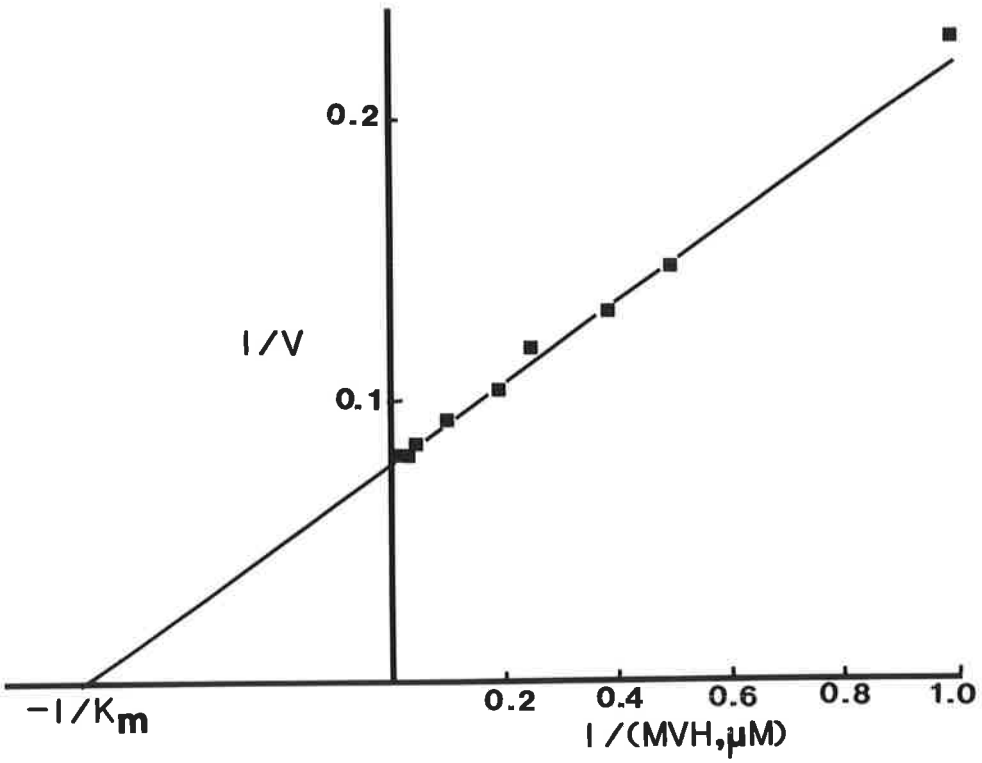
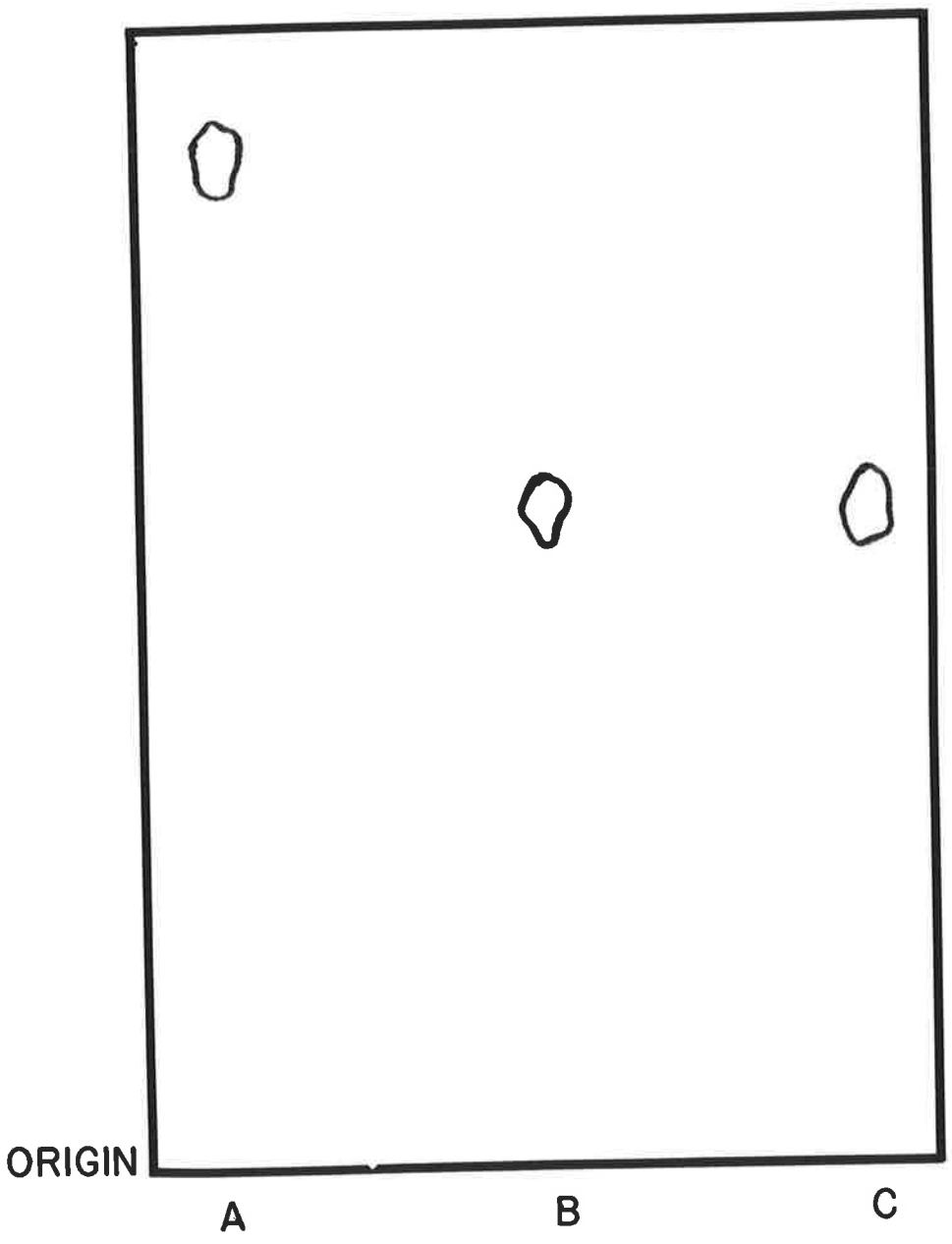


FIG. 7: Separation of the flavin component of the purified nitrate reductase by paper chromatography.

Flavin was isolated from the purified enzyme (fraction 3, Table 1) as described in Section 2.2.8. The isolated flavin along with authentic standards of FMN and FAD were subjected to a paper chromatography.

A = standard FMN (50µg).
B = flavin isolated from nitrate reductase.
C = standard FAD (50µg).

FIG 7



3.1.3 Inhibitor studies

The inhibitory effects of several compounds were tested with NADPH and MVH as reductants for the purified enzyme (fraction 3, Table 1).

Thus azide (0.1 mM), thiocyanate (0.5 mM) and sodium diethyldithiocarbamate (1.0 mM) markedly inhibited MVH-dependent nitrate reductase activity (Table 3) whereas cyanide (2.0 mM), 8-hydroxyquinoline (1.0 mM) and α,α'-dipyridyl (2.0 mM) restricted the enzyme to a lesser extent.

Similarly, the NADPH-dependent activity was strongly retarded by azide (90% inhibition at 0.5 mM) and to a lesser extent by cyanide (Table 4).

Sulphydryl-group inhibitors such as p-CMB and N-ethylmaleimide also restricted enzyme activity. The extent of inhibition by p-CMB (0.5 mM) on the NADPH-dependent activity was similar to that for azide, but it was less for the MVH-linked reaction (Table 5). Rotenone (0.1 mM) and amytal (0.5 mM), flavoprotein inhibitors, restricted enzyme activity when NADPH was the reductant, whereas amytal at the same concentration was not very inhibitory in the MVH-linked reaction.

Double reciprocal plots of the effects of various concentrations of either chlorate or bromate at different levels of nitrate on the MVH-dependent nitrate reductase activity are illustrated in Figs. 8A and 8B. These two compounds inhibited the reaction competitively with respect to nitrate. The apparent K_i values estimated from Dixon plots were 18.1 mM for chlorate (Fig. 9) and 15.6 mM for bromate (Fig. 10).

Nitrite, the product of nitrate reduction, also inhibited the NADPH-dependent nitrate reductase activity competitively with respect to nitrate and the K_i value for nitrite was 90 μM (Figs. 11A and 11B).

TABLE 3: Effects of metal inhibitors on the MVH-dependent nitrate reductase activity

The reaction mixture was as described in Section 2.2.1.1. The enzyme preparation (fraction 3, Table 1) was pre-incubated with the inhibitors for 15 min prior to starting the reaction.

The results are expressed as % inhibition with respect to the control (without inhibitor). The specific activity of the control was 1386 nmole NO_2^- produced/min/mg protein.

Inhibitors	Final Concentration (mM)	Inhibition (%)
NaN_3	0.10	87
	0.25	89
	0.50	91
KSCN	0.10	59
	0.25	74
	0.50	83
KCN	0.40	18
	0.80	30
	2.00	41
DIECA	0.25	34
	0.50	56
	1.00	78
8-Hydroxyquinoline	0.25	30
	0.50	36
	1.00	40
α,α'-dipyridyl	0.50	23
	1.00	26
	2.00	33

TABLE 4: Effects of inhibitors on NADPH-dependent nitrate reductase activity

The reaction mixture was as described in Section 2.2.1.1. Inhibitors, at the concentrations indicated, were preincubated with the purified enzyme (fraction 3, Table 1) for 15 min prior to starting the reaction. The results are expressed as % inhibition of the control (without inhibitor). Activity of the control was 552 nmole NO_2^- produced/min/mg protein.

Inhibitors	Final Concentration (mM)	Inhibition (%)
NaN_3	0.10	60
	0.25	76
	0.50	90
KCN	0.40	24
	0.80	36
	2.00	52
p-CMB	0.10	68
	0.20	76
	0.50	90
NEM	0.50	32
	1.00	48
	2.50	60
Rotenone	0.01	30
	0.05	38
	0.10	62
Amytal	0.10	15
	0.25	28
	0.50	45

TABLE 5: Effects of inhibitors of sulphydryl-groups and flavin respectively on MVH-nitrate reductase activity

The enzyme preparation (fraction 3, Table 1) was preincubated with the inhibitors for 15 min prior to starting the reaction. The assay mixture was as described in Section 2.2.1.1. The results are expressed as % inhibition of the control (without inhibitor). The activity of the control was 1386 nmole NO_2^- produced/min/mg protein.

Inhibitors	Final Concentration (mM)	Inhibition (%)
Iodoacetamide	0.5	9
	1.0	13
	2.5	14
NEM	0.5	14
	1.0	16
	2.5	30
p-CMB	0.1	16
	0.2	50
	0.5	64
$HgCl_2$	0.1	47
	0.2	71
	0.5	90
Amytal	0.5	9
	1.0	16
	2.0	31

FIG. 8: Inhibitory effects of chlorate and bromate on MVH-nitrate reductase activity.

The activity of the enzyme (fraction 3, Table 1) was determined as described in Section 2.2.1.1, except that various concentrations of either chlorate or bromate were added at defined levels of nitrate.

A: A Lineweaver-Burk plot of the effects of chlorate at various levels of nitrate. The concentrations of chlorate were:

(△) 0 mM; (●) 2.5 mM; (▲) 5 mM; and (○) 10 mM.
Inset: Inhibition by chlorate with nitrate at (◆) 2.5 mM; (▲) 5 mM; and (■) 10 mM.

V = activity (μmole NO_2^- produced/min/mg protein).

B: A Lineweaver-Burk plot of the effects of bromate at various levels of nitrate. The concentrations of bromate were:

(△) 0 mM; (●) 2.5 mM; (▲) 5 mM; and (○) 10 mM.
Inset: Inhibition by bromate with nitrate at (◆) 2.5 mM; (▲) 5 mM; and (■) 10 mM.

V = activity (μmole NO_2^- produced/min/mg protein).

FIG 8A

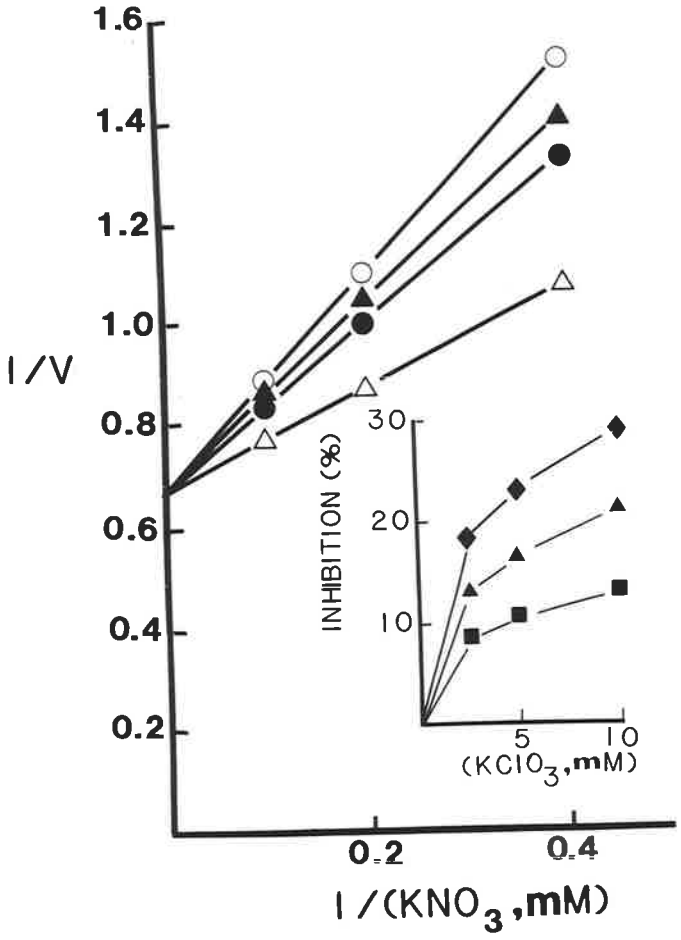

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/V
0.2
0.4
1/(KNO3,mM)
INHIBITION (%)
30
20
10
5
10
(KClO3,mM)

FIG 8B

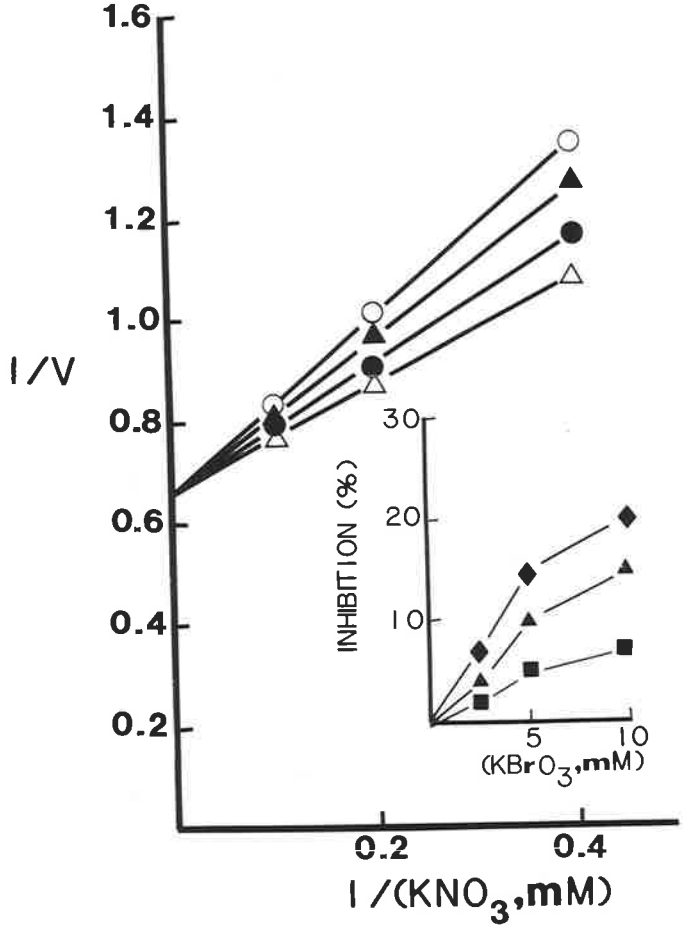

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/V
0.2
0.4
1/(KNO3,mM)
INHIBITION (%)
30
20
10
5
10
(KBrO3,mM)

FIG. 9: A Dixon plot of the inhibitory effects of various concentrations of chlorate on the MVH-nitrate reductase activity.

Nitrate reductase (fraction 3, Table 1) activity was determined as described in Section 2.2.1.1, except that various concentrations of nitrate were used as well as the addition of chlorate at the concentrations indicated.
The concentrations of nitrate were:

(■) 2.5 mM;
(▲) 5 mM;
(◆) 10 mM.

V = activity (μmole NO_2^- produced/min/mg protein).

FIG 9

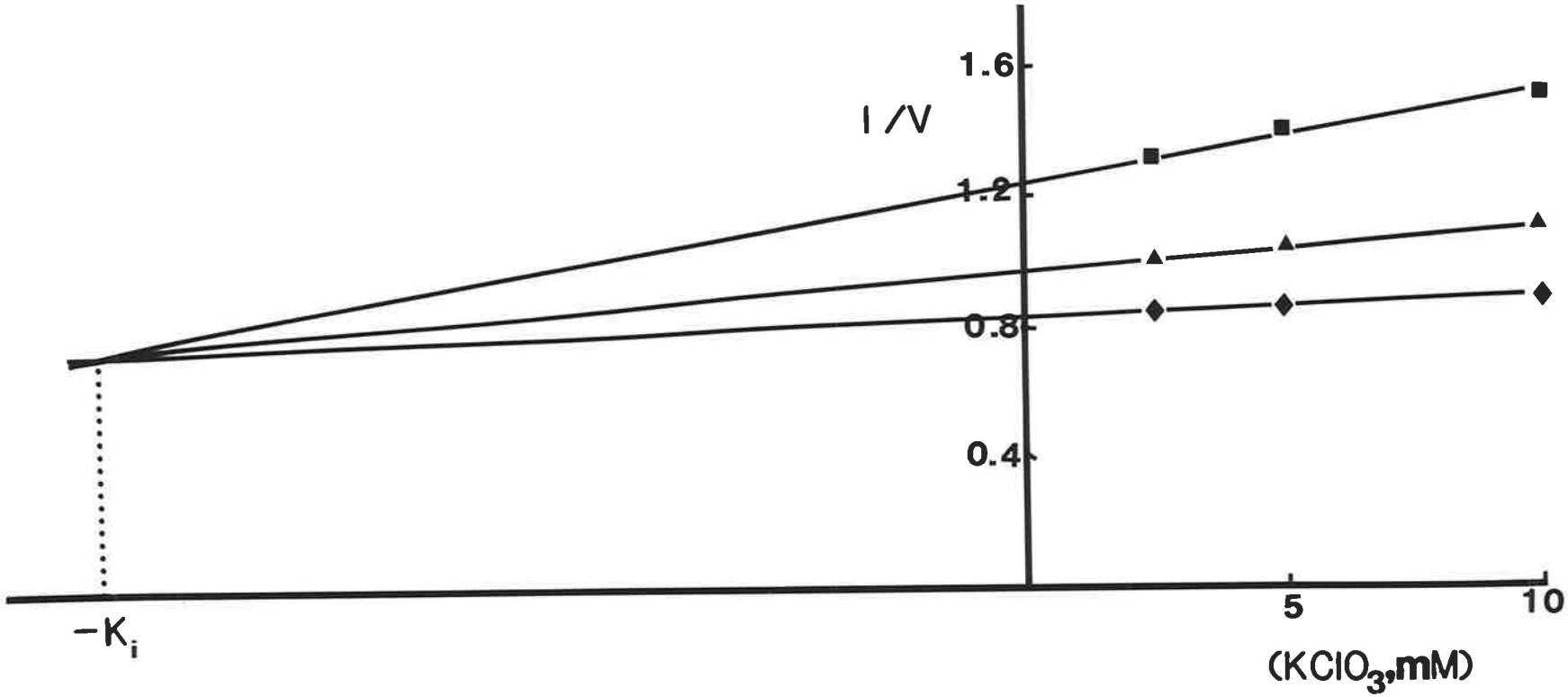


FIG. 10: A Dixon plot of the inhibitory effects of various concentrations of bromate on the MVH-nitrate reductase activity.

Nitrate reductase activity of the purified enzyme (fraction 3, Table 1) was determined as described in Section 2.2.1.1, except that various concentrations of nitrate were used as well as the addition of bromate at the concentrations indicated. The concentrations of nitrate were:

(■) 2.5 mM;
(▲) 5 mM; and
(◆) 10 mM.

V = activity (μmole NO_2^- produced/min/mg protein).

FIG 10

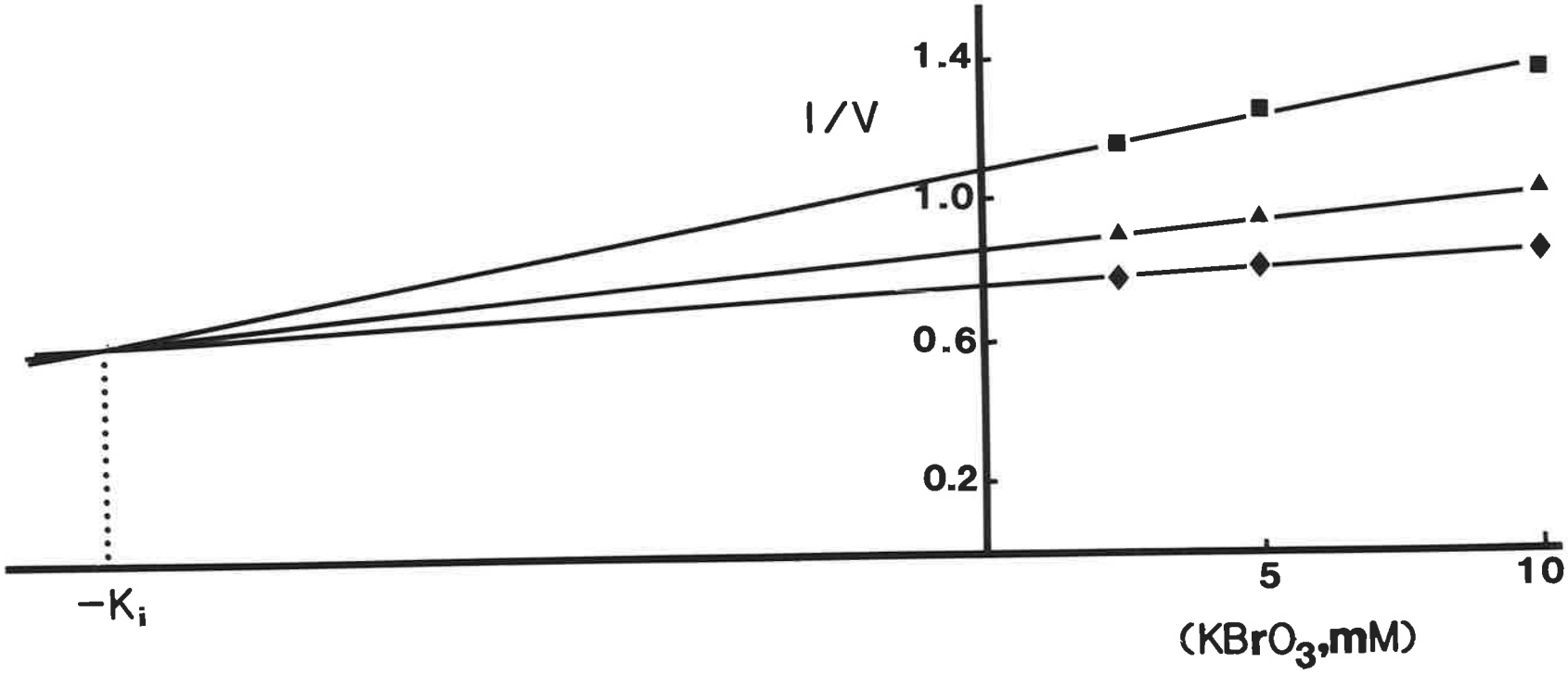


FIG. 11: Effects of various concentrations of nitrite on NADPH-nitrate reductase activity.

The activity of the enzyme (fraction 3, Table 1) was measured by following the rate of nitrate-dependent NADPH oxidation in a 1 cm quartz cell at 340 nm using a Varian Techtron Spectrophotometer. The reaction mixture (in a 3 ml final volume) contained: phosphate buffer (pH 7.1), 50 mM; NADPH, 0.2 mM; FAD, 10 µM; KNO_3 and KNO_2 at concentrations indicated; and enzyme (10 µg protein).

A: A Lineweaver-Burk plot of the effects of various concentrations of nitrite at defined levels of nitrate. The concentrations of nitrite were: (△) 0 µM; (●) 50 µM; (▲) 100 µM; and (○) 200 µM.

Inset: Inhibitory effects of nitrite at various levels of nitrate:
(▲) 50 µM; (◆) 100 µM; and (■) 200 µM.

B: A Dixon plot of the effects of various concentrations of nitrite at defined levels of nitrate. The concentrations of nitrate were:
(□) 50 µM; (△) 100 µM; and (○) 200 µM.

V = activity (µmole NADPH oxidized/min/mg protein).

FIG 11A

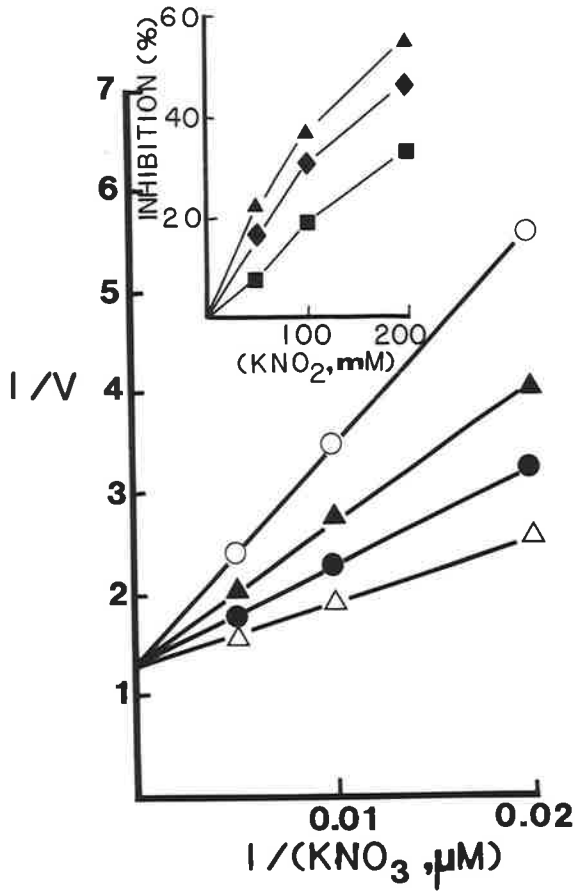
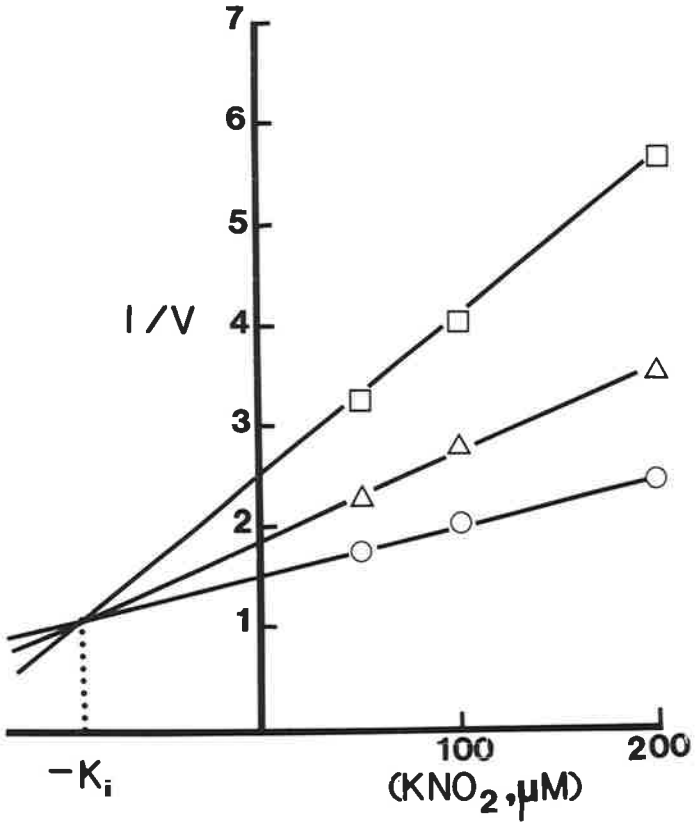


FIG 11B



3.2 Nitrite reductase

3.2.1 Purification

All operations during the purification were carried out at 4°C. The preparation of the mycelial felts from cultures is described in Section 2.1.2.

In a typical preparation, 25g of fresh mycelial felts were homogenized using a glass homogenizer in 75 ml of cold 0.1M phosphate buffer (pH 7.5) containing 5 mM cysteine, 5 mM EDTA and 10 µM FAD. The homogenate was passed twice through a French pressure cell at 20,000 p.s.i. and then centrifuged at 27,000 *g* for 20 min. Nitrite reductase was recovered in the supernatant (S_{27}) which is used as crude extract.

To this extract, solid $(NH_4)_2SO_4$ was gradually added with constant stirring under argon until a 35% $(NH_4)_2SO_4$ saturation was achieved. During the addition of $(NH_4)_2SO_4$ the pH of the extract was maintained at 7.5 by adding cold 0.1M KOH. After standing in ice for 30 min it was centrifuged at 20,000 *g* for 15 min. Following the same procedure, the supernatant obtained from the previous step was brought to 60% $(NH_4)_2SO_4$ saturation. After centrifugation, the pellet was dissolved in the preparation buffer (minus FAD) and dialyzed for 4 hr against 3L of 0.1M phosphate buffer (pH 7.5). The dialyzed solution was then loaded onto a Blue Sepharose CL-6B column (140 x 15 mm). Prior to loading the enzyme, 100 ml of BSA (2 mg/ml in 0.1M phosphate buffer (pH 7.5) containing 0.2 mM EDTA and 0.1 mM DTT) was passed through the column which was then washed with 200 ml of the same buffer. After loading the enzyme, the column was again washed with 25 ml of 0.1M phosphate buffer (pH 7.5) containing 0.5 mM ATP and 0.5 mM $NADP^+$ and then with the same buffer. The enzyme was eluted from the column with 0.1M phosphate buffer (pH 7.5) containing 0.5 mM NADPH.

This fraction represented a 54-fold purification with a specific activity of 869 nmoles NADPH oxidized/min/mg protein and a 21% recovery. The summary of the purification procedure is presented in Table 6.

Associated with purified NAD(P)H-dependent nitrite reductase were NAD(P)H-linked hydroxylamine reductase and NAD(P)H-diaphorase activities. The ratios of nitrite reductase to hydroxylamine reductase activities in each fraction during purification (Table 6) were constant (0.8) indicating that these are associated with the same enzyme rather than separate and distinct enzyme proteins.

The NAD(P)H-diaphorase utilized either ferricyanide, 2,6'-dichlorophenolindophenol (DCPIP) or cytochrome *c* (horse heart) as an electron acceptor with either NADPH or NADH as an electron donor. The purified nitrite reductase preparation (fraction 3, Table 6) had no sulphite reductase activity with either NADPH or NADH as the reductant.

3.2.2 Properties

3.2.2.1 Effect of pH and time of incubation

Nitrite reductase activity was active over a range of pH values with a maxima of 7.0 for NADPH- and 7.3 for MVH-linked reaction (Fig. 12A). The optimum pH for NADPH-dependent hydroxylamine reductase activity was also similar to that for nitrite reductase (Fig. 12B).

The enzyme activity determined by following either NO_2^- utilization or NH_3 formation was linear up to 15 min (Fig. 13). Thus an incubation time of 10 min was routinely used for assaying enzyme activity when nitrite utilization was measured.

TABLE 6: Purification of nitrite reductase

The enzyme activity from each fraction was measured by following the initial rate of NADPH oxidation in the presence of either nitrite (values without brackets) or hydroxylamine (values in brackets) as an electron acceptor. The reaction mixtures were as described in Section 2.2.2.2.

One unit of enzyme activity corresponds to 1 nmole NADPH oxidized/min.

Fraction	Activity (units)	Protein (mg)	Specific Activity (units/ mg protein)	Ratio Nitrite: hydroxylamine reductases activity	Purification (-fold)	Recovery (%)
1.Crude extract (S_{27})	5760 (7360)	360	16 (20)	0.8	-	100
2.Precipitate from 35-60% $(NH_4)_2SO_4$ saturation, dialysed against 0.1M phosphate buffer (pH 7.0) for 4 hr	1770 (2100)	96.8	18 (22)	0.8	1	31
3.Fraction 2 loaded onto a Blue Sepharose CL-6B column (14x1.5cm) and eluted with 0.1M phosphate buffer (pH 7.5) containing 0.5 mM NADPH	1480 (1910)	1.7	869 (1124)	0.8	54	21

FIG. 12: Effects of pH on nitrite reductase and hydroxylamine reductase activities.

A: The NADPH-dependent (▲) and MVH-linked (■) nitrite reductase activities were assayed at various pH values. The activities of the enzyme (fraction 3, Table 6) were determined by measuring the utilization of nitrite in the reaction mixture as described in Section 2.2.2.1.

B: The activities of the enzyme with either nitrite (□) or hydroxylamine (△) as an electron acceptor were determined at various pH values by following the initial rates of NADPH oxidation as described in Section 2.2.2.2.

FIG 12 A

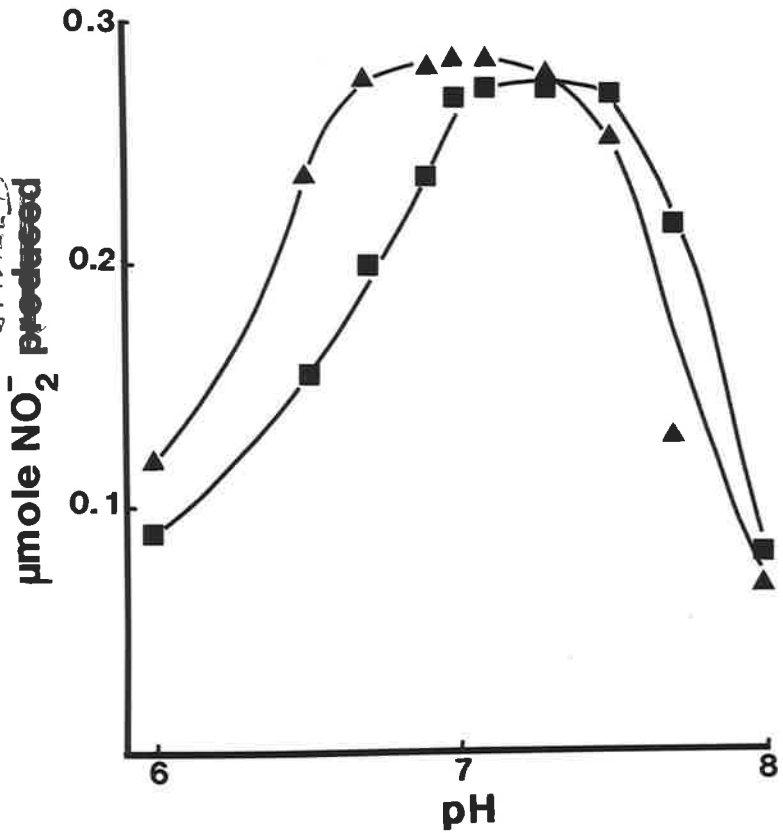


FIG 12B

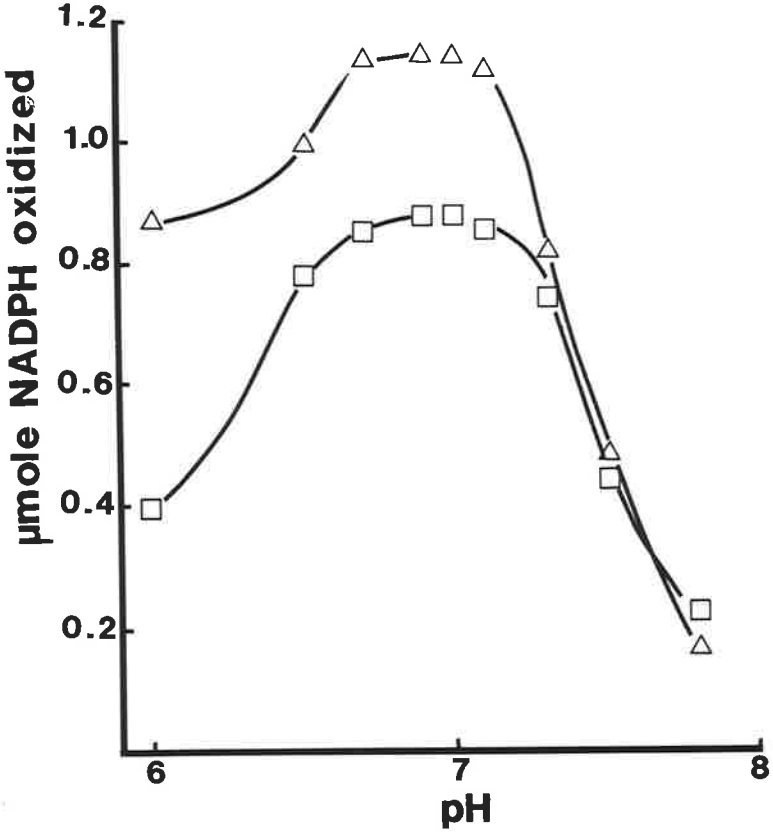
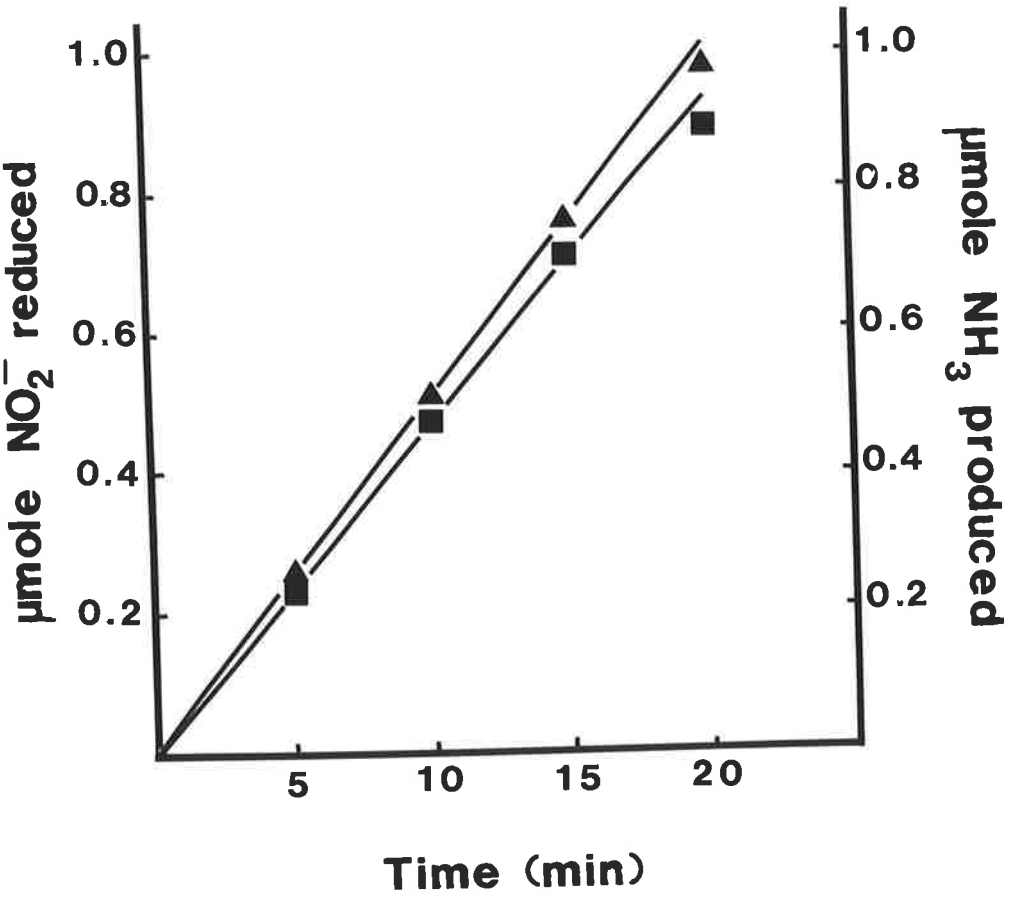


FIG. 13: Effects of incubation times on nitrite reduction.

The NADPH-dependent nitrite reductase activity was assayed in the purified preparation (fraction 3, Table 6) by following either NO_2^- utilization (▲) or NH_3 formation (■) as described in Section 2.2.2.1.

FIG 13


1.0
0.8
0.6
0.4
0.2
µmole NO_2^- reduced
1.0
0.8
0.6
0.4
0.2
µmole NH_3 produced
5
10
15
20
Time (min)

3.2.2.2 Electron donors

A variety of compounds functioned as an electron donor for nitrite reduction by the purified enzyme (fraction 3, Table 6). The results in Table 7 indicate that both NADPH and NADH act as reductants but NADPH was more effective. In addition viologen dyes and flavins, chemically reduced with $Na_2S_2O_4$, also functioned as electron donors but their activities were less than for NADPH.

3.2.2.3 Requirement for flavin

For either NADPH or NADH-linked nitrite reductase, FAD was required for maximal activity. Similar results were observed for NAD(P)H-hydroxylamine reductase and NAD(P)H-cytochrome *c* reductase (Table 8). Only 14% of the maximal NADPH-dependent nitrite reductase activity was detected without added FAD; FMN did not replace FAD since only 24% of the maximal activity for FAD was recorded for FMN (10 μM).

In contrast the diaphorase activity with either ferricyanide or dichlorophenolindophenol (DCPIP) as the electron acceptor, exogenous FAD was not required for maximal activity.

3.2.2.4 K_m values for substrates, reductants and cofactors

The effect of varying concentration of substrates, reductants and cofactors on NADPH-dependent nitrite reductase and NADPH-dependent hydroxylamine reductase activities are illustrated in Figs. 14A, 14B, 15A, 15B, 16A and 16B. Based on the Lineweaver-Burk plots of the data, the apparent K_m values for nitrite, NADPH and FAD of nitrite reductase activity were (μM): 17, 40 and 0.11, respect-

TABLE 7: Effects of various electron donors on nitrite reductase activity

The enzyme preparation (fraction 3, Table 6) was used after dialyzing against 3L of 50 mM phosphate buffer (pH 7.0) for 4 hr. The assay conditions were as described in Section 2.2.2.1. When either NADPH or NADH was used as an electron donor, the reaction mixture contained (in 1 ml final volume):

50 mM phosphate buffer (pH 7.0); 0.1 mM KNO_2; 0.2 mM NADPH or NADH; 10 µM FAD and enzyme containing 17 µg protein.

The reaction mixture with MVH, BVH, $FMNH_2$ and $FADH_2$ respectively, as the reductant was:

50 mM phosphate buffer (pH 7.3); 0.1 mM KNO_2; 0.2 mM MV, BV, FMN or FAD; 1 mM $Na_2S_2O_4$ (in 1% (w/v) $NaHCO_3$, freshly prepared) and the purified enzyme.

The results are expressed as % activity of the control (using NADPH as an electron donor) which is 0.29 µmole NO_2^- reduced/min/mg protein.

Electron donors	Activity (%)
NADPH	100
NADH	56
MVH	96
BVH	77
$FMNH_2$	41
$FADH_2$	26

TABLE 8: Effects of flavins on NAD(P)H-nitrite reductase and its associated diaphorase activities

The enzyme preparation (fraction 3, Table 6) was used after dialyzing against 3L of 50 mM phosphate buffer (pH 7.0) for 4 hr. The activity of the enzyme was determined as described in Section 2.2.2.2 except that, where indicated, FAD was omitted from the reaction mixture or FMN was substituted for FAD. In the diaphorase activities, nitrite was omitted and the electron acceptor was:

hydroxylamine, 12 mM; $K_3F_e(CN)_6$, 0.2 mM; DCPIP, 0.5 mM or cytochrome *c* (horse heart), 0.05 mM.

Electron acceptors	Flavin	Enzyme activity	
		NADPH-dependent (µmole NADPH oxidized/min/ mg protein)	NADH-dependent (µmole NADH oxidized/min/ mg protein)
Nitrite	FAD	0.87	0.49
	FMN	0.21	0.20
	None	0.13	0.15
Hydroxylamine	FAD	1.15	0.61
	FMN	0.79	0.20
	None	0.40	0.10
Ferricyanide	FAD	11.82	7.07
	FMN	11.36	7.19
	None	11.36	6.84
DCPIP	FAD	12.98	11.13
	FMN	12.98	11.13
	None	12.98	10.20
Cytochrome *c* (horse heart)	FAD	1.03	0.58
	FMN	0.25	0.19
	None	0.21	0.13

FIG. 14: Effects of various concentrations of substrates on NADPH-nitrite reductase and NADPH-hydroxylamine reductase activities.

The activity of the enzyme (fraction 3, Table 6) were determined as described in Section 2.2.2.2.

A: Double reciprocal plot of the effects of various concentrations of nitrite on NADPH-dependent nitrite reductase activity.

B: Double reciprocal plot of the effects of various concentrations of hydroxylamine on NADPH-linked hydroxylamine reductase activity.

V = activity (µmole NADPH oxidized/min/mg protein).

FIG 14A

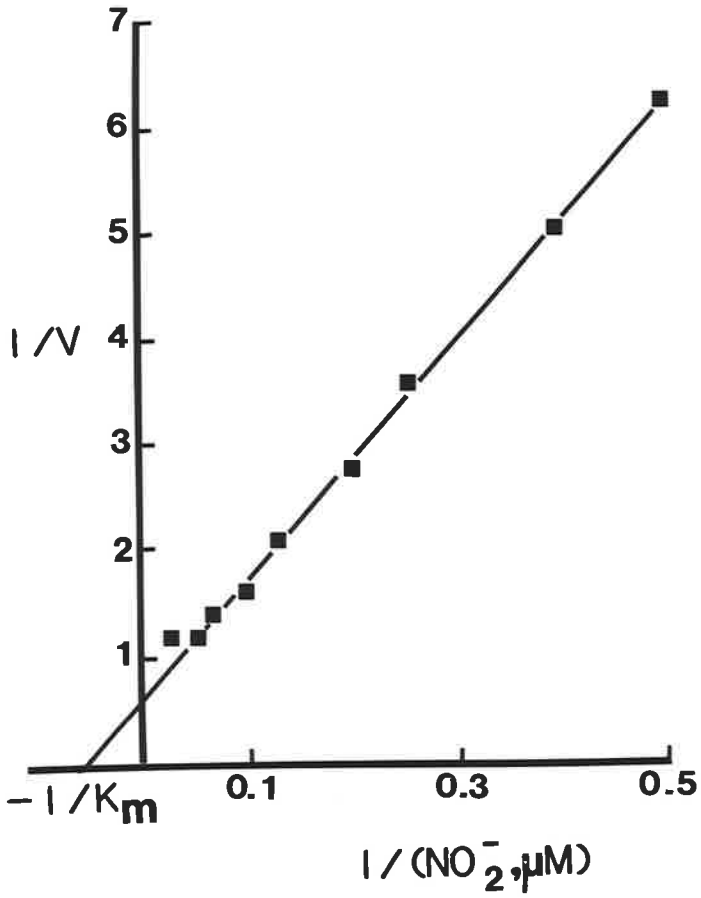


FIG 14B

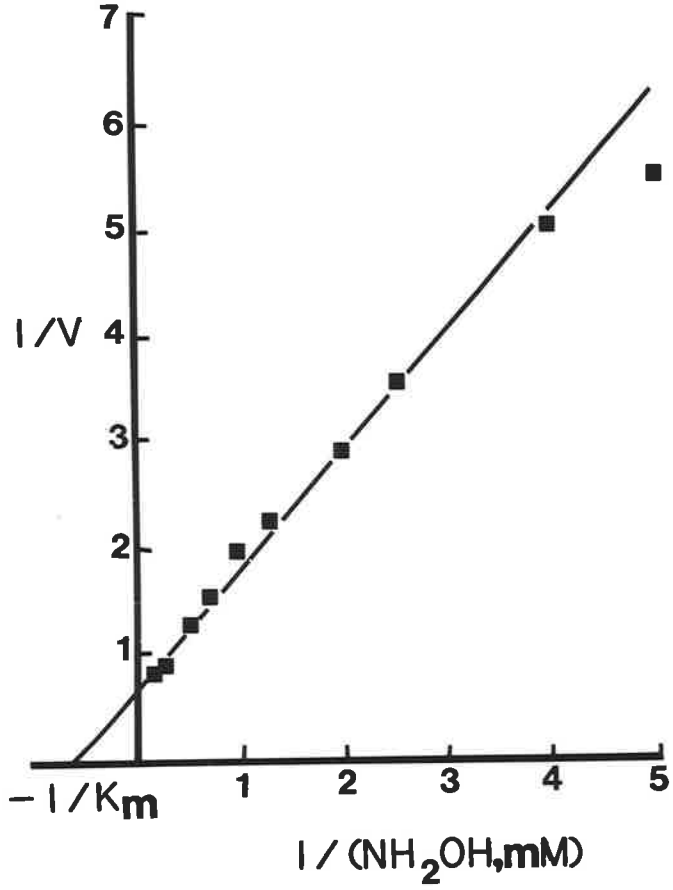


FIG. 15: Double reciprocal plots of the effects of various concentrations of NADPH on NADPH-dependent nitrite reductase and NADPH-dependent hydroxylamine reductase activities.

A: NADPH-dependent nitrite reductase.

B: NADPH-dependent hydroxylamine reductase.

The enzyme preparation (fraction 3, Table 6) was used after dialyzing against 3L of 50 mM phosphate buffer (pH 7.0) for 4 hr. The enzyme activities were determined as described in Section 2.2.2.2, except that the concentration of reductant was varied as indicated.

V = activity (μmole NADPH oxidized/min/mg protein).

FIG 15A

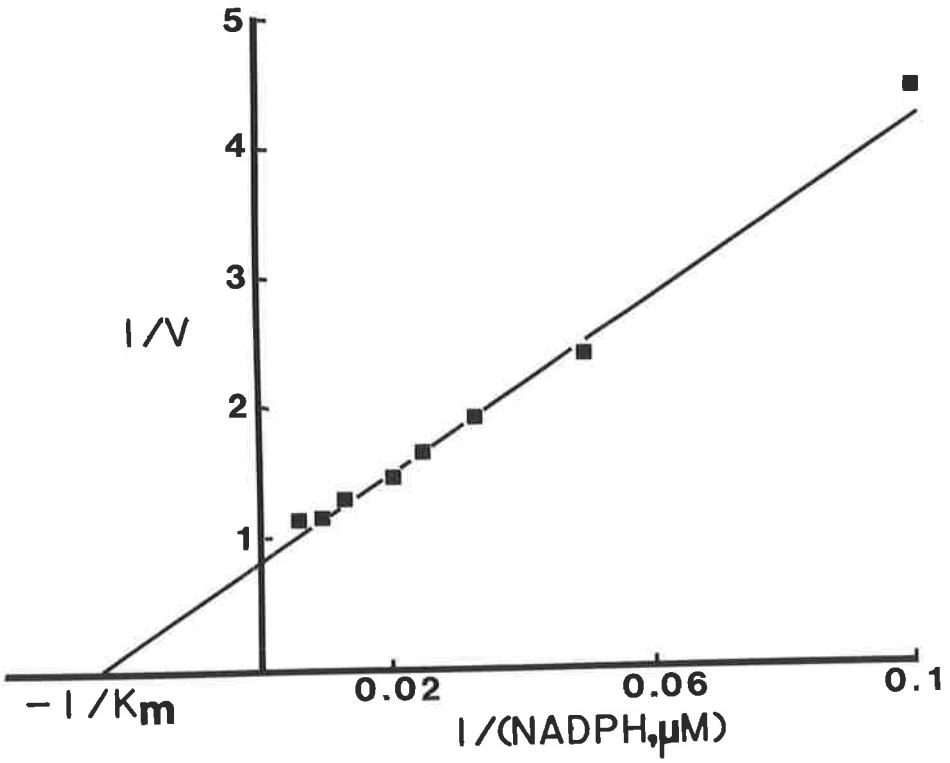


FIG 15B

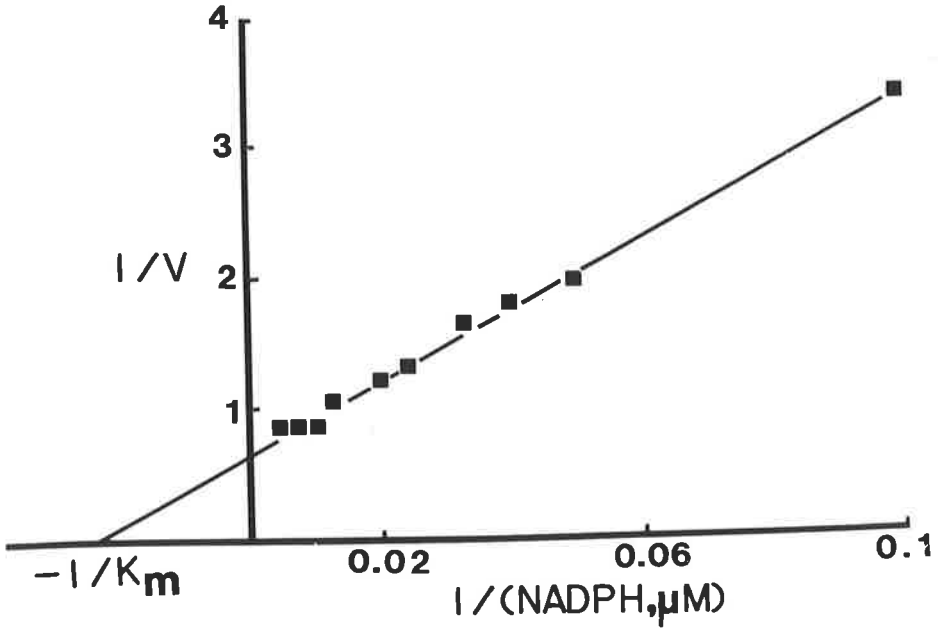


FIG. 16: Double reciprocal plots of the effects of various concentrations of FAD on NADPH-dependent nitrite reductase and NADPH-dependent hydroxylamine reductase activities.

A: nitrite reductase activity.

B: hydroxylamine reductase activity.

The activity of the enzyme (fraction 3, Table 6) was determined as described in Section 2.2.2.2, except that the concentrations of FAD was varied as indicated.

V = activity (µmole NADPH oxidized/min/mg protein).

FIG 16A

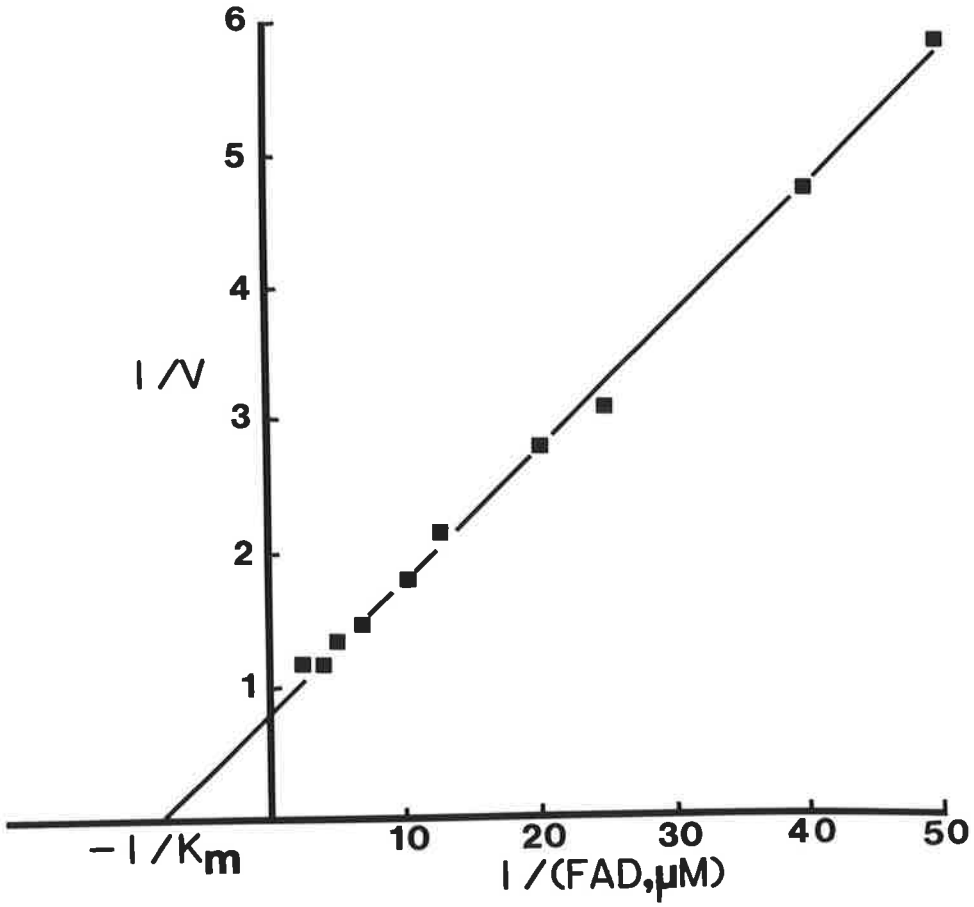
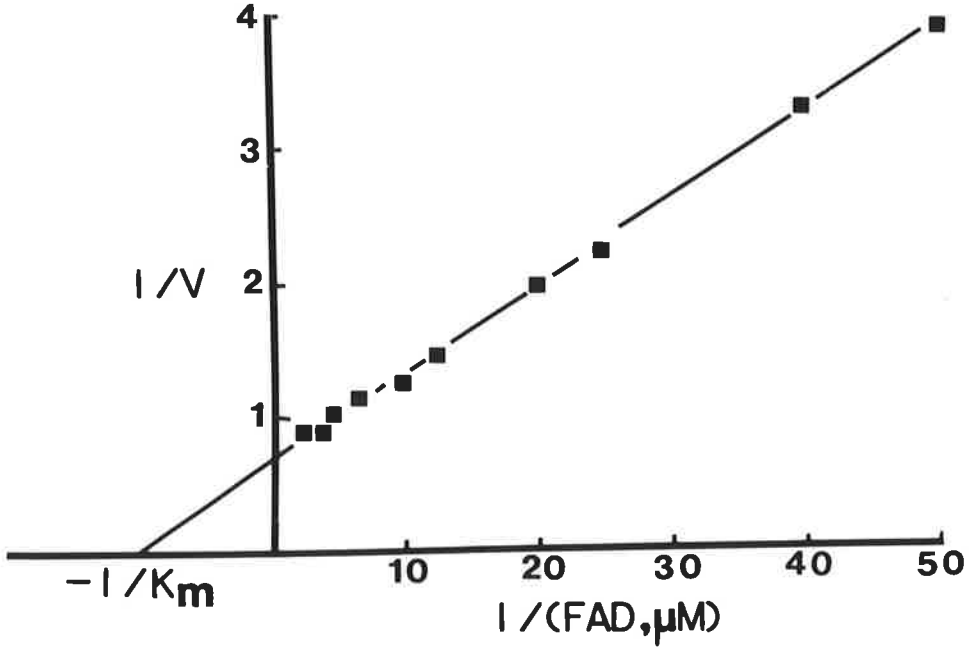


FIG 16B



ively; whereas the K_m values for hydroxylamine, NADPH and FAD of hydroxylamine reductase activity were 1.67 mM, 40 µM and 0.10 µM, respectively.

3.2.2.5 Stoichiometries for NADPH, nitrite and ammonia

The results of stoichiometry studies for the purified enzyme (fraction 3, Table 6) are presented in Table 9. The ratio of NADPH oxidized to nitrite reduced and ammonia produced was 3:1:1. In the hydroxylamine-dependent reaction the ratio of NADPH oxidized to ammonia formed was 1:1.

3.2.3 Inhibitor studies

The effects of metal binding agents on NADPH-dependent nitrite reductase activity are shown in Table 10. Thus α,α'-dipyridyl at 5.0 mM was without effect whereas O-phenanthroline (5.0 mM) and diethyldithiocarbamate (1.0 mM) inhibited the enzyme activity by about a half. Cyanide was a strong inhibitor and at 0.5 mM it completely inhibited the NADPH-dependent reaction. Other compounds such as azide and arsenite restricted enzyme activity to a lesser extent.

The inhibitory effects of reagents which react with sulphydryl groups are presented in Table 11. Thus p-CMB at 0.05 mM completely inhibited NADPH-dependent activity; its inhibitory effect was completely reversed by adding 0.1 mM cysteine. N-ethylmaleimide also restricted enzyme activity.

With NADPH as an electron donor (Table 11) nitrite reduction was inhibited by amytal (5.0 mM), rotenone (2.0 mM) and mepacrine (0.5 mM) by 58, 35 and 62%, respectively. The inhibition of the enzyme activity by mepacrine was reversed by adding 10 µM FAD.

TABLE 9: Stoichiometries for NADPH, NO_2^- and NH_3 for nitrite reductase

Nitrite reductase and hydroxylamine reductase activities were determined as described in Section 2.2.2.1. The enzyme (fraction 3, Table 1) contained 17 µg protein with a specific activity of 0.87 and 1.12 µmole NADPH oxidized/min/mg protein for nitrite reductase and hydroxylamine reductase, respectively.

Reaction incubation time	NADPH oxidized (n mole)	NO_2^- reduced (n mole)	NH_3 produced (n mole)	Ratio NADPH: NO_2^-	Ratio NADPH: NH_3
Nitrite reductase					
5 min	74.8	24.8	23.1	3.0	3.2
10 min	149.5	49.5	47.3	3.0	3.1
Hydroxylamine reductase					
5 min	95.8	-	87.0	-	1.1
10 min	193.4	-	161.3	-	1.2

TABLE 10: Effects of metal binding agents on NADPH-dependent nitrite reductase

Nitrite reductase activity was determined as described in Section 2.2.2.2, except that inhibitor was added at concentration indicated. The enzyme preparation (fraction 3, Table 6) was preincubated with the inhibitor for 15 min prior to starting the reaction. The results are expressed as % inhibition of the control (without inhibitor). The activity of the control was 0.89 µmole NADPH oxidized/min/mg protein.

Inhibitors	Final Concentration (mM)	Inhibition (%)
KCN	0.1	39
	0.2	53
	0.5	100
$NaAsO_2$	0.1	11
	0.5	38
	1.0	58
NaN_3	0.1	5
	0.5	26
	1.0	39
DIECA	0.1	5
	0.5	22
	1.0	42
O-phenanthroline	1.0	24
	5.0	45

TABLE 11: Effects of inhibitors of sulphydryl groups and flavin on NADPH-nitrite reductase

The enzyme activity was determined as described in Section 2.2.2.2. Inhibitors were preincubated for 15 min with the enzyme (fraction 3, Table 6) prior to starting the reaction. The results are expressed as % inhibition of the control (without inhibitor). The activity of the control was 0.89 µmole NADPH oxidized/min/mg protein.

Inhibitors	Final Concentration (mM)	Inhibition (%)
p-CMB	0.02	76
	0.05	100
p-CMB + cysteine	0.05 + 0.1	0
NEM	2.0	13
	5.0	43
Amytal	2.0	20
	5.0	58
Rotenone	0.5	19
	1.0	35
Mepacrine	0.1	19
	0.2	38
	0.5	62
Mepacrine + FAD	0.5 + 0.01	5

Double reciprocal plots of the effects of various concentrations of either cyanide or sulphite at defined levels of nitrite on NADPH- dependent nitrite reduction (Figs. 17A and 18A) indicate that both compounds are competitive inhibitors with respect to nitrite. The apparent K_i values estimated from Dixon plots were 35 µM for cyanide (Fig. 17B) and 10.75 mM for sulphite (Fig. 18B).

3.2.4 Inactivation of nitrite and hydroxylamine reductases by NADPH in the presence of FAD

Nitrite reductase and hydroxylamine reductase activities assayed with NADPH as an electron donor were inactivated by preincubation of the enzyme for 15 min at 4°C with NADPH and FAD (Table 12). Preincubation of the enzyme with either NADPH or FAD did not restrict enzyme activity. Similar results were obtained when $NADP^+$ was added to the preincubation mixture instead of NADPH.

When the electron acceptor, either nitrite or hydroxylamine, was included in the preincubation mixture the enzyme was protected against this inactivation.

3.3. Incorporation of [^{15}-N]-labelled $(NH_4)_2SO_4$ into cell-nitrogen

L-methionine-DL-sulphoximine (MSX) and azaserine are inhibitors of glutamine synthetase and glutamate synthase, respectively, but do not affect glutamate dehydrogenase (Brenchley, 1973; Miflin and Lea, 1976). The inhibitory effects of these compounds on the incorporation of ^{15}N-labelled $(NH_4)_2SO_4$ into washed felts are shown in Table 13. The results indicate that in the absence of inhibitors the amount of ^{15}N-labelled $(NH_4)_2SO_4$ incorporated into washed felts after 1 hr incubation was 4.4 µg N/mg protein. Thus MSX (2 mM) and azaserine (1 mM) inhibited the

FIG. 17: Effects of various concentrations of cyanide on NADPH-dependent nitrite reductase activity.

The activity of the enzyme (fraction 3, Table 6) was determined by measuring nitrite utilization in the assay mixture as described in Section 2.2.2.1, except that cyanide was added at concentration indicated.

A: A Lineweaver-Burk plot of the effects of various concentrations of cyanide at defined levels of nitrite. The concentrations of cyanide were:
(▲) 0 μM; (■) 25 μM; (△) 50 μM; and (□) 100 μM.

B: A Dixon plot of the effects of various concentrations of cyanide at defined levels of nitrite. The concentrations of nitrite were:
(□) 25 μM; (△) 50 μM; and (○) 100 μM.

V = activity (μmole NO_2^- reduced/min/mg protein).

FIG 17B

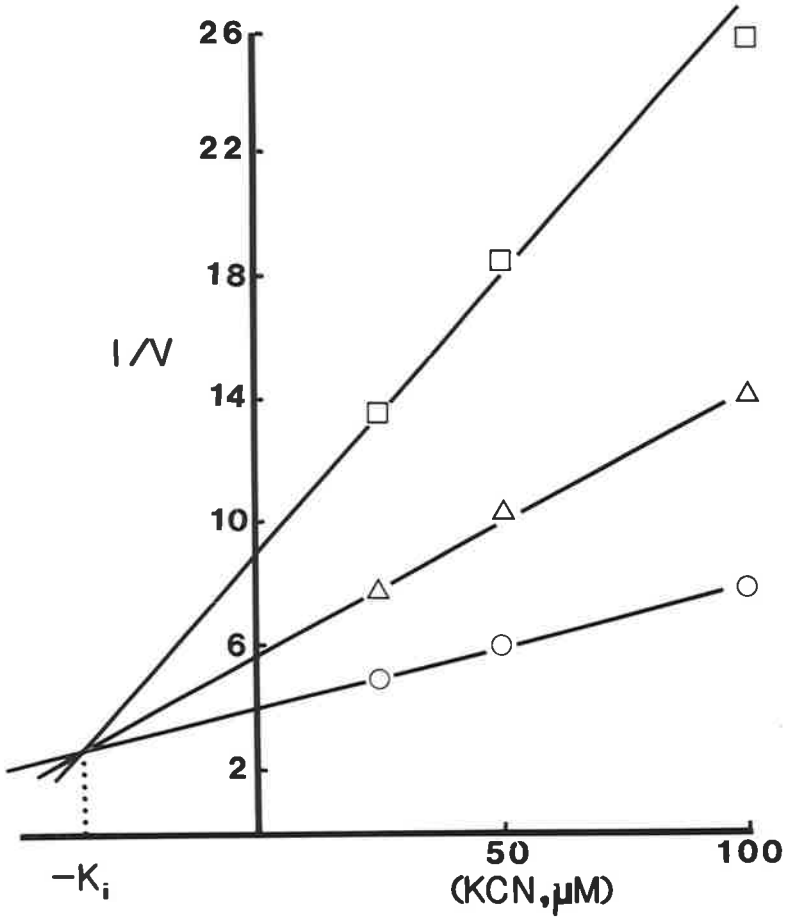


FIG 17A

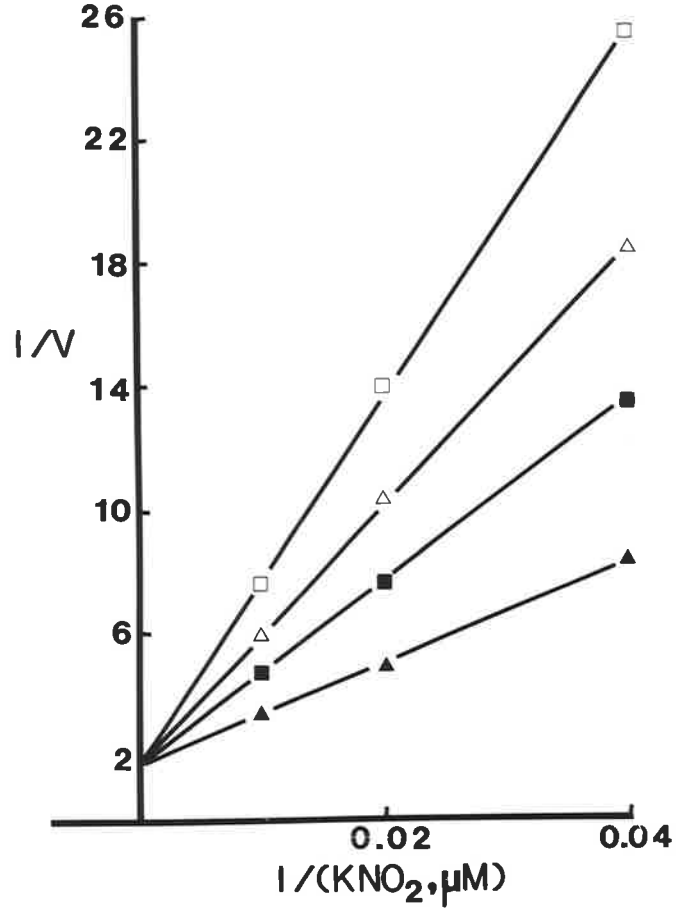


FIG. 18: Effects of various concentrations of sulphite on NADPH-dependent nitrite reduction.

The activity of the enzyme (fraction 3, Table 6) was determined by measuring nitrite utilization in the assay mixture as described in Section 2.2.2.1, except that sulphite was added at concentration indicated.

A: A Lineweaver-Burk plot of the effects of various concentrations of sulphite at defined level of nitrite. The concentrations of sulphite were: (▲) 0 mM; (■) 5 mM; (△) 10 mM; and (□) 20 mM.

B: A Dixon plot of the effects of various concentrations of sulphite at defined levels of nitrite. The concentrations of nitrite were: (□) 25 μM; (△) 50 μM; and (○) 100 μM.

V = activity (μmole NO_2^- reduced/min/mg protein).

FIG 18B

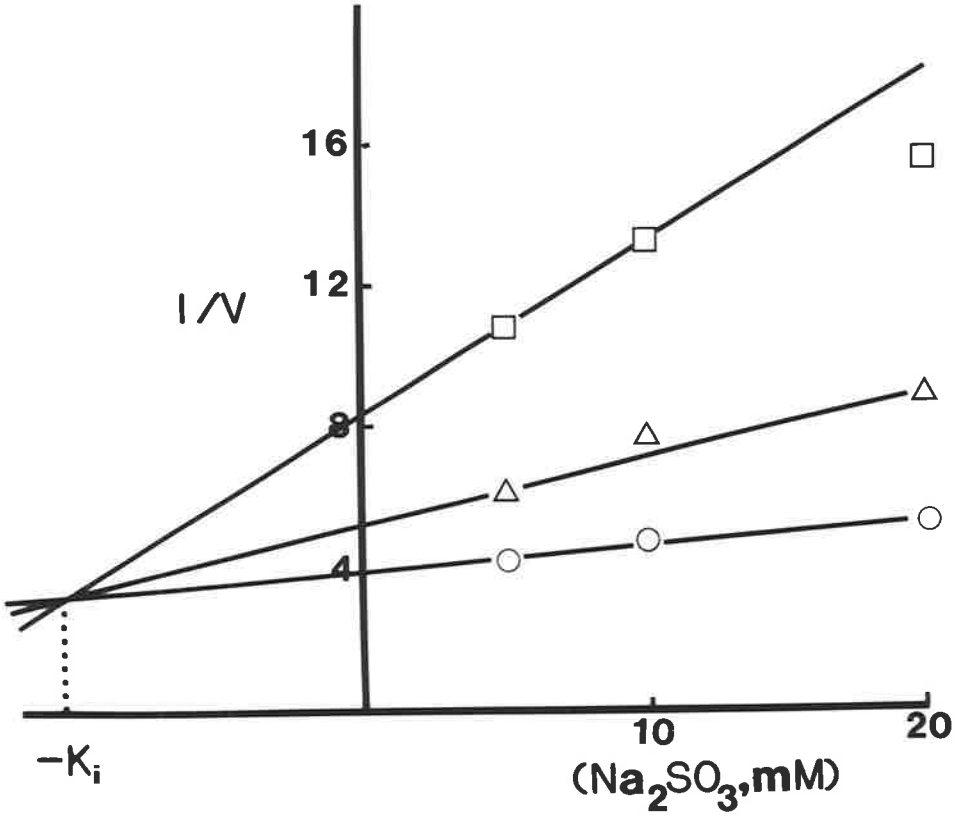


FIG 18A

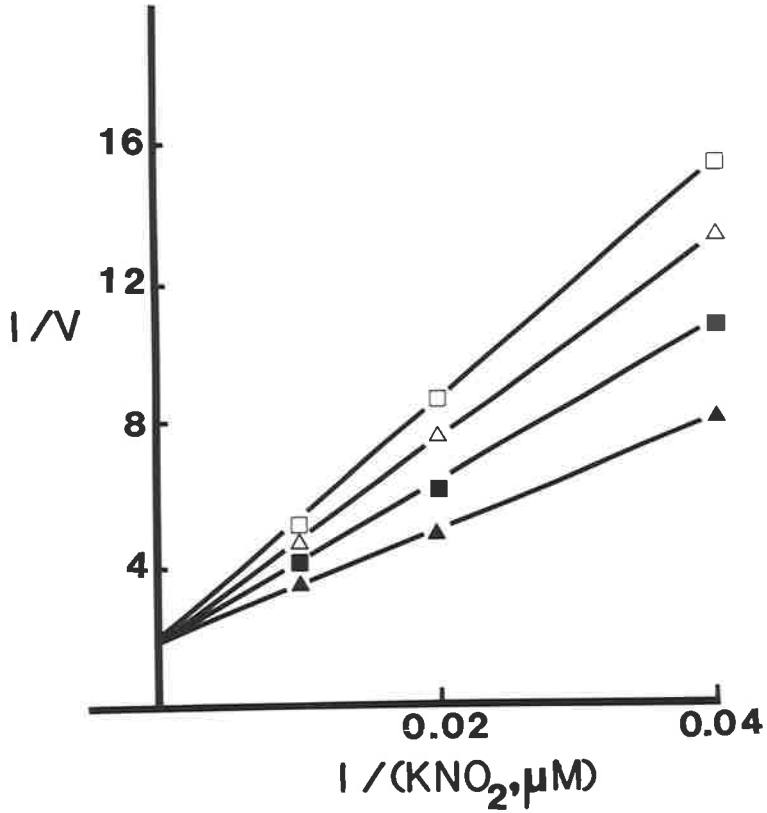


TABLE 12: Effects of preincubation of nitrite reductase with reductant, cofactor and/or substrate on its activity

The enzyme (fraction 3, Table 6) was used after dialyzing against 3L of 50 mM phosphate buffer (pH 7.0) for 4 hr. Aliquots of the enzyme containing 100 µg protein were pre-incubated in a final volume of 0.5 ml with 50 mM phosphate buffer (pH 7.0) and (where indicated):

0.5 mM NADPH; 0.5 mM $NADP^+$; 20 µM FAD; and either 1 mM $NaNO_2$ or 20 mM NH_2OH.

After a 15 min incubation at 4°C, the activities were measured by adding 0.1 ml of each preincubation system to the reagents of standard assay mixture as described in Section 2.2.2.2.

Preincubation conditions	NADPH-nitrite reductase activity (µmole NADPH oxidized/min/mg protein)	NADPH-hydroxylamine reductase activity (µmole NADPH oxidized/min/mg protein)
Enzyme	0.87	1.13
+FAD	0.87 (100)	1.11 (98)
+NADPH	0.84 (97)	1.09 (96)
$+NADP^+$	0.87 (100)	1.15 (101)
$+NO_2^-$	0.88 (101)	1.09 (96)
$+NH_2OH$	0.92 (106)	1.13 (100)
+FAD + NADPH	0.36 (42)	0.51 (45)
+FAD + $NADP^+$	0.87 (100)	1.13 (100)
+FAD + NADPH + NO_2^-	0.63 (72)	0.77 (68)
+FAD + NADPH + NH_2OH	0.68 (78)	0.84 (74)
+FAD + $NADP^+$ + NO_2^-	0.86 (99)	0.94 (83)
+FAD + $NADP^+$ + NH_2OH	0.97 (111)	1.13 (100)

Figures in brackets represent % of activity of control (enzyme alone).

TABLE 13: Effects of L-methionine-DL-sulphoximine (MSX) and azaserine on the incorporation of ^{15}N-labelled $(NH_4)_2SO_4$ into washed cells

Washed cells grown with either nitrate or ammonia as the sole nitrogen source were prepared as described in Section 2.1.1. Mycelial suspensions (1g fresh weight in 20 ml N-free fresh culture medium) were incubated with and without MSX and azaserine, respectively, for 2 hr at 30°C prior to adding ^{15}N-labelled $(NH_4)_2SO_4$ (5 mM final concentration). The incubation was continued for a further 1 hr. Samples for ^{15}N enrichment analysis were prepared and analysed as described in Section 2.3.1.

Nitrogen source for growth	^{15}N incorporation (µg N/hr/mg protein)		
	without inhibitor	+ MSX (2 mM)	+ azaserine (1 mM)
Nitrate	4.4	2.1 (52)	3.0 (31)
Ammonia	4.6	2.6 (57)	3.2 (30)

Figures in brackets represent % inhibition.

incorporation of the labelled compound into cell-nitrogen by 52% and 31%, respectively. The inhibitory effects were similar for felts grown with either nitrate or ammonia as the sole nitrogen source.

3.4 Glutamine synthetase

3.4.1 Purification

The enzyme was purified to homogeneity by the procedure described below. All operations were carried out at 4°C. In a typical preparation 18g frozen mycelial felts were ground in a chilled porcelain mortar and pestle in 60 ml cold 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM $MnCl_2$ (the buffer) and then macerated further in a glass homogenizer. The homogenate passed through a French pressure cell (3 times) at 20,000 p.s.i. was centrifuged at 20,000 *g* for 20 min. The supernatant fraction (S_{20}) containing glutamine synthetase activity was used as the crude extract.

The crude extract was loaded onto a Blue Sepharose CL-6B column (115 x 15 mm) equilibrated with the buffer. The column was then washed with approximately 200 ml of the buffer. The enzyme was eluted from the column in the buffer containing 2 mM ADP. Fractions containing glutamine synthetase activity were pooled and then loaded onto a DEAE-cellulose (DE-52) column (200 x 25 mm) pre-equilibrated with the buffer. The column was then washed with 250 ml of the buffer. Glutamine synthetase was separated by a linear gradient of 0-500 mM NaCl in the buffer. The total gradient volume was 150 ml and the flow rate was 50 ml/hr. The enzyme was eluted between 300 and 375 mM NaCl. Active fractions were pooled and dialyzed for 16 hr against 3L of the buffer. The dialyzed solution was then subjected to a second Blue Sepharose CL-6B chromatography as described above. A summary of the purification is presented in Table 14.

TABLE 14: Purification of glutamine synthetase

The transferase activity of the enzyme from each fraction was determined as described in Section 2.2.3.1. One unit of enzyme activity corresponds to 1 μmole γ-glutamylhydroxamate produced/min.

Fraction	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification (-fold)	Recovery (%)
1.Crude extract (S_{20})	20.80	156.80	0.13	-	100
2.Fraction 1 loaded onto a Blue Sepharose column (115x15mm) and eluted with 50mM Tris-HCl buffer (pH 7.0) containing 1mM $MnCl_2$ and 2mM ADP	18.00	12.60	1.43	11	87
3.Fraction 2 loaded onto a DE-52 column (200x 25mM) and eluted with a linear gradient of 0-500mM NaCl in 50mM Tris-HCl (pH 7.0) containing 1mM $MnCl_2$	16.20	8.40	1.93	15	78
4.Fraction 3, after dialyzing against 3L of 50mM Tris-HCl buffer (pH 7.0) containing 1mM $MnCl_2$ for 16hr, loaded onto a Blue Sepharose column, and eluted as in Step 2	11.48	0.82	14.00	108	55

3.4.2 Properties

3.4.2.1 Molecular weight

The molecular weight of the native enzyme was determined by gel filtration on a Sepharose 6B column as described in Section 2.2.7.1. The purified enzyme (fraction 4, Table 14) has a molecular weight of 490 kDa (Fig. 19A). The enzyme subunit separated by SDS-polyacrylamide gel electrophoresis appeared as a single protein band with a molecular weight of 60 kDa. Thus the enzyme is composed of 8 subunits of similar size.

3.4.2.2 Effect of pH and incubation time

The effects of pH on transferase and biosynthetic activities of the enzyme are shown in Fig. 20. The transferase reaction was active over a pH range of 6.6 - 7.4 with a maximum at 7.0. Similar results were obtained for the biosynthetic activity.

The effects of time of incubation on both transferase and biosynthetic activities were linear at least up to 15 min (Fig. 21). Thus a 15 min incubation time was routinely used for assaying the enzyme activity.

3.4.2.3 Divalent cation requirement

Since a divalent cation was required for maximal activity, the effects of various cations on the transferase and biosynthetic activities were studied. The results in Table 15 indicate that the maximum activity of the transferase reaction was observed with Mn^{2+} at 5 mM. At this concentration low activities were recorded (% of

FIG. 19A: Molecular weight determination of glutamine synthetase by gel filtration.

The purified enzyme (fraction 4, Table 14) was passed through a Sepharose 6B column as described in Section 2.2.7.1. Elution buffer was 50 mM Tris-HCl (pH 7.0) containing 1 mM $MnCl_2$ and 0.1M NaCl. The K_{av} values were calculated from $K_{av} = (V_e-V_o)/(V_t-V_o)$. The proteins used as markers were:

(□) thyroglobulin;
(○) ferritin;
(■) catalase; and
(●) aldolase.

FIG. 19B: Estimation of subunit molecular weight of the purified enzyme by SDS-polyacrylamide gel electrophoresis.

The molecular weight of the enzyme subunit (fraction 4, Table 14) was determined by polyacrylamide (12.5%) slab gel electrophoresis in the presence of 0.1% (w/v) SDS, as described in Section 2.2.7.2. Relative mobility (R_f) of proteins were calculated by dividing the migration distance of the proteins with the movement of the tracking dye at the end of the run. The standard proteins used were:

(○) phosphorylase *b*;
(□) albumin;
(△) ovalbumin;
(●) carbonic anhydrase;
(■) trypsin inhibitor; and
(▲) α-lactalbumin.

FIG 19A

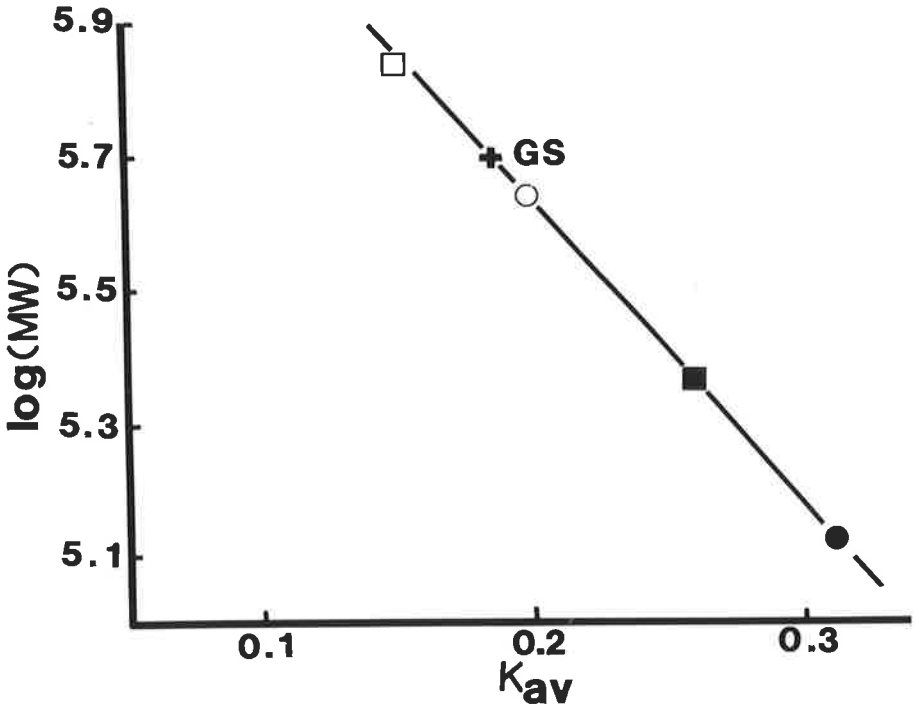


FIG 19B

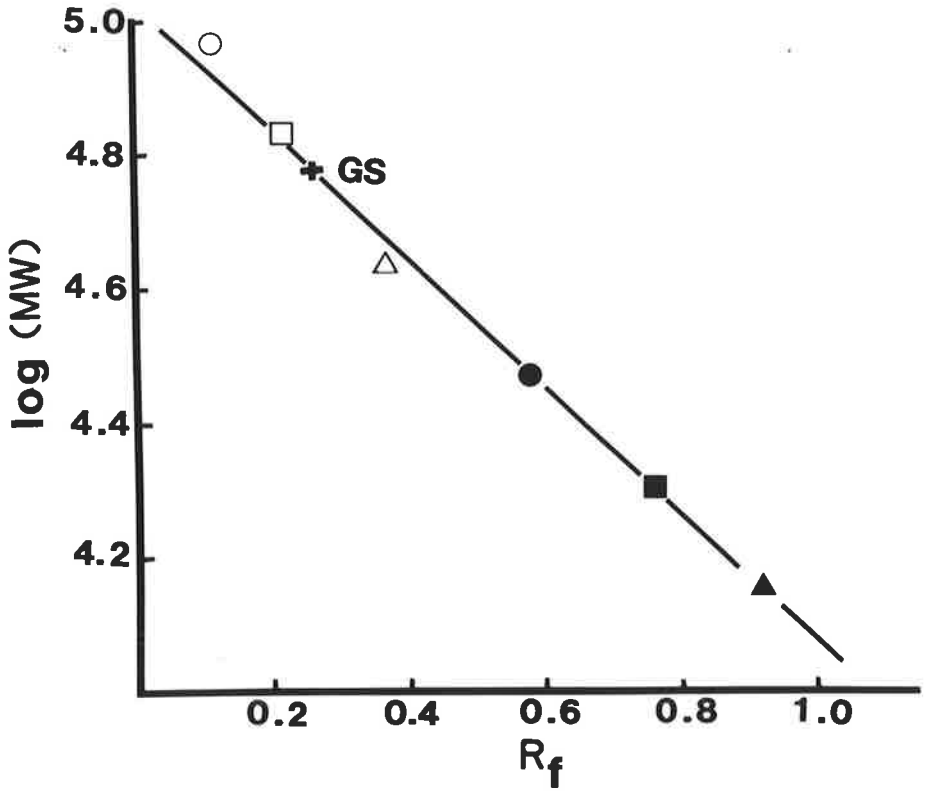


FIG. 20: Effects of pH on glutamine synthetase activity.

Transferase and biosynthetic activities of the purified enzyme (fraction 4, Table 14) were determined as described in Sections 2.2.3.1 and 2.2.3.2, except that the pH of the reaction mixture was varied as indicated.

(●) transferase activity.
(■) biosynthetic activity.

FIG 20

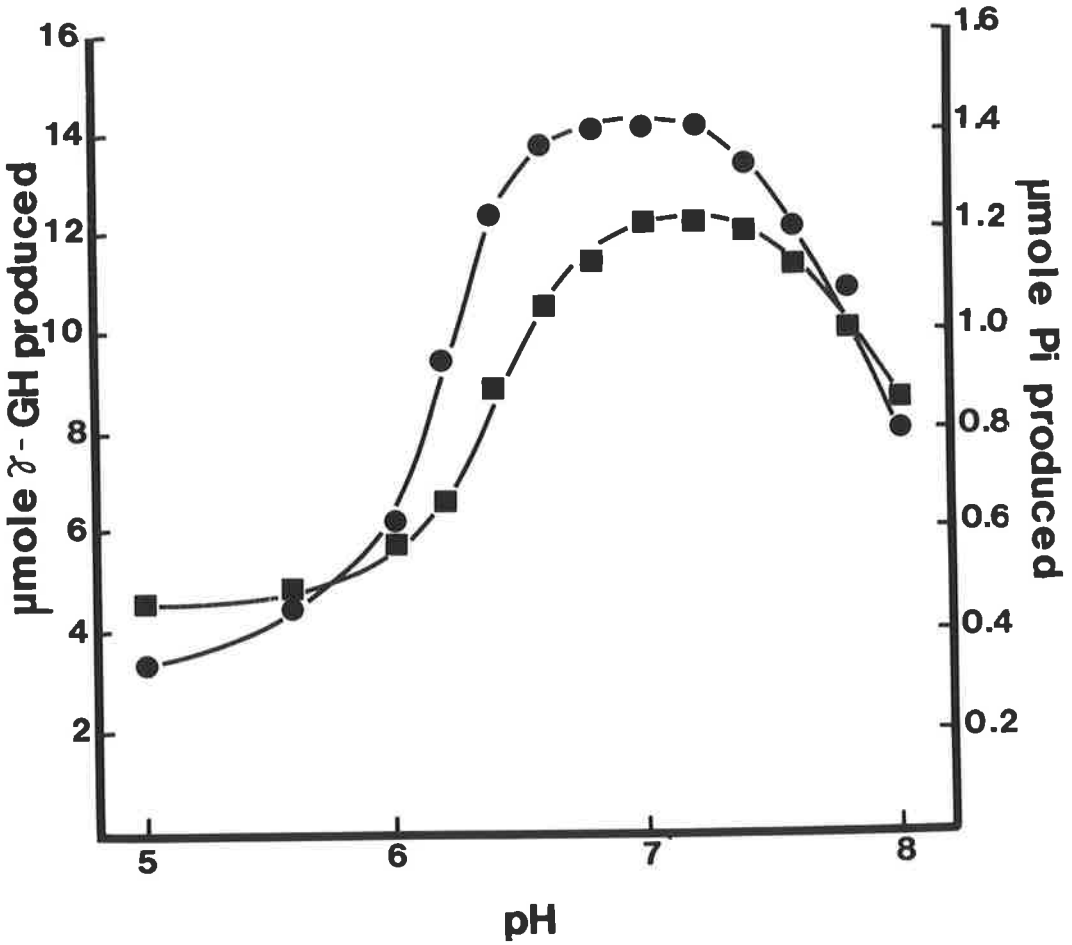

µmole γ- GH produced
µmole Pi produced
pH
16
14
12
10
8
6
4
2
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5
6
7
8

FIG. 21: Effects of incubation time on glutamine synthetase activity.

The activities of the transferase and biosynthetic enzyme (fraction 4, Table 14) were determined as described in Sections 2.2.3.1 and 2.2.3.2, except that the incubation period was varied as indicated.

(●) transferase activity.
(■) Mg^{2+}-dependent biosynthetic activity.
(▲) Mn^{2+}-dependent biosynthetic activity.

FIG 21

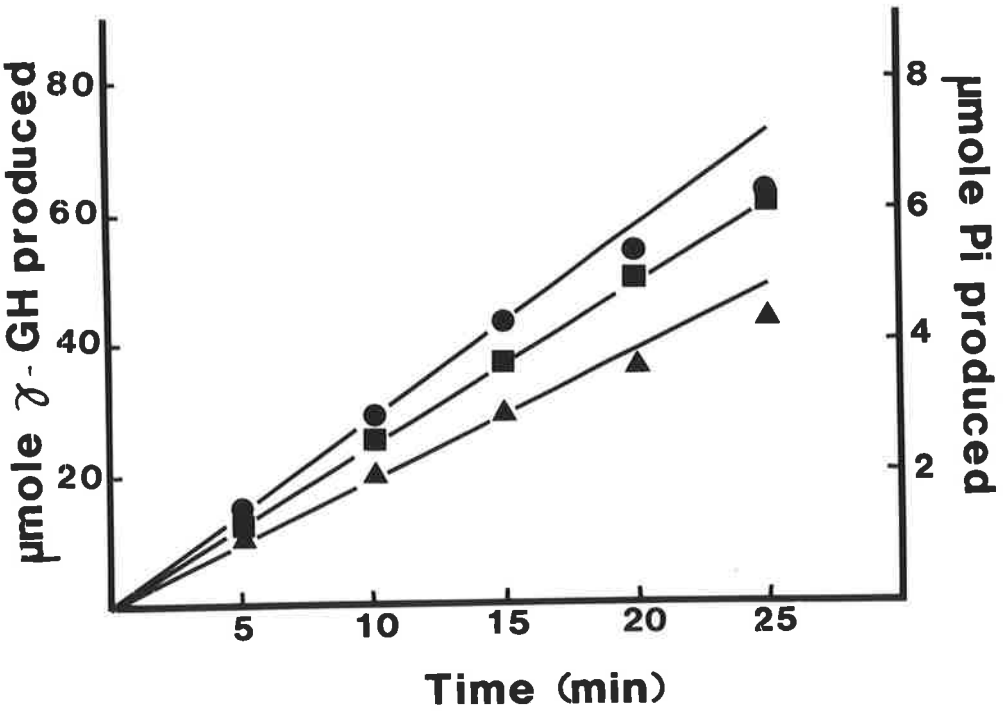

µmole γ-GH produced
80
60
40
20
µmole Pi produced
8
6
4
2
5
10
15
20
25
Time (min)

TABLE 15: Transferase and biosynthetic activities with various divalent cations

Purified enzyme (fraction 4, Table 14) was used after dialyzing againt 4L of 50 mM Tris-HCl buffer (pH 7.0) for 16 hr. The transferase and biosynthetic activities of the enzyme were determined as described in Sections 2.2.3.1 and 2.2.3.2, respectively, except that divalent cations used were as indicated. The concentrations of divalent cations were 0.5 and 5 mM for transferase activity and 5 or 50 mM for the biosynthetic reaction. The results are expressed as % activity of either transferase activity (in the presence of 5 mM Mn^{2+}) or biosynthetic activity (in the presence of 50 mM Mg^{2+}). The activities of the control were:

14.9 μmole γ-glutamylhydroxamate produced/min/mg protein (for transferase activity) and 1.2 μmole P_i produced/min/mg protein (for biosynthetic reaction).

Cations	Transferase activity (%)		Biosynthetic activity (%)	
	0.5 mM	5 mM	5 mM	50 mM
Mn^{2+}	94	100	76	71
Co^{2+}	23	19	87	89
Cu^{2+}	16	9	13	41
Mg^{2+}	13	2	91	100
Ni^{2+}	12	0	10	13
Ca^{2+}	12	0	10	12
Fe^{2+}	11	0	12	33
Zn^{2+}	10	0	12	26

that for Mn^{2+}): Co^{2+} (19); Cu^{2+} (9) and Mg^{2+} (2) whereas Ni^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} were without effect. At lower concentration (0.5 mM) Mn^{2+} was also more effective than other divalent cations. In contrast to the transferase reaction, the maximum activity for biosynthetic assay was obtained with Mg^{2+} at 50 mM. Co^{2+} and Mn^{2+} substituted for Mg^{2+} to a lesser extent; 89 and 71% respectively. The order of effectiveness of the various cations at both concentrations (5 and 50 mM) was the same; $Mg^{2+} > Co^{2+} > Mn^{2+} > Cu^{2+} > Fe^{2+} > Zn^{2+} > Ni^{2+} > Ca^{2+}$.

3.4.2.4 Nucleotide specificity

A range of nucleotide diphosphates and triphosphates were tested for their effects on the transferase and biosynthetic activities of the purified enzyme (fraction 4, Table 14). The results in Table 16 indicate that maximum transferase activity was recorded with ADP. Other nucleotide diphosphates; CDP, GDP, IDP and UDP functioned at lower rates whereas nucleotide triphosphates were relatively ineffective. In contrast, ATP was the most effective nucleotide for the biosynthetic activity; whereas nucleotide disphosphates were relatively ineffective. The Mg^{2+}-dependent biosynthetic activity with other nucleotides was 10% or less of that with ATP, whereas CTP, GTP and ITP substituted for ATP more effectively in the Mn^{2+}-dependent biosynthetic reaction; 65, 62 and 54% respectively, of that with ATP.

3.4.2.5 K_m values for substrates

Effects of various concentrations of substrates of the Mn^{2+}-dependent transferase activity and the Mg^{2+}-dependent biosynthetic

TABLE 16: Effects of various nucleotides on transferase and biosynthetic activities

The purified enzyme (fraction 4, Table 14) was used after dialyzing against 4L of 50 mM Tris-HCl buffer (pH 7.0) for 16 hr. The enzyme activities were determined as described in Sections 2.2.3.1 and 2.2.3.2, except that either ADP or ATP were replaced by other nucleotides as indicated. The nucleotide concentrations were 0.4 mM for transferase assay and 7.5 mM for the biosynthetic reaction. The enzyme activity of the control for transferase activity (with ADP) was 14.9 µmole γglutamyl hydroxamate produced/min/mg protein; for Mg^{2+}-dependent and Mn^{2+}-dependent biosynthetic activities were 1.23 µmole and 0.95 µmole P_i produced/min/mg protein, respectively.

Nucleotides	Activity of the control (%)		
	Transferase	Biosynthetic	
		Mg^{2+}-dependent	Mn^{2+}-dependent
ADP	100	4	4
CDP	42	2	2
GDP	38	5	2
IDP	42	0	3
UDP	29	0	0
ATP	5	100	100
CTP	3	8	65
GTP	4	10	62
ITP	1	6	54
UTP	3	5	25

reaction were studied. The effects of glutamine concentrations over a range of 0-30 mM on transferase activity are illustrated in Fig.22A (inset). The K_m value of 4.5 mM for glutamine was calculated from double reciprocal plot of glutamine concentrations against enzyme activity (Fig. 22A).

The effects of hydroxylamine at a fixed concentration of glutamine (30 mM) on transferase activity are shown in Fig. 22B. The enzyme activity increased up to 8 mM hydroxylamine and the apparent K_m value for hydroxylamine was 2.2 mM, calculated from double reciprocal plot of the data.

The effects of various concentrations of ADP on the transferase activity (Fig. 24A) indicate that the enzyme activity increased up to 0.25 mM ADP. From double reciprocal plot of the data, the K_m value for ADP was calculated to be 0.14 mM.

The effects of glutamate, over a range of 0-10 mM, on the biosynthetic reaction are illustrated in Fig. 23A (inset). The K_m values for glutamate calculated from a double reciprocal plot was 2.0 mM (Fig. 23A) and for NH_4Cl was 0.6 mM (Fig. 23B). Enzyme activity increased up to 2 mM NH_4Cl (Fig. 23B, inset). The effects of various concentrations of ATP on the biosynthetic activity are presented in Fig. 24B. The enzyme activity increased up to 2 mM ATP and the apparent K_m value for ATP was 1.25 mM as calculated from double reciprocal plot of the data.

3.4.3 Inhibitor studies

The effects of various concentrations of L-methionine-DL-sulphoximine, an analogue of glutamine, on transferase activity are presented in Table 17.

FIG. 22: Effects of various concentrations of substrates on glutamine synthetase-transferase activity.

The transferase activity of the purified enzyme (fraction 4, Table 14) was determined as described in Section 2.2.3.1, except that the concentrations of substrates were varied as indicated.

A: Double reciprocal plot of the effects of various concentrations of glutamine on transferase activity.

B: Double reciprocal plot of the effects of various concentrations of hydroxylamine on transferase activity.

V = activity (μmole γ-glutamylhydroxamate produced/min/mg protein).

FIG 22A

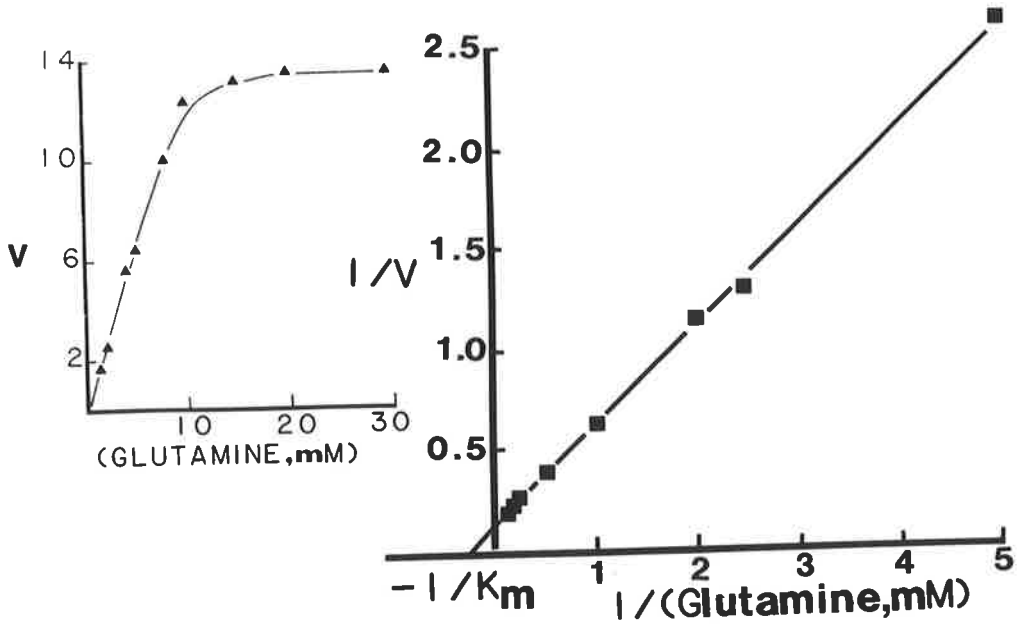


FIG 22B

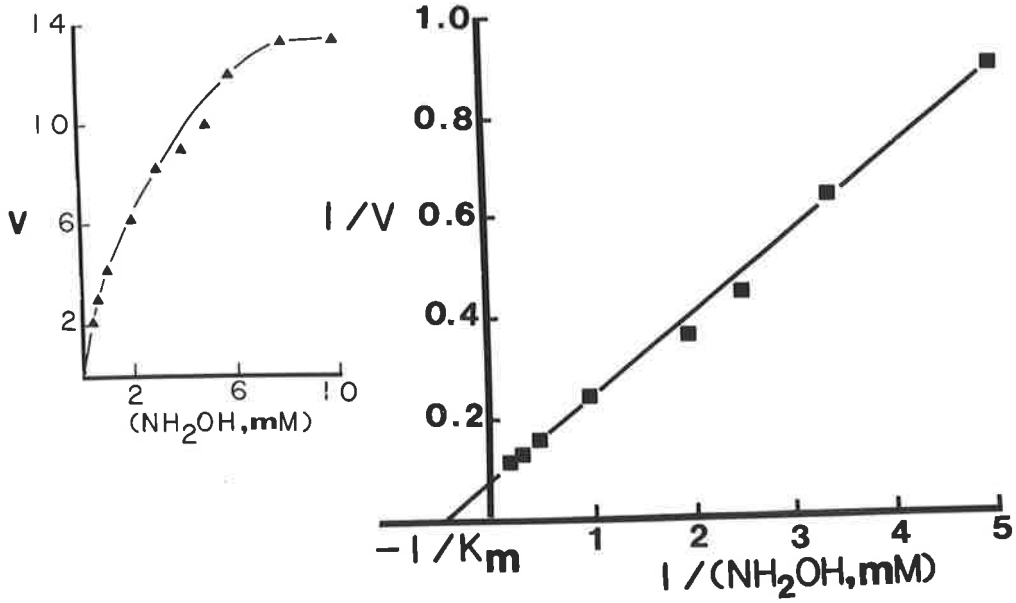


FIG. 23: Effects of various concentrations of substrates on glutamine synthetase-biosynthetic activity.

The biosynthetic activity of the purified enzyme (fraction 4, Table 14) was determined as described in Section 2.2.3.2, except that the concentrations of substrates were varied as indicated.

A: Double reciprocal plot of the effects of various concentrations of glutamate on biosynthetic activity.

B: Double reciprocal plot of the effects of various concentrations of NH_4Cl on biosynthetic activity.

V = activity (μmole P_i produced/min/mg protein).

FIG 23A

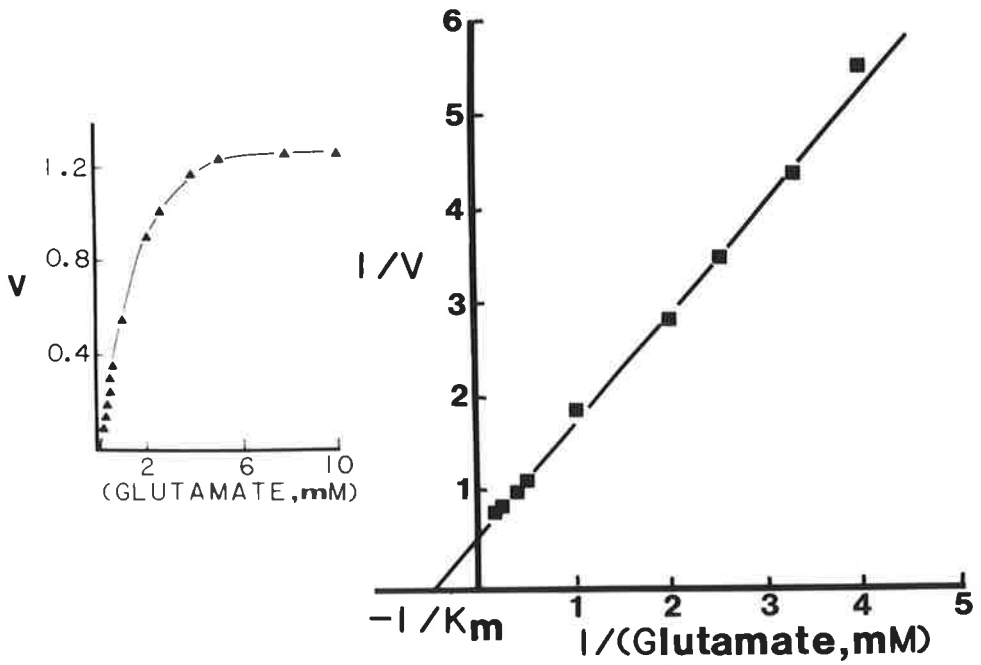


FIG 23B

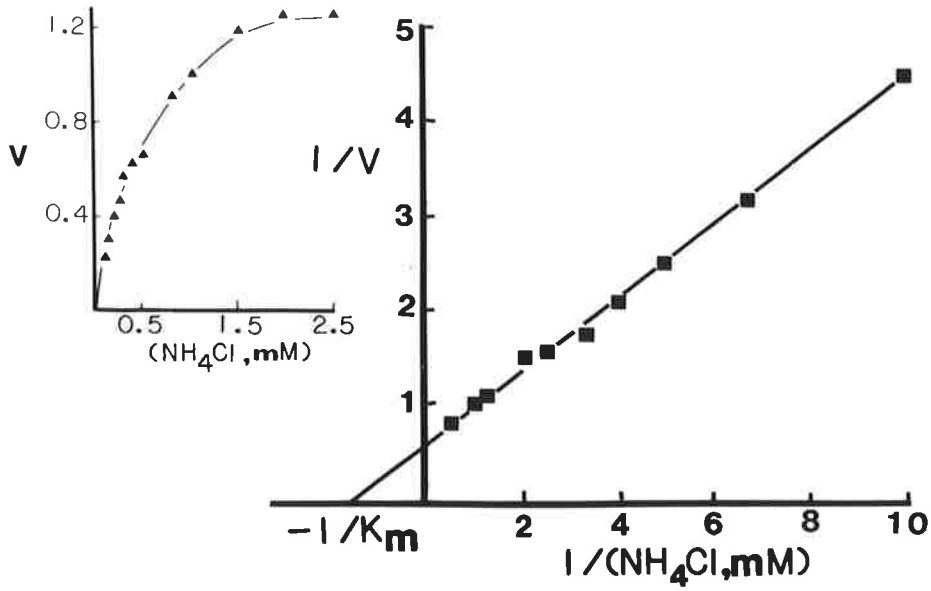


FIG. 24: Effects of various concentrations of ADP and ATP on glutamine synthetase activity.

The purified enzyme (fraction 4, Table 14) was used after dialyzing in 4L of 50 mM Tris-HCl buffer (pH 7.0) for 16 hr. The transferase and biosynthetic activities of the enzyme preparation were determined as described in Sections 2.2.3.1 and 2.2.3.2, except that ADP and ATP respectively, was added at various concentrations.

A: Double reciprocal plot of the effects of various concentrations of ADP on transferase activity.

V = activity (μmole γ-glutamylhydroxamate produced/min/mg protein).

B: Double reciprocal plot of the effects of various concentrations of ATP on biosynthetic activity.

V = activity (μmole P_i produced/min/mg protein).

FIG 24A

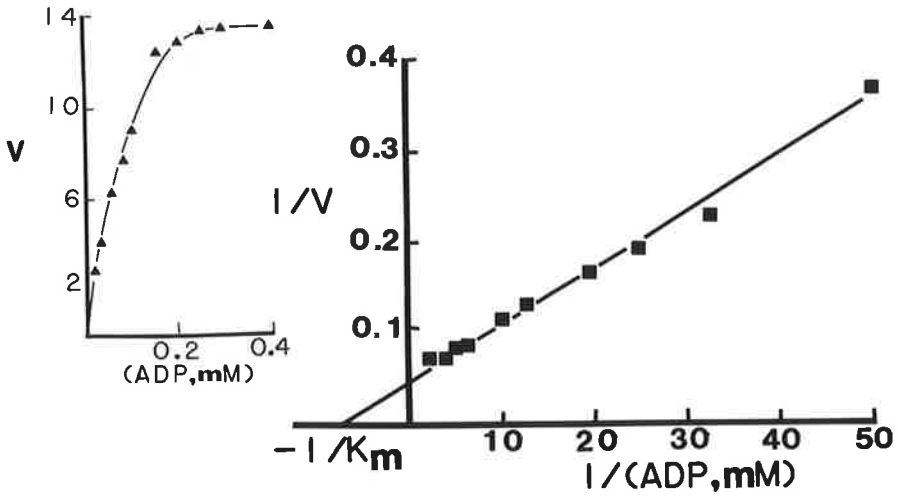


FIG 24B

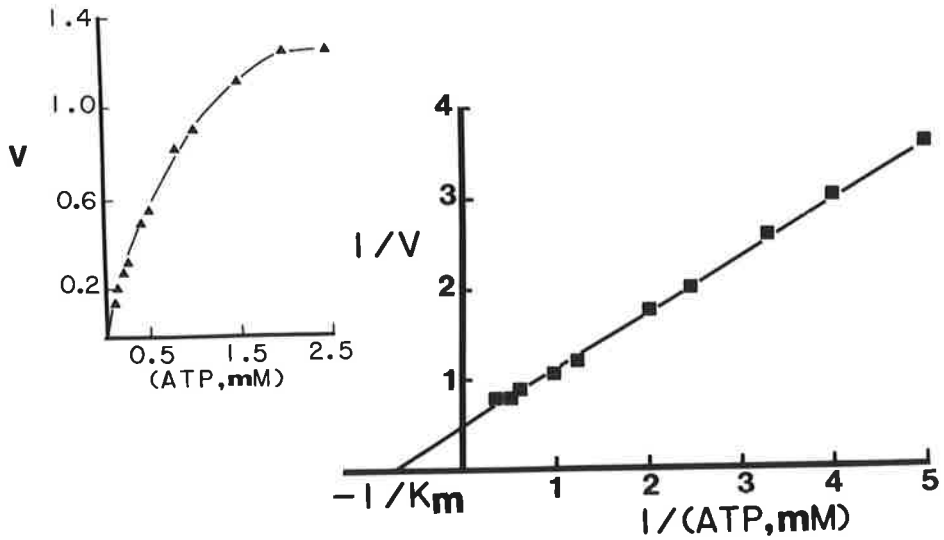


TABLE 17: Effects of various concentrations of L-methionine-DL-sulphoximine on transferase activity

The transferase activity of the purified enzyme (fraction 4, Table 14) was determined as described in Section 2.2.3.1, except that MSX was added at concentration indicated. The results are expressed as % inhibition of control (without MSX). The activity of the control was 14 µmole γ-glutamylhydroxamate produced/min/mg protein.

Final concentration of MSX (µM)	Inhibition (%)
1	27
2	40
3	49
4	60
5	67
10	84
15	90
20	93

The enzyme was markedly inhibited by MSX; 93% inhibition was recorded at 20 μM.

Various amino acids were tested for their inhibitory effects on the purified enzyme activity. The results in Table 18 indicate that cysteine, alanine, glycine, serine, isoleucine, threonine, proline, phenylalanine and valine each at 10 mM markedly inhibited transferase activity whereas the biosynthetic reaction was restricted by cysteine, isoleucine and tryptophan.

The effects of some organic acids on the Mn^{2+}-dependent transferase and Mg^{2+}-dependent biosynthetic activities are shown in Table 19. Oxalate (10 mM) markedly inhibited transferase activity by 81% and oxalacetate and α-ketoglutarate by 69 and 40% respectively; whereas malate, lactate and pyruvate were without effect. The biosynthetic activity was restricted by oxalate, α-ketoglutarate and oxalacetate (each at 10 mM) by 49, 49 and 42% respectively, whereas malate, lactate, citrate, pyruvate and succinate did not affect the enzyme activity.

Double reciprocal plots of the effects of various concentrations of glutamate and NH_4Cl (substrates for the biosynthetic activity) on the transferase reaction (Figs. 25A and 25B) show that both substrates are competitive inhibitors with respect to glutamine. At 20, 10 and 5 mM glutamine respectively, glutamate (20 mM) inhibited the transferase reaction by 12, 23 and 28%; while NH_4Cl at the same concentration restricted the enzyme to a lesser extent. The apparent K_i values estimated from Dixon plots were 34.5 mM for glutamate (Fig. 26) and 38 mM for NH_4Cl (Fig. 27).

3.4.4 Adenylylation/deadenylylation

In some bacteria the extent of the transferase activity in the presence of 60 mM $MgCl_2$ has been used to indicate the extent of adenylylation of

TABLE 18: Effects of various amino acids on glutamine synthetase activity

Transferase and biosynthetic activities of the enzyme (fraction 4, Table 14) were determined as described in Sections 2.2.3.1 and 2.2.3.2, except that amino acid was added at 10 mM final concentration. The results are expressed as % inhibition of the control (without amino acid). Activity of the control was 14.9 µmole γ-glutamylhydroxamate produced/min/mg protein (for transferase reaction) and 1.2 µmole P_i produced/min/mg protein (for biosynthetic assay).

Amino acids (10 mM final concentration)	Inhibition (%)	
	Transferase activity	Biosynthetic activity
Cysteine	94	80
Alanine	89	40
Glycine	78	48
Serine	74	42
Isoleucine	71	62
Threonine	70	40
Proline	66	58
Phenylalanine	66	52
Valine	63	49
Lysine	47	30
Histidine	42	52
Methionine	41	28
Asparagine	41	32
Tryptophan	33	60
Leucine	26	40
Arginine	13	16

TABLE 19: Effects of organic acids on glutamine synthetase activity

Transferase and biosynthetic activities of the enzyme (fraction 4, Table 14) were determined as described in Sections 2.2.3.1 and 2.2.3.2, except that the organic acid was added at 10 mM final concentration. The results are expressed as % inhibition of the control (without organic acid). Activity of the control was 14.9 µmole γ-glutamylhydroxamate produced/min/mg protein (for transferase reaction) and 1.2 µmole P_i produced/min/mg protein (for biosynthetic assay).

Organic acids (10 mM final concentration)	Inhibition (%)	
	Transferase activity	Biosynthetic activity
Oxalate	81	49
Oxalacetate	69	42
α-ketoglutarate	40	49
Citrate	5	0
Succinate	3	0

FIG. 25: Inhibitory effects of glutamate and NH_4Cl on glutamine synthetase-transferase activity.

The transferase activity of the enzyme (fraction 4, Table 14) was determined as described in Section 2.2.3.1, except that various concentrations of either glutamate or NH_4Cl were added at defined levels of glutamine.

A: A Lineweaver-Burk plot of the effects of glutamate at various levels of glutamine. The concentrations of glutamate were: (□) 0 mM; (△) 5 mM; (■) 10 mM; and (▲) 20 mM.

Inset: Inhibition by glutamate with glutamine at (◆) 5 mM; (▲) 10 mM; and (■) 20 nM.

B: A Lineweaver-Burk plot of the effects of NH_4Cl at various levels of glutamine. The concentrations of NH_4Cl were: (□) 0 mM; (△) 5 mM; (■) 10 mM; and (▲) 20 mM.

Inset: Inhibition by NH_4Cl with glutamine at (◆) 5 mM; (▲) 10 mM; and (■) 20 mM.

V = activity (μmole γ-glutamylhydroxamate produced/min/mg protein).

S = substrate (glutamine, mM).

FIG 25A

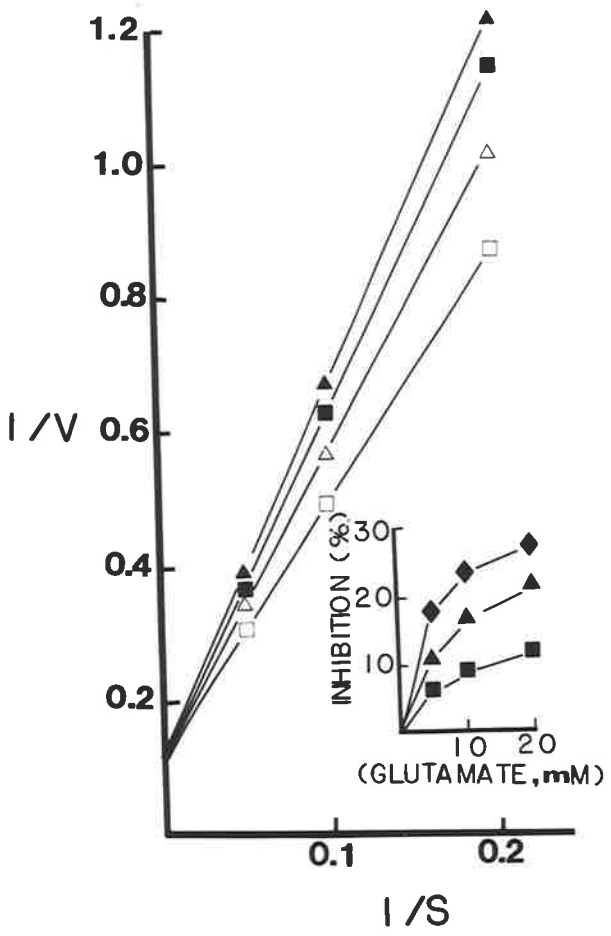


FIG 25B

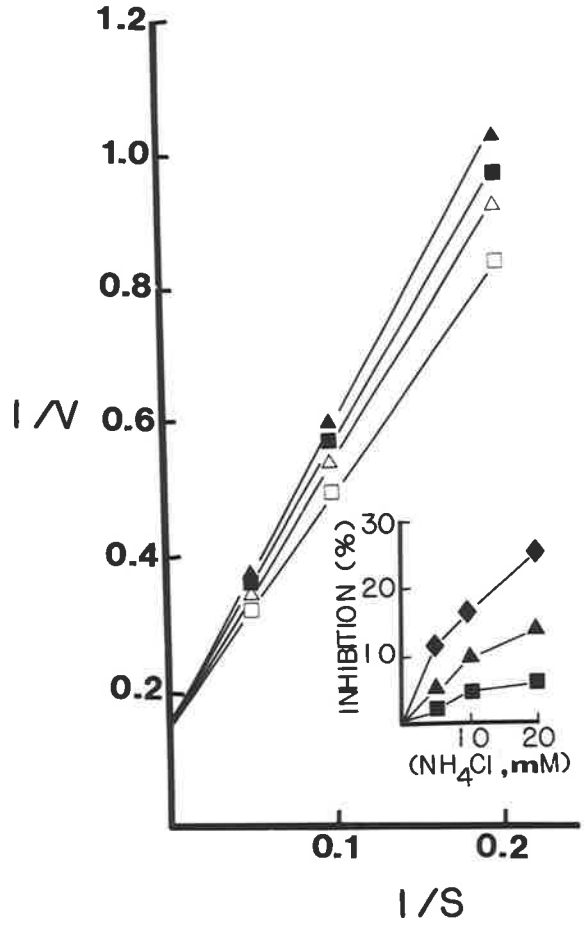


FIG. 26: A Dixon plot of the effects of various concentrations of glutamate on glutamine synthetase-transferase activity

The transferase activity of the enzyme (fraction 4, Table 14) was determined as described in Section 2.2.3.1, except that various concentrations of glutamine were used as well as the addition of glutamate at the concentrations indicated. The concentrations of glutamine were:

(■) 5 mM;
(▲) 10 mM; and
(●) 20 mM.

V = activity (μmole γ-glutamylhydroxamate produced/min/mg protein).

FIG 26

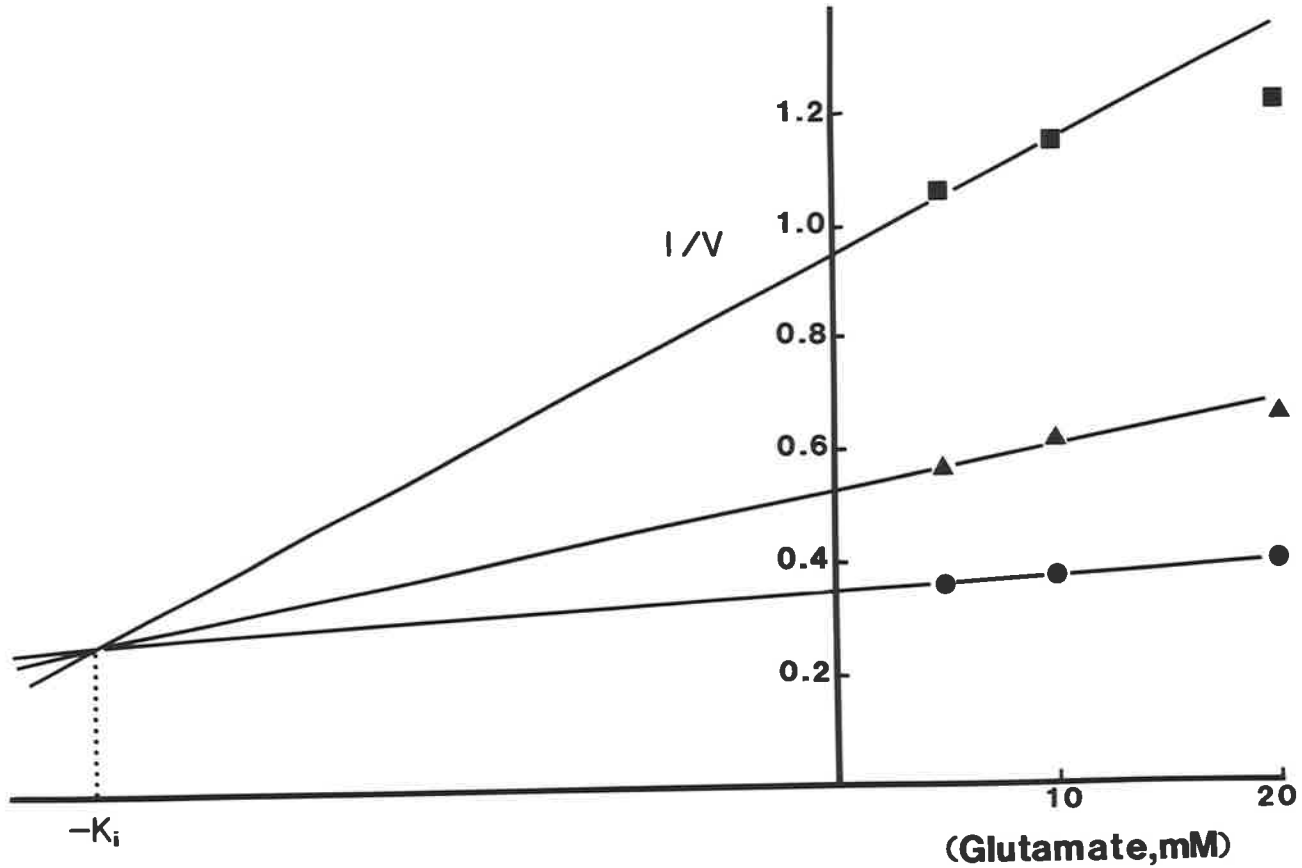


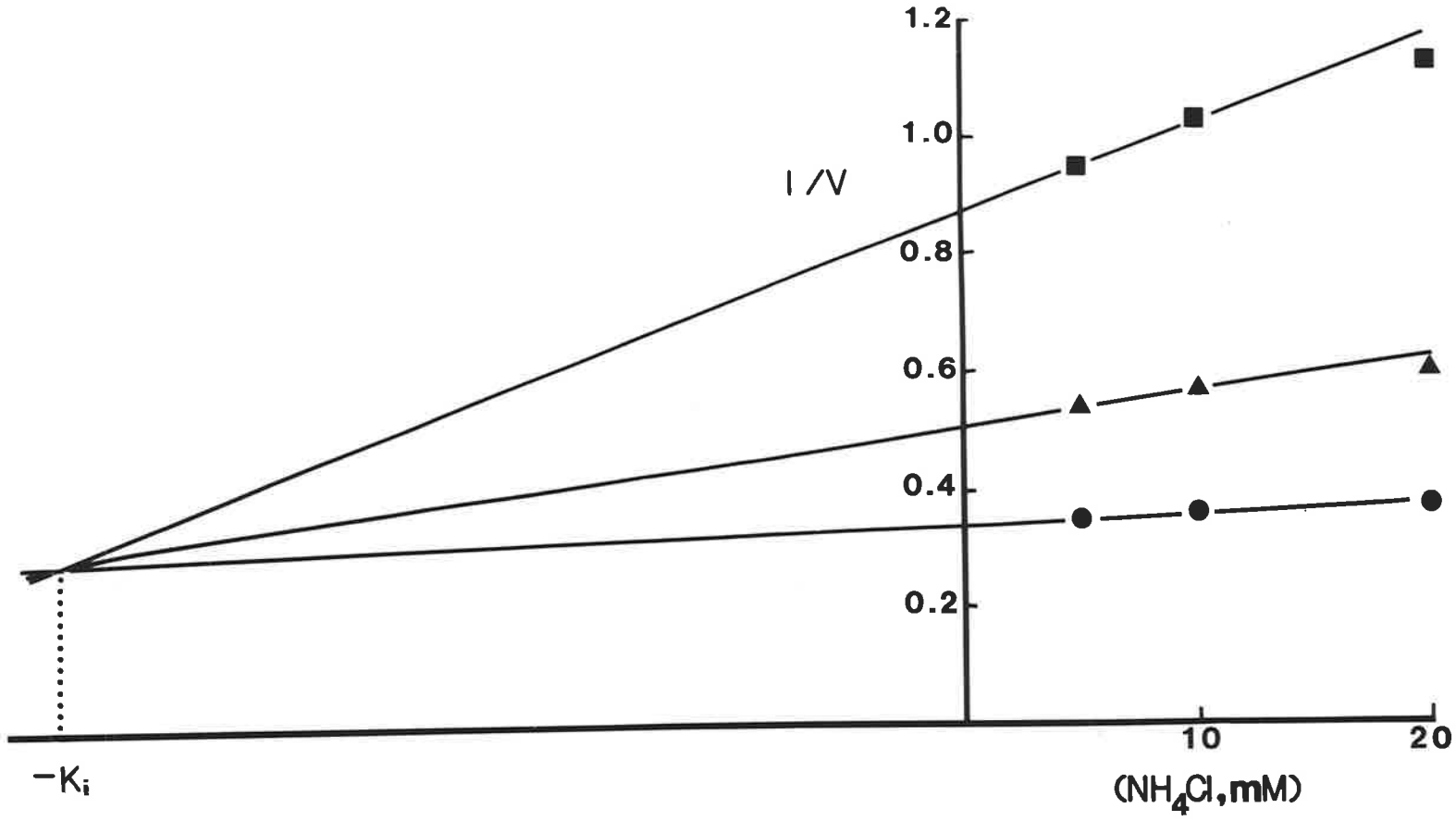
FIG. 27: A Dixon plot of the effects of various concentrations of ammonium chloride on glutamine synthetase-transferase activity.

The transferase activity of the enzyme (fraction 4, Table 14) was determined as described in Section 2.2.3.1, except that various concentrations of glutamine were used as well as the addition of NH_4Cl at concentrations indicated. The concentrations of glutamine were:

(■) 5 mM;
(▲) 10 mM; and
(●) 20 mM.

V = activity (μmole γ-glutamylhydroxamate produced/min/mg protein).

FIG 27



glutamine synthetase (Alef and Zumft, 1981; Michalski *et al.*, 1983; Kumar and Nicholas, 1984). The fully adenylylated enzyme is relatively inactive in the presence of Mg^{2+}, whereas the deadenylylated enzyme is not affected. The effects of snake venom phosphodiesterase treatment on the transferase activity of the purified enzyme (fraction 4, Table 14) in the presence and absence of 60 mM $MgCl_2$ are presented in Table 20. The percentage of adenylylation was reduced from 74% to 25% on treating the purified enzyme with snake venom phosphodiesterase as described in Section 2.2.9, and the A_{260} values were decreased by about 20%.

More direct evidence for the cleavage of AMP from the adenylylated enzyme was achieved by separating the SVD-treated enzyme in polyacrylamide gels electrophoresis as described in Section 2.2.9. The AMP was detected in the gels by UV light soon after electrophoresis with reference to appropriate authentic markers.

3.5 Glutamate synthase

3.5.1 Purification

The enzyme was purified to about a 100-fold with a recovery of 38% (Table 21) by the following procedure. Cell-free extracts were prepared at 4°C and subsequent purification of the enzyme was carried out at this temperature.

Frozen mycelial felts were ground in a chilled porcelain mortar and pestle and homogenized in 50 ml Tris-HCl buffer (pH 7.5), 1g mycelial felts to 3 ml of the buffer, using a glass homogenizer. The homogenate passed, three times, through a French pressure cell at 20,000 p.s.i. was centrifuged at 20,000 *g* for 20 min.. The supernatant fraction (S_{20}) containing glutamate synthase activity was used as the crude extract.

TABLE 20: Effects of snake venom phosphodiesterase on glutamine synthetase activity

Snake venom phosphodiesterase (500 µg) was added to 10 ml of the purified enzyme preparation (fraction 4, Table 14). After incubation for 1 hr at 37°C the reaction mixture was loaded onto a Sephadex G-10 column to separate AMP which was cleaved from the adenylylated enzyme (G-10 fraction). The transferase activity of the SVD-treated and untreated enzyme was determined as described in Section 2.2.3.1, either with or without 60 mM $MgCl_2$.

Treatment	A_{260}	Transferase activity (units/mg protein)		Adenylylation (%)
		$-Mg^{2+}$	$+Mg^{2+}$	
Without SVD (fraction 4, Table 14)	0.64	14.92	3.94	74
With SVD (G-10 fraction)	0.54	14.75	11.04	25

To the crude extract (S_{20}), $MnCl_2$ (32 ml of a M solution per litre of crude extract) was slowly added with constant stirring. The mixture was immediately centrifuged at 20,000 *g* for 15 min and the supernatant (S_{20}, $MnCl_2$) was collected. Solid $(NH_4)_2SO_4$ was then gradually added to the supernatant (S_{20}, $MnCl_2$) with constant stirring to produce 25% $(NH_4)_2SO_4$ saturation. During addition of $(NH_4)_2SO_4$, the pH of solution was maintained at 7.5 by adding cold 50 mM Tris. After standing for 30 min, the mixture was centrifuged at 20,000 *g* for 15 min. The supernatant collected was then brought to 65% $(NH_4)_2SO_4$ saturation and allowed to stand for 30 min before centrifuging at 20,000 *g* for 15 min. The pellet re-dissolved in 50 mM Tris-HCl buffer (pH 7.5) was recentrifuged at 20,000 *g* for 10 min to remove insoluble material.

The clarified solution dialyzed for 16 hr against 3L of 50 mM Tris-HCl buffer (pH 7.5) was then loaded onto a DE-32 column (140 x 35 mm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The column was then washed with 300 ml of the same buffer. Glutamate synthase was separated by a linear gradient of NaCl (0 to 500 mM) in the buffer. The total gradient volume was 250 ml and the flow rate was 50 ml/hr. Active fractions eluted between 200 and 300 mM NaCl were pooled and dialyzed for 16 hr against 3L of 50 mM Tris-HCl buffer (pH 7.5).

The dialyzed enzyme was loaded onto a Blue Sepharose CL-6B column (80 x 15 mm) which had been pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and then washed with 150 ml of the same buffer. The enzyme eluted in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM NADPH had a specific activity of 7.6 µmole NADPH oxidized/min/mg protein. A summary of the purification is given in Table 21.

TABLE 21: Purification of glutamate synthase

The enzyme activity of each fraction was measured by following the oxidation of NADPH in the reaction mixture as described in Section 2.2.4. One unit of enzyme activity corresponds to 1 µmole NADPH oxidized/min.

Fraction	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification (-fold)	Recovery (%)
1.Crude extract (S_{20})	12.30	164.0	0.075	-	100
2.Precipitate from $(NH_4)_2SO_4$ 25-65% saturation dialyzed for 16hr against 3L of 50mM Tris-HCl buffer (pH 7.5)	12.78	37.2	0.344	5	103
3.Fraction 2 loaded ontc a DE-32 column (140x35mm) and eluted with 0-0.5M NaCl in 50mM Tris-HCl buffer (pH 7.5) and then dialyzed for 16hr against 3L of the same buffer	7.03	5.8	1.216	16	57
4.Fraction 3 loaded onto a Blue Sepharose CL-6B column (80x15mm) and eluted with 50mM Tris-HCl buffer (pH 7.5) containing 1mM NADPH	4.66	0.6	7.618	102	38

3.5.2 Properties

3.5.2.1 Molecular weight

The purified glutamate synthase (fraction 4, Table 21) had a molecular weight of 220 kDa as determined by gel filtration on a Sepharose 6B column (Fig. 28A). A subunit molecular weight was established for the enzyme by SDS-polyacrylamide gel electrophoresis; one major protein band was detected with a molecular weight of 53.7 kDa. Thus the enzyme is composed of 4 subunits of similar size.

3.5.2.2 Effect of pH

The effect of pH on enzyme activity was determined as described in Section 2.2.4. The results in Fig. 29 indicate that the optimum pH for enzyme activity was between 8.0 and 8.4 and it retained more than 50% of the activity over the pH range 7.4 - 9.0.

3.5.2.3 Substrate requirement

The results in Table 22 for the purified enzyme (fraction 4, Table 21) indicate that glutamine, α-ketoglutarate and NADPH are essential for activity. The requirement for these compounds was specific since NADH could not substitute for NADPH; neither oxalate nor pyruvate for α-ketoglutarate; and neither asparagine nor NH_4Cl for glutamine.

3.5.2.4 K_m values for substrates and NADPH

Effect of various concentrations of substrates on enzyme activity were determined. The effects of glutamine concentrations over a range of 0-6 mM are illustrated in Fig. 30A. The K_m value

FIG. 28A: Molecular weight determination of glutamate synthase by gel filtration.

The purified enzyme (fraction 4, Table 21) was passed through a Sepharose 6B column as described in Section 2.2.7.1. Elution buffer was 50 mM Tris-HCl (pH 7.5) containing 0.1M NaCl. The K_{av} values were calculated from $K_{av} = (V_e-V_o)/(V_t-V_o)$. The proteins used as markers were:

(□) ferritin;
(●) catalase;
(▲) aldolase; and
(■) albumin.

FIG. 28B: Estimation of subunit molecular weight of the purified enzyme by SDS-polyacrylamide gel electrophoresis.

The molecular weight of the enzyme subunit (fraction 4, Table 21) was determined by polyacrylamide (12.5%) slab gel electrophoresis in the presence of 0.1% (w/v) SDS, as described in Section 2.2.7.2. Relative mobility (R_f) of proteins were calculated by dividing the migration distance of the proteins with the movement of the tracking dye at the end of the run. The standard proteins used were:

(■) phosphorylase *b*;
(▲) albumin;
(●) ovalbumin;
(□) carbonic anhydrase;
(△) trypsin inhibitor; and
(○) α-lactalbumin.

FIG 28A

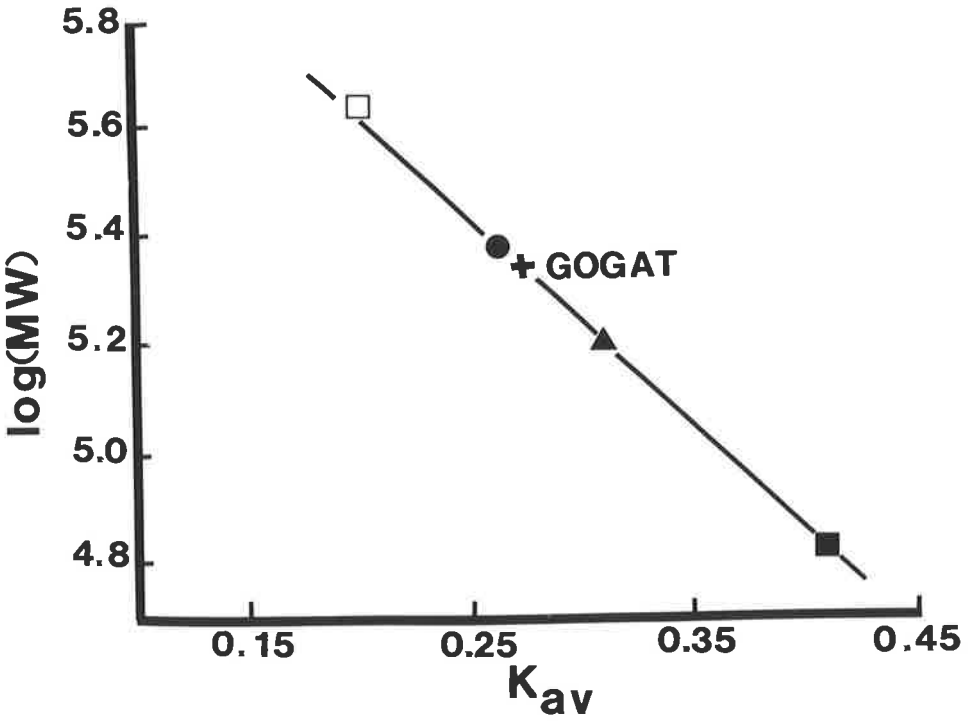


FIG 28B

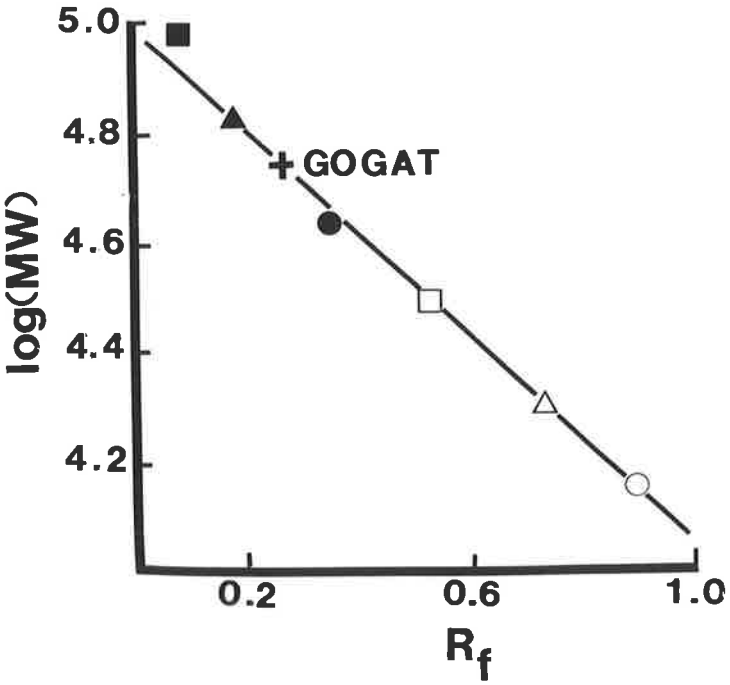


FIG. 29: Effects of pH on glutamate synthase activity.

The activity of the purified enzyme was determined as described in Section 2.2.4, except that the pH of reaction mixture was varied as indicated.

FIG 29

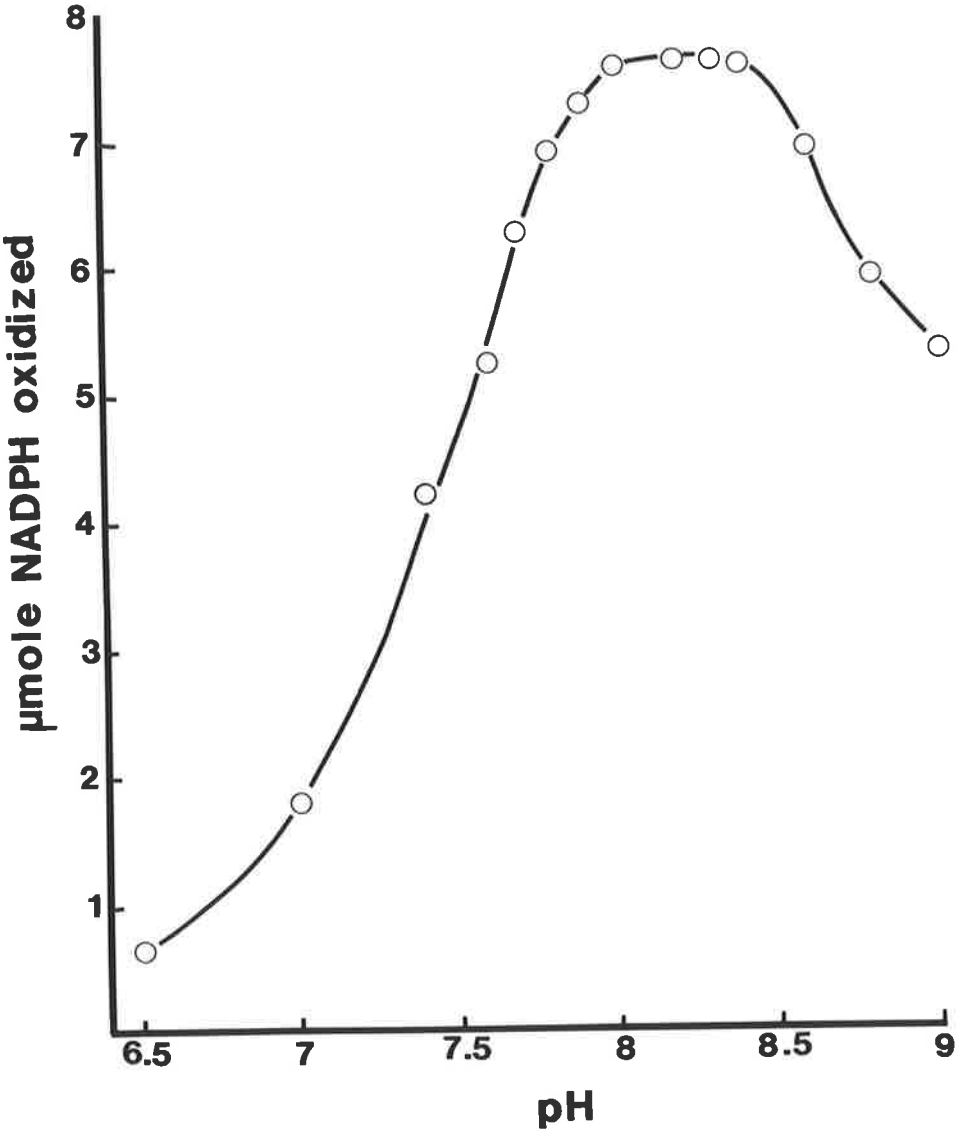


TABLE 22: Substrates and NADPH requirements for glutamate synthase activity

The purified enzyme (fraction 4, Table 21) was used after dialyzing for 16 hr against 3L of 50 mM Tris-HCl buffer (pH 7.5). The enzyme activity was measured as described in Section 2.2.4. The results are expressed as % of control (complete assay mixture). The activity of the control was 7.6 µmole NADPH oxidized/min/mg protein.

Assay conditions	Activity (%)
Complete	100
Omit NADPH	0
Omit NADPH, add NADH	0
Omit α-ketoglutarate	2
Omit α-ketoglutarate, add oxalacetate	2
Omit α-ketoglutarate, add pyruvate	0
Omit glutamine	4
Omit glutamine, add asparagine	3
Omit glutamine, add NH_4Cl	2

of 2.6 mM was calculated from double reciprocal plot of glutamine concentrations against enzyme activity (Fig. 30B).

The effects of various concentrations of α-ketoglutarate on enzyme activity are shown in Fig. 31A. Enzyme activity increased as the concentration of α-ketoglutarate increased up to 1.5 mM. From double reciprocal plot of the data (Fig. 31B), the K_m value for α-ketoglutarate was calculated to be 0.35 mM.

The effects of various concentrations of NADPH showed that the enzyme activity increased up to 75 μM NADPH (Fig. 32A). The K_m value for NADPH was 35 μM as calculated from double reciprocal plot of the data.

3.5.3 Inhibitor studies

The effects of a range of compounds on enzyme activity were studied. The results in Table 23 indicate that the enzyme was markedly inhibited by phenylalanine (78% at 10 mM) and to a lesser extent (<45%) by arginine, leucine, valine and glutamate but it was not restricted by either histidine or asparagine (each at 10 mM). Valine, isoleucine, aspartate, alanine, glycine and threonine (1 mM) had no effect on enzyme activity.

The inhibitory effects of various metabolites are presented in Table 24. Thus oxalate, malate and fumarate (at 5 mM) inhibited enzyme activity by 48, 40 and 29%, respectively. Enzyme activity was also restricted by 47, 29 and 31% respectively, by ATP, ADP and AMP (5 mM).

Azaserine, an analogue of glutamine, markedly inhibited the enzyme (Table 25). Thus at 1 mM, it restricted enzyme activity by 68%.

The results in Table 26 indicate that metal-binding agents also inhibited glutamate synthase activity. Thus O-phenanthroline (at 10 mM)

FIG. 30: Effects of various concentrations of glutamine on glutamate synthase activity.

The activity of the purified enzyme (fraction 4, Table 21) was determined as described in Section 2.2.4, except that the concentrations of glutamine were varied as indicated.

A: Glutamate synthase activity at various concentrations of glutamine.

B: Double reciprocal plot of the effects of various concentrations of glutamine on enzyme activity.

V = activity (µmole NADPH oxidized/min/mg protein).

FIG 30A

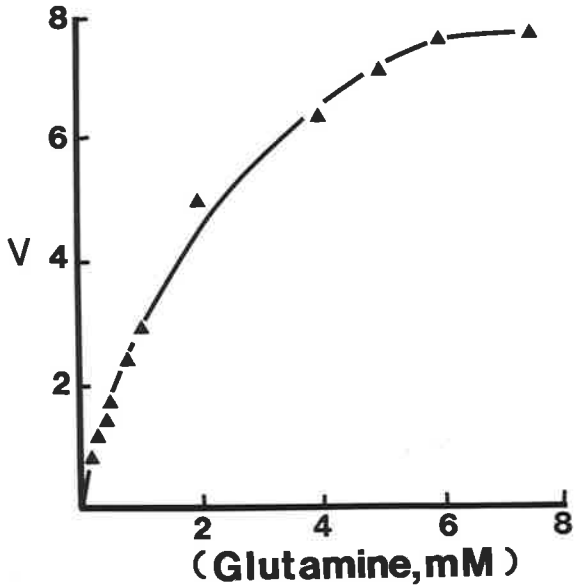


FIG 30B

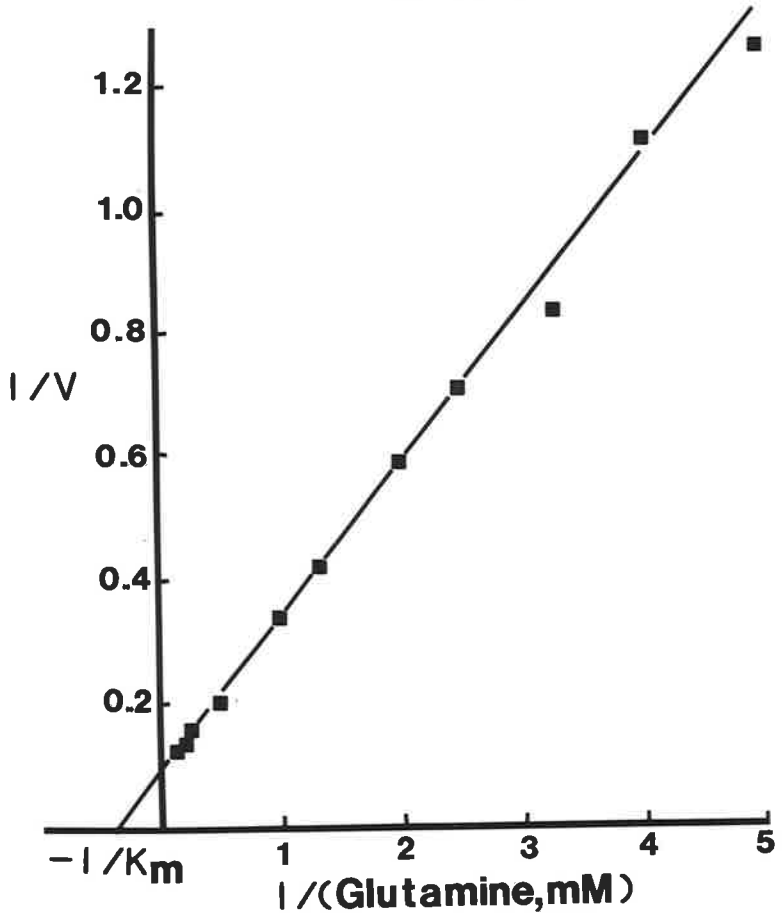


FIG. 31: Effects of various concentrations of α-ketoglutarate on glutamate synthase activity.

The activity of the purified enzyme (fraction 4, Table 21) was determined as described in Section 2.2.4, except that the concentrations of α-ketoglutarate were varied as indicated.

A: Glutamate synthase activity at various concentrations of α-ketoglutarate.

B: Double reciprocal plot of the effects of various concentrations of α-ketoglutarate on enzyme activity.

V = activity (μmole NADPH oxidized/min/mg protein).

FIG 31A

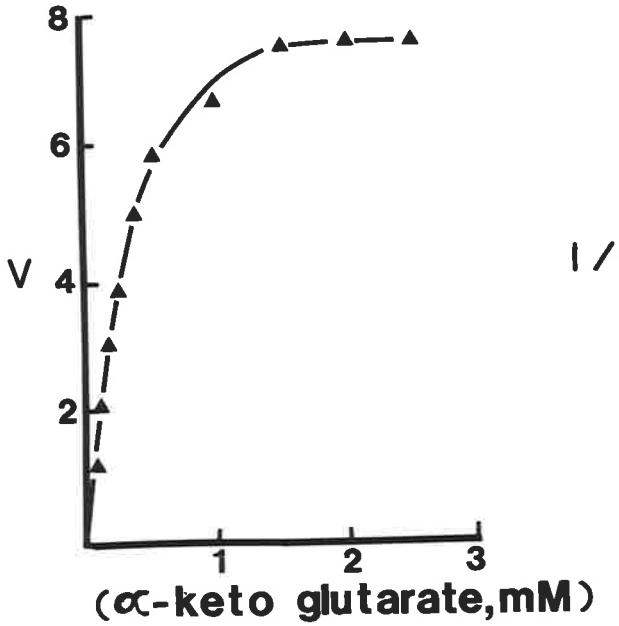


FIG 31B

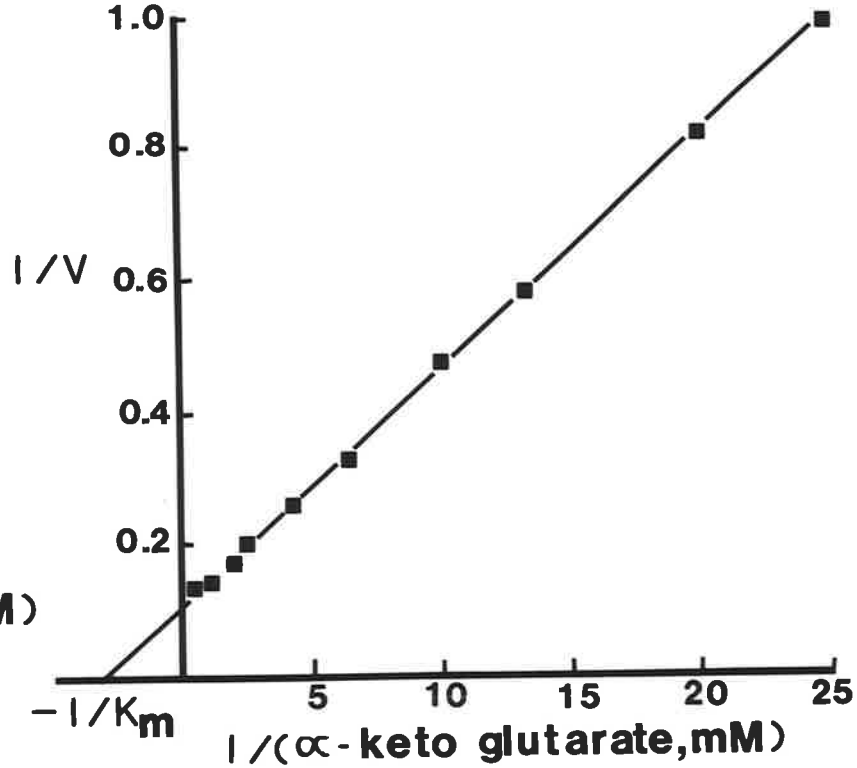


FIG. 32: Effects of various concentrations of NADPH on glutamate synthase activity.

The purified enzyme (fraction 4, Table 21) was used after dialyzing for 16 hr against 3L of 50 mM Tris-HCl buffer (pH 7.5). The enzyme activity was determined as described in Section 2.2.4, except that the concentrations of NADPH were varied as indicated.

A: Glutamate synthase activity at various concentrations of NADPH.

B: A Lineweaver-Burk plot of the effects of various concentrations of NADPH on enzyme activity.

V = activity (μmole NADPH oxidized/min/mg protein).

FIG 32A

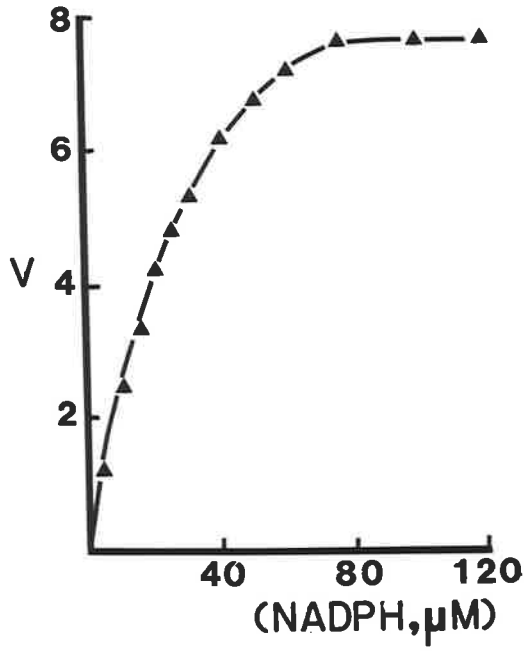


FIG 32B

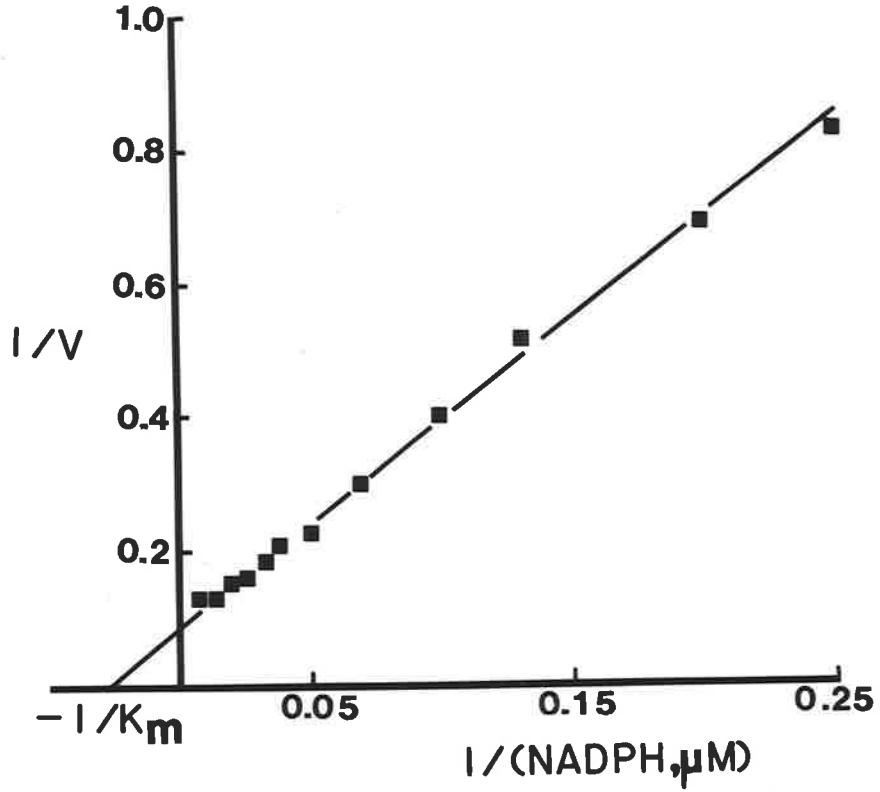


TABLE 23: Effects of amino acids on glutamate synthase activity

The activity of the enzyme (fraction 4, Table 21) was determined as described in Section 2.2.4, except that amino acid was added at the concentration indicated. The results are expressed as % inhibition of control (without inhibitor). The activity of the control was 7.6 µmole NADPH oxidized/min/mg protein.

Amino acids	Inhibition (%)		
	1 mM	5 mM	10 mM
Phenylalanine	14	28	78
Arginine	9	9	43
Leucine	4	5	36
Valine	0	6	26
Glutamate	9	16	25
Isoleucine	0	22	23
Aspartate	0	20	22
Serine	6	14	21
Tryptophan	5	5	20
Threonine	0	5	19
Methionine	8	13	16
Cysteine	9	9	14
Lysine	8	8	14

TABLE 24: Effects of various metabolites on glutamate synthate activity

The activity of the purified enzyme (fraction 4, Table 21) was determined as described in Section 2.2.4, except that metabolite was added at the concentration indicated. The results are expressed as % inhibition of control (without addition of metabolite). Activity of the control was 7.6 µmole NADPH oxidized/min/mg protein.

Metabolites	Inhibition (%)	
	1 mM	5 mM
c-AMP	0	5
AMP	0	31
ADP	18	29
ATP	0	47
Pyruvate	14	20
Citrate	8	16
Succinate	9	14
Fumarate	16	29
Malate	11	40
Oxalacetate	15	20
Oxalate	26	48

TABLE 25: Effects of various concentrations of azaserine on glutamate synthase activity

The activity of the purified enzyme (fraction 4, Table 21) was determined as described in Section 2.2.4, except that azaserine was added at concentration indicated. The results are expressed as % inhibition of control (without azaserine). The activity of control was 7.6 µmole NADPH oxidized/min/mg protein.

Azaserine concentration (mM)	Inhibition (%)
0.2	14
0.4	26
0.6	42
0.8	60
1.0	68

TABLE 26: Effects of various inhibitors on glutamate synthase activity

The activity of the enzyme (fraction 4, Table 21) was determined as described in Section 2.2.4, except that the inhibitor was added at the concentrations indicated. The enzyme was preincubated with the inhibitor for 5 min at 30°C before initiating the reaction. The results are expressed as % inhibition of control (without inhibitor). The activity of the control was 7.6 µmole NADPH oxidized/min/mg protein.

Inhibitors	Final concentrations (mM)	Inhibition (%)
Sodium arsenite	4	9
	10	57
Sodium azide	10	5
	25	15
Potassium cyanide	10	4
	25	94
α,α'-dipyridyl	1	4
	5	43
	10	88
O-phenanthroline	1	8
	4	68
	10	100
p-CMB	0.01	46
	0.05	96
	0.10	100
p-CMB + Cysteine	0.1 + 0.2	0

completely restricted activity, and α,α'-dipyridyl and arsenite (each at 10 mM) inhibited the enzyme by 88% and 57% respectively.

The enzyme was also markedly inhibited by p-CMB at 0.1 mM and this effect was reversed by the addition of 0.2 mM cysteine.

4. DISCUSSION

4.1 Nitrate reductase

Nitrate reductase, the first enzyme involved in nitrate assimilation is regulated in many organisms (Beevers and Hageman, 1969). This enzyme has been characterized and its properties and regulation studied in bacteria (Nicholas and Nason, 1955; Lowe and Evans, 1964; Guerrero *et al.*, 1973; Herrera and Nicholas, 1974; Villalobo *et al.*, 1977; Rasul Chaudry and MacGregor, 1983), fungi (Nicholas and Nason, 1954a; 1954b; Garrett and Nason, 1969; McDonald and Coddington, 1974; Guerrero and Gutierrez, 1977; Renosto *et al.*, 1981; Horner, 1983), algae (Solomonson, 1975; Solomonson *et al.*, 1975; Manzano *et al.*, 1976; Gewitz *et al.*, 1981; Howard and Solomonson, 1982; Schlee *et al.*, 1985), and in higher plants (Notton *et al.*, 1977; Campbell and Smarelli, 1978; Lewis *et al.*, 1982; Oji *et al.*, 1982; Nakagawa *et al.*, 1985; Streit *et al.*, 1985).

Nitrate reductase from *S. sclerotiorum* studied in this thesis has a molecular weight of 210 kDa and is composed of two subunits with a molecular weight of 107 and 123 kDa. These results are comparable with subunits of 115 and 130 kDa for an enzyme of 230 kDa purified from *N. crassa* (Pan and Nason, 1978), subunits of 97 and 98 kDa for an enzyme of 199 kDa in *P. chrysogenum* (Renosto *et al.*, 1981), the enzyme from *R. glutinis* with a molecular weight of 230 kDa which is composed of two identical subunits of 118 kDa (Guerrero and Gutierrez, 1977) and the enzyme from *Spinacia oleracea* (molecular weight of 270 kDa) with subunits of 110 to 120 kDa (Nakagawa *et al.*, 1985). The molecular weight of nitrate reductase from eukaryotic organisms calculated by a variety of techniques including sucrose density gradient and gel filtration is in the range 160 to 520 kDa (reviewed by Hewitt and Notton, 1980). Nitrate reductases from fungi,

green algae and higher plants are usually composed of subunits which vary in number and size. Nitrate reductases from *N. crassa*, *P. chrysogenum*, *Asp. nidulans* and *C. utilis* have dissimilar size subunits (Sims *et al.*, 1968; Pan and Nason, 1978; Renosto *et al.*, 1981; Minagawa and Yoshimoto, 1982) whereas the enzyme from *R. glutinis* and *Sp. oleracea* have two identical subunits (Guerrero and Gutierrez, 1977; Nakagawa *et al.*, 1985).

In the present study the NADPH-dependent and MVH-linked nitrate reductase activities from *S. sclerotiorum* had a pH optima around 7.1 and 7.5, respectively (Fig. 2), in agreement with results for these activities in various microorganisms. The NAD(P)H-dependent nitrate reductase activities from *Thiobacillus denitrificans*, *R. glutinis* and *Asp. nidulans* have pH optima between 7 and 8 (Sawhney and Nicholas, 1977; Guerrero and Gutierrez, 1977; Minagawa and Yoshimoto, 1982). The pH optima of the MVH-linked reaction in *Rhizobium japonicum* and *R. glutinis* respectively, were 7.0 and 7.5 (Lowe and Evans, 1964; Guerrero and Gutierrez, 1977). In contrast, a very high pH value of 10.5 for maximum activity of the MVH-dependent nitrate reductase was recorded for the purified enzyme from *Anacystis nidulans* (Manzano *et al.*, 1976).

The results reported herein show that both NADPH and NADH serve as electron donors for nitrate reductase activity in *S. sclerotiorum* but NADPH was more effective than NADH; in contrast to nitrate reductases from *Ustilago maydis* (Lewis and Fincham, 1970), *Ch. vulgaris* (Solomonson and Vennesland, 1972) and from higher plants (Dunn-Coleman *et al.*, 1984) where the reverse was found. The preference for NADPH over NADH as a reductant for enzyme activity has been reported for *N. crassa* (Nason and Evans, 1953; Garrett and Nason, 1969), *Asp. nidulans* (Cove and Coddington, 1965;

Minagawa and Yoshimoto, 1982), *P. chrysogenum* (Renosto *et al.*, 1981) and *R. glutinis* (Guerrero and Gutierrez, 1977).

Dithionite-reduced methyl viologen was the most effective electron donor for the enzyme from *S. sclerotiorum* (Table 2). The maximal MVH-linked activity was not dependent on exogenous FAD, in agreement with the results for the enzyme from *Asp. nidulans* and *R. glutinis* (Guerrero and Gutierrez, 1977; Minagawa and Yoshimoto, 1982). Assimilatory nitrate reductases from *N. crassa*, *T. nitratophila* and *P. chrysogenum* have also been shown to utilize reduced viologen dyes as reductants (Garrett and Nason, 1969; Rivas *et al.*, 1973; Amy *et al.*, 1977; Pan and Nason, 1978; Renosto *et al.*, 1981). In the present work, dithionite-reduced flavin nucleotides functioned as electron donors for nitrate reduction; $FMNH_2$ was more effective than $FADH_2$. The results confirm those recorded for *N. crassa*(Garrett and Nason, 1969), *P. chrysogenum*(Renosto *et al.*, 1982), *R. glutinis* (Guerrero and Gutierrez, 1977) and *Acinetobacter calcoaceticus* (Villalobo *et al.*, 1977). Although the reduced flavin nucleotides appear to be generally effective as reductants, its physiologically availability in cells is uncertain (Schrader *et al.*, 1968; Amy *et al.*, 1977; Pan and Nason, 1978).

Unlike the MVH-linked activity, when either NADPH or NADH was the electron donor then FAD was required for maximal activity. This requirement for FAD in the NAD(P)H-dependent nitrate reductase from *S. sclerotiorum* (Table 2) is similar to that reported for *Asp. nidulans* (Minagawa and Yoshimoto, 1982) and *T. nitratophila* (Rivas *et al.*, 1973). This requirement results from a dissociation of FAD from the enzyme protein during purification. The flavin (FAD) was also readily dissociated during purification of the enzyme from *N. crassa* (Nicholas and Nason, 1954a;

Garrett and Nason, 1967). Crude extracts or slightly purified preparations of the spinach enzyme were not activated by added FAD (Hewitt and Notton, 1980). In the present work, FAD was present in the purified enzyme from *S. sclerotiorum*, so that the flavin component was not completely dissociated during purification. A flavin component was also found in purified nitrate reductases from *Asp. nidulans* (McDonald and Coddington, 1974; Minagawa and Yoshimoto, 1982) and *Ch. vulgaris* (Solomonson *et al.*, 1975). As reported herein, the NADPH-dependent nitrate reductase assay in *S. sclerotiorum* showed no activity when FAD was replaced by FMN, in contrast to the enzyme from *N. crassa*, *Asp. nidulans* and *P. chrysogenum* where FMN to varying degrees substituted for FAD (Nason and Evans, 1953; Nicholas and Nason, 1954a; Cove and Coddington, 1965; Renosto *et al.*, 1981; 1982).

Kinetic data for nitrate reductase from *S. sclerotiorum* reported herein indicate that the K_m values for nitrate (33 µM) and for NADPH (40 µM) are comparable with those for the enzyme from *Asp.nidulans*, 60 and 10 µM, respectively (McDonald and Coddington, 1974), *N. crassa*, 200 and 62 µM (Garrett and Nason, 1969), *R. glutinis*, 125 and 20 µM (Guerrero and Gutierrez, 1977) and *Cyan. caldarium*, 84 and 37 µM (Rigano and Aliotta, 1975). Similarly the K_m value for FAD (0.2 µM) in the NADPH-dependent reaction is in agreement with that for the enzyme from *P. chrysogenum* namely 0.17 µM (Renosto *et al.*, 1982). The K_m values for nitrate of the MVH-, $FMNH_2$- and $FADH_2$-linked nitrate reductase activity at 1700, 150 and 71 µM respectively, were markedly higher than that of the NADPH-dependent activity. These results indicate that NADPH functions as an electron donor under physiological conditions.

Nitrate reductases from microorganisms are inhibited by various

compounds. Inhibitions of either NADPH-dependent or MVH-linked nitrate reductase activity in *S. sclerotiorum* by azide and cyanide (Tables 3 and 4) is in agreement with the results for *N. crassa*, *R. glutinis* and *Asp. nidulans* (Nicholas and Nason, 1954a; McDonald and Coddington, 1974; Guerrero and Gutierrez, 1977; Pan and Nason, 1978; Minagawa and Yoshimoto, 1982). The inhibitory effects of these compounds on enzyme activity reported herein were similar when either NADPH or MVH was used as the electron donor, indicating that the primary site of action of metal binding agents is probably molybdenum, as shown by Nicholas and Nason (1954b) and McDonald and Coddington (1974).

Sulphydryl-group inhibitors such as p-CMB and NEM restricted the enzyme activity (Tables 4 and 5). The extent of inhibition of the NADPH-dependent activity by p-CMB was similar to that of azide, but it was less for the MVH-linked reaction. Amytal and rotenone also restricted the NADPH-dependent nitrate reductase activity in *S. sclerotiorum*. Either sulphydryl-group or flavin inhibitors showed less inhibition with MVH than with NADPH as an electron donor. This confirms the results for the purified enzyme from *N. crassa* (Nicholas and Nason, 1954a; Garrett and Nason, 1969; Pan and Nason, 1978) where electrons are transferred from MVH directly to molybdenum whereas NADPH donates reducing equivalents first to FAD. The involvement of sulphydryl-group(s) in the transfer of electron from NADPH to flavin component is in agreement with the data for *N. crassa* (Nicholas and Nason, 1954a; Amy *et al.*, 1977).

The results presented in this thesis show that chlorate and bromate are competitive inhibitors of nitrate reductase activity with respect to nitrate (Fig. 8), in agreement with the results for *Asp. nidulans* and *R. glutinis*. McDonald and Coddington (1974) found that chlorate was

bound to nitrate reductase from *Asp. nidulans* at the same site as nitrate. Similarly, chlorate functioned as an electron acceptor for the nitrate reductase from *R. glutinis* (Guerrero and Gutierrez, 1977). However, since the K_i values for chlorate and bromate (18.1 and 15.6 mM, respectively) were about 10 times higher than the K_m value for nitrate, it is unlikely that either chlorate or bromate would inhibit nitrate reduction *in vivo*.

As reported herein, nitrite, the product of nitrate reduction, also inhibited NADPH-dependent reduction of nitrate competitively (Fig. 11). In *S. sclerotiorum* the K_i value for nitrite (90 µM) was 3-fold greater than the K_m value for nitrate, in agreement with the data for *Chlorella* enzyme (Solomonson and Vennesland, 1972) indicating that nitrite is unlikely to inhibit the enzyme *in vivo*. However, the K_i values for nitrite determined in *R. glutinis* (Guerrero and Gutierrez, 1977) and *Asp. nidulans* (McDonald and Coddington, 1974) were similar to the K_m value for nitrate so that nitrite might well inhibit nitrate reductase activity *in vivo*.

4.2 Nitrite reductase

The second enzyme in the nitrate assimilation pathway is nitrite reductase which has been purified and characterized from various microorganisms (Nason *et al.*, 1954; Medina and Nicholas, 1957; Nicholas *et al.*, 1960; Rivas *et al.*, 1973; Vega *et al.*, 1973; 1975; Lafferty and Garrett, 1974; Garrett and Amy, 1978; Greenbaum *et al.*, 1978; Prodouz and Garrett, 1981).

The 54-fold purified nitrite reductase from *S. sclerotiorum* reported herein has a specific activity up to 5 times greater than that reported by Nicholas *et al.* (1960) for their 50-fold purified enzyme from *N. crassa*.

The higher specific activity may be a result of including chelating and sulphydryl-protecting agents and FAD in the preparation buffers. The omission of any one of those compounds from the preparation buffers resulted in a low nitrite reductase activity in crude extracts. The enzyme from *S. sclerotiorum* is similar to nitrite reductases from other non-photosynthetic organisms in its instability *in vitro* (Kemp and Atkinson, 1966; Lafferty and Garrett, 1974).

The results presented in this thesis show that NAD(P)H-hydroxylamine reductase was associated with the purified NAD(P)H-nitrite reductase. The activity ratios of nitrite reductase to hydroxylamine reductase were constant during purification (Table 6), in agreement with the data for *N. crassa* (Lafferty and Garrett, 1974). The results indicate that both nitrite- and hydroxylamine-reductases are associated with the same enzyme rather than separate enzyme proteins. The assimilatory NAD(P)H-nitrite reductase from *S. sclerotiorum* also had an associated NAD(P)H-diaphorase activity which utilize either ferricyanide, DCPIP or cytochrome (horse heart) as an electron acceptor (Table 8). The results are similar to those reported for *N. crassa* (Vega, 1976).

The NAD(P)H-dependent nitrite and hydroxylamine reductases activities reported herein were active over a pH range of 6.5 to 7.5 with a maximum at 7.0 (Fig. 12). These results are comparable to the pH optimum for the enzyme activity from *N. crassa* (Nicholas *et al.*, 1960; Lafferty and Garrett, 1974).

In the present study, the results of experiments on the stoichiometry of the nitrite reduction (Table 9) essentially confirm previous observations with *E. coli*, *Az. chroococcum*, *T. nitratophila* and *N. crassa* (Kemp and Atkinson, 1966; Vega *et al.*, 1973; Rivas *et al.*, 1973; Lafferty

and Garrett, 1974; Greenbaum *et al.*, 1978). The ratio of NADPH oxidized to nitrite reduced and ammonia formed in the NADPH-dependent reaction was 3:1:1, as expected for a six-electron transfer reaction (Lafferty and and Garrett, 1974). Hydroxylamine could replace nitrite in the enzyme reaction *in vitro*, also forming ammonia as the product. Thus the ratio of NADPH oxidized to ammonia produced in the hydroxylamine reductase-mediated reaction was 1:1, in agreement with the data for the enzyme from *N. crassa* (Greenbaum *et al.*, 1978).

Nitrite reductase from *S. sclerotiorum* utilized NADPH as the most effective electron donor whereas NADH functioned to a lesser extent (56% of that with NADPH), in agreement with the enzyme from *Asp. nidulans* and *N. crassa* (Pateman *et al.*, 1967; Lafferty and Garrett, 1974; Vega *et al.*, 1975; Greenbaum *et al.*, 1978; Prodouz and Garrett, 1981). However, Rivas *et al.* (1973) showed that nitrite reductase from *T. nitratophila* used NADPH, but not NADH, as an electron donor. The enzyme from *S. sclerotiorum* also utilized dithionite-reduced viologen dyes as reductants but they were less effective than NADPH, in accord with the results for the enzyme from *N. crassa* and *T. nitratophila* (Rivas *et al.*, 1973; Lafferty and Garrett, 1974). As reported herein flavin nucleotides (FAD and FMN) chemically reduced with dithionite, functioned as reductants in contrast to the results for the enzyme from *T. nitratophila* and *Az. chroococcum* (Rivas *et al.*, 1973; Vega *et al.*, 1973) where $FMNH_2$ and $FADH_2$ were ineffective electron donors.

The maximal activities *in vitro* of the NAD(P)H-dependent nitrite-, hydroxylamine- and cytochrome *c*-reductases from *S. sclerotiorum* were obtained when FAD was added to the reaction mixture (Table 8), in agreement

with results for these enzyme activities in *N. crassa* (Nason *et al.*, 1954; Medina and Nicholas, 1957; Nicholas *et al.*, 1960; Lafferty and Garrett, 1974; Vega, 1976; Garrett and Amy, 1977). In nitrite reductase from *N. crassa* FMN substituted for FAD, but FMN was less effective for the enzyme from *S. sclerotiorum*. In contrast to the results reported herein, FMN added as a cofactor was without effect on the enzyme activity in *T. nitratophila* and *Az. chroococcum* (Rivas *et al.*, 1973; Vega *et al.*, 1973). It is noteworthy that in the NAD(P)H-diaphorase activity of the enzyme from *S. sclerotiorum* using ferricyanide and DCPIP as electron acceptors, neither FAD nor FMN was required for maximal activity.

The results presented in this thesis show that the K_m values of nitrite reductase activity from *S. sclerotiorum* for nitrite, NADPH and FAD respectively, were 17, 40 and 0.11 μM whereas the K_m values for hydroxylamine, NADPH and FAD of hydroxylamine reductase activity were 1670, 40 and 0.11 μM, respectively (Figs 14-16). The results are comparable to those for nitrite reductase from *N. crassa* and *E. coli* (Kemp and Atkinson, 1966; Lafferty and Garrett, 1974). Since the K_m value for hydroxylamine is about 100-fold greater than that for nitrite, the physiological substrate of the enzyme from *S. sclerotiorum* is more likely to be nitrite. Hydroxylamine was not a product of nitrite reductase as was found for the enzyme from *E. coli*, *B. subtilis* and *N. crassa* (Kemp and Atkinson, 1966; Prakash and Sadana, 1972; Lafferty and Garrett, 1974).

Nitrite reductases from microorganisms have been shown to be inhibited by various compounds (Nason *et al.*, 1954; Medina and Nicholas, 1957; Nicholas *et al.*, 1960; Vega *et al.*, 1973; Lafferty and Garrett, 1974). The NADPH-linked enzyme from *S. sclerotiorum* was sensitive to cyanide, arsenite, azide and DIECA (Table 10). The results indicate that a metal

component is essential for the enzyme activity, in agreement with the results for the enzyme from *N. crassa*, *T. nitratophila* and *Az. chroococcum* (Nason *et al.*, 1954; Nicholas *et al.*, 1960; Rivas *et al.*, 1973; Vega *et al.*, 1973). Since cyanide inhibited the enzyme activity competitively with respect to nitrite (Fig. 17), it seems likely that this chelating agent inhibits by reversibly binding at an essential site on the enzyme as suggested by Vega *et al.*, (1973) for the enzyme from *Az. chroococcum*.

The NADPH-dependent nitrite reductase activity was markedly inhibited by p-CMB and this effect was reversed by cysteine (Table 11). This indicates a requirement for sulphydryl-groups for enzyme activity, in agreement with the data for the enzyme from *N. crassa* (Medina and Nicholas, 1957; Nicholas *et al.*, 1960; Lafferty and Garrett, 1974) and *Az. chroococcum* (Vega *et al.*, 1973). Mepacrine, a flavin inhibitor, restricted the NADPH-dependent nitrite reduction and this effect was reversed by adding FAD. Amytal and rotenone also inhibited the enzyme activity. The results indicate the presence of flavin as shown by Nicholas *et al.* (1960) for the ezyme from *N. crassa*.

The inhibition of NADPH-dependent reaction by sulphite was competitive with respect to nitrite (Fig. 18), in agreement with the data for nitrite reductase from *E. coli* and *N. crassa* (Kemp and Atkinson, 1966; Lafferty and Garrett, 1974). In *E. coli* and yeast, sulphite reductase catalyzed the reduction of nitrite and hydroxylamine to ammonia, in addition to reducing sulphite to sulphide (Prabhakararao and Nicholas, 1970; Siegel *et al.*,1982; Janick *et al.*, 1983). The K_m values for sulphite, nitrite and hydroxylamine respectively, were 12, 1500 and 10500 µM for sulphite reductase from *E. coli* (Siegel *et al.*, 1982) and 38,180 and 4500 µM for the enzyme from yeast (Prabhakararao and Nicholas, 1970). The nitrite reductase enzyme from

S. sclerotiorum did not reduce sulphite to sulphide, in agreement with the results for the enzyme from *N. crassa* (Greenbaum *et al.*, 1978). In the present study, the K_i value for sulphite (10.75 mM) is three orders of magnitude more than the K_m value for nitrite (17 μM) indicating that sulphite is unlikely to inhibit nitrite reductase *in vivo*. This confirms that nitrite is the physiological substrate for the enzyme.

The NADPH-dependent nitrite and hydroxylamine reductases from *S. sclerotiorum* were inactivated upon preincubation with NADPH in the presence of FAD (Table 12). When the enzyme was preincubated with $NADP^+$, no inactivation was observed. These results indicate that only the reduced form (NADPH) inactivated the enzyme, in agreement with the results for the enzyme from *N. crassa* (Lafferty and Garrett, 1974; Vega *et al.*, 1975) and *Az. chroococcum* (Vega *et al.*, 1973). The enzyme was protected against inactivation by NADPH by preincubating the enzyme with either nitrite or hydroxylamine. The results are similar to those reported for nitrite reductase from *N. crassa* (Lafferty and Garrett, 1974; Vega *et al.*, 1975), *E. coli* (Kemp and Atkinson, 1966) and *Az. chroococcum* (Vega *et al.*, 1973). Contrary to the results for the enzyme from *S. sclerotiorum*, the activity of the nitrite reductase from *E. coli* was, however, enhanced by preincubation with nitrite (Kemp and Atkinson, 1966). Moreover, the inactivation of the enzyme from *Az. chroococcum* was reversed by the substrate, which differs with the results for nitrite reductase from *S. sclerotiorum*.

4.3 Pathway of ammonia assimilation

The assimilation of ammonia into glutamate in microorganisms and plants is achieved either *via* glutamate dehydrogenase or the glutamine

synthetase/glutamate synthase pathway. In the present study, the incorporation of ^{15}N-labelled $(NH_4)_2SO_4$ into washed felts with and without MSX or azaserine was employed to determine the pathway of ammonia assimilation. Consequently, either of the inhibitors should restrict ammonia assimilation *via* the glutamine synthetase/glutamate synthase pathway, but they have no effect on glutamate dehydrogenase.

The results indicate that the incorporation of ^{15}N-labelled $(NH_4)_2SO_4$ into washed felts of *S. sclerotiorum* was markedly inhibited by MSX (>50%) and to a lesser extent (30%) by azaserine (Table 13). These results contrast to those for *Nitrobacter agilis* where glutamate dehydrogenase was the key enzyme for the assimilation of ammonia (Kumar and Nicholas, 1982). In *S. sclerotiorum* the inhibitory effects were similar for felts grown with either nitrate or ammonia as the sole nitrogen source. The purified glutamine synthetase and glutamate synthase were also inhibited by MSX and azaserine, respectively. Since glutamate dehydrogenase activity was not detected in cell-free preparations, this is further evidence that the glutamine synthetase/glutamate synthase pathway is the main route for the incorporation of ammonia into amino compounds in *S. sclerotiorum*. The synthesis of glutamate also proceeds *via* the glutamine synthetase/glutamate synthase pathway when *Cb. vibrioforme f. thiosulphatophilum* was grown on low concentrations of ammonia up to 30 mM (Khanna and Nicholas, 1983b).

4.3.1 Glutamine synthetase

Glutamine synthetase is a key enzyme for ammonia assimilation in *S. sclerotiorum*. This enzyme has been purified and its properties and regulation studied in many organisms (Shapiro and Stadtman, 1970;

Ginsburg and Stadtman, 1973; Sims *et al.*, 1974; Palacios, 1976; Kleinschmidt and Kleiner, 1978; Bhandari *et al.*, 1983; Mitchell and Magasanik, 1983; Kimura *et al.*, 1984; Kumar and Nicholas, 1984; Beudeker and Tabita, 1985; Wang and Nicholas, 1985).

Purified glutamine synthetase from *S. sclerotiorum* reported in this thesis has a molecular weight of 490 kDa and is composed of 8 identical subunits of 60 kDa in agreement with the results from other microorganisms. Bhandari *et al.* (1983) and Murrell and Dalton (1983) showed that the enzyme from *Rh. japonicum* and *Methylococcus capsulatus* also has subunits with a molecular weight of 60 kDa. Similar results have been reported for the enzyme from other eukaryotic microorganisms (Sims *et al.*, 1974; Palacios, 1976; Lin and Kapoor, 1978; Beudeker and Tabita, 1985) except that glutamine synthetase from *Sac. cerevisiae* contains 10-12 subunits (Mitchell and Magasanik, 1983).

In the present study, two assays namely biosynthetic and γ-glutamyltransferase have been used to determine the properties of glutamine synthetase (Shapiro and Stadtman, 1970). The transferase and biosynthetic reactions in *S. sclerotiorum* were active over a pH range 6.6 - 7.4 with a maximum at 7.0, in agreement with data for the enzyme from other microorganisms (Shapiro and Stadtman, 1970; Deuel and Stadtman, 1970; Sawhney and Nicholas, 1978b; Florencio and Ramos, 1985; Wang and Nicholas, 1985).

Kinetic data for transferase activity of the enzyme from *S. sclerotiorum* show that the K_m values for glutamine (4.5 mM), hydroxylamine (2.2 mM) and ADP 0.14 mM) are lower than those for the enzyme from *An. flos-aqua*, *M. capsulatus*, *Rh. japonicum* and *Cb. vibrioforme* f. *thiosulphatophilum* (McMaster *et al.*, 1980; Murrell and Dalton, 1983; Khanna and Nicholas, 1983b; Bhandari and Nicholas, 1984). In the biosynthetic assay, the K_m

values for glutamate, ammonia and ATP, respectively, were 2.0, 0.6 and 1.25 mM. These results are comparable with those reported for *An. cylindrica* (Sawhney and Nicholas, 1978b) and *Cb. vibrioforme* f. *thiosulphatophilum* (Khanna and Nicholas, 1983b).

The inhibition of transferase activity by glutamate and NH_4Cl, the substrates for the biosynthetic reaction was competitive for glutamine (Fig. 25), in agreement with the results for *An. cylindrica* (Sawhney and Nicholas, 1978b), *Rh. japonicum* (Bhandari *et al.*, 1983 and *Cb. vibrioforme* f. *thiosulphatophilum* (Khanna and Nicholas, 1983b). The results indicate that glutamine interacts with the enzyme, so that its NH_2 group occupies the ammonia binding site while the oxygen binding site, to which glutamate is bound, is required for the attachment of the oxygen atom of glutamine as postulated by Gass and Meister (1970).

In the present study, the biosynthetic reaction required Mg^{2+} for maximum activity whereas transferase activity was Mn^{2+}-dependent (Table 15), in accord with the results for glutamine synthetase from other micro-organisms (Hubbard and Stadtman, 1967; Sawhney and Nicholas, 1978b; Bhandari and Nicholas, 1981; Khanna and Nicholas, 1983b). The requirement for Mn^{2+} and arsenate for enzyme activity indicates that the production of γ-glutamylhydroxamate is catalyzed by transferase type reaction rather than a reaction catalyzed by either aminohydrase (Hubbard and Stadtman, 1967) or glutaminase (Meister, 1974).

Glutamine synthetase from *S. sclerotiorum* reported herein showed a marked dependence on ATP in the Mg^{2+}-dependent biosynthetic activity (Table 16), in agreement with data for *Anabaena* sp (Stacey *et al.*, 1979). This result differs from those reported by Woolfolk *et al.* (1966) and Murrell and Dalton (1983) who showed that the enzyme from *E. coli* and

M. capsulatus, respectively, utilized other nucleotides, especially GTP, in the Mg^{2+}-dependent biosynthetic reaction. However, in *S. sclerotiorum* when Mn^{2+} was used instead of Mg^{2+} in the enzyme assay, then other nucleotide triphosphates functioned more effectively in the biosynthetic reaction, in accord with the enzyme from *B. stearothermophilus* (Hachimori *et al.*, 1974). The transferase activity was maximal with ADP whereas other nucleotide diphosphates had low activities, in agreement with the enzyme from *E. coli* (Woolfolk *et al.*, 1966) and *M. capsulatus* (Murrell and Dalton, 1983).

Purified enzyme from *S. sclerotiorum* was sensitive to various feedback inhibitors (Tables 18 and 19), in accord with the results for the enzyme from other organisms. Thus alanine, glycine and serine inhibited the transferase activity of the *S. sclerotiorum* enzyme as for the enzyme from *An. flos-aqua* (McMaster *et al.*, 1980), *Rhodopseudomonas palustris* (Alef and Zumft, 1981), *Cb. vibrioforme* f. *thiosulphatophilum* (Khanna and Nicholas, 1983b), *Ac. nidulans* (Florencio and Ramos, 1985) and *D. gummosa* (Wang and Nicholas, 1985). In addition cysteine, isoleucine, threonine, phenylalanine, valine, proline and methionine restricted the enzyme from *S. sclerotiorum* in contrast to the enzyme from *Cb. vibrioforme* f. *thiosulphatophilum* (Khanna and Nicholas, 1983b) where the last three amino acids were without effect. The inhibition of biosynthetic activity by tryptophan, histidine, alanine, leucine and lysine is similar to the results for the enzyme from *B. subtilis* (Brown, 1980) and *Ac. nidulans* (Florencio and Ramos, 1985). In contrast the enzyme from *An. cylindrica* and *E. coli* were not affected by lysine (Woolfolk and Stadtman, 1964; Sawhney and Nicholas, 1978a). As with alanine and glycine, lysine is not a direct product of glutamine metabolism (Stadtman *et al.*, 1968), so that

they are derived by transaminase reactions in *S. sclerotiorum*. As reported herein, glutamine synthetase was also inhibited by oxalate, oxalacetate and α-ketoglutarate, in agreement with the enzyme from *B. stearothermophilus* (Hachimori *et al.*, 1974), *Nitrosomonas europaea* (Bhandari and Nicholas, 1981) and *Rh. japonicum* (Bhandari *et al.*, 1983).

In the present study, glutamine synthetase from *S. sclerotiorum* grown with nitrate was partially adenylylated (Table 20), in agreement with the results for a range of bacteria grown on various nitrogen sources (Kleinschmidt and Kleiner, 1978; Khanna and Nicholas, 1983a; Murrell and Dalton, 1983; Bhandari and Nicholas, 1984; Kumar and Nicholas, 1984; Kimura *et al.*, 1984; Masters and Madigan, 1985; Wang and Nicholas, 1985). Since the adenylylated form of glutamine synthetase from *S. sclerotiorum* was deadenylylated by treatment with snake venom phosphodiesterase, the enzyme from this fungus is regulated by adenylylation/deadenylylation system. It is of interest that these eukaryotic cells also have this regulatory mechanism as do prokaryotes.

4.3.2 Glutamate synthase

Glutamate synthase which catalyzes the reductive transfer of the amino group from glutamine to α-ketoglutarate, has been purified and characterized from a range of bacteria (Miller and Stadtman, 1972; Adachi and Suzuki, 1977; Hemmila and Mantsala, 1978; Khanna and Nicholas, 1983b; Wang and Nicholas, 1985), fungi (Roon *et al.*, 1974; Masters and Rowe, 1979; Hummelt and Mora, 1980b) and from higher plants (Boland and Benny, 1977; Wallsgrove *et al.*, 1977; Hirasawa and Tamura, 1984; Suzuki *et al.*, 1984).

The enzyme from *S. sclerotiorum* has a molecular weight of 220 kDa which is in the range of those reported for other microorganisms (Miller

and Stadtman, 1972; Adachi and Suzuki, 1977; Hemmila and Mantsala, 1978; Masters and Rowe, 1979; Hummelt and Mora, 1980b; Yelton and Yoch, 1981; Galvan *et al.*, 1984). As compared with glutamate synthase from *S. sclerotiorum* which has 4 similar subunits of 53.7 kDa, the enzyme from *N. crassa* is composed of a single monomer with a molecular weight of >200 kDa (Hummelt and Mora, 1980b) and the enzyme from *Sac. cerevisiae* consists of two subunits of molecular weight 169 and 61 kDa. However, the enzyme from *E. coli*, *B. megaterium* and *Rhodospirillum rubrum* has molecular weight around 800 kDa and is composed of 8 subunits: 4 large (135 kDa) and 4 small (53 kDa) (Miller and Stadtman, 1972; Hemmila and Mantsala, 1978; Yelton and Yoch, 1981).

Glutamate synthase has a pH optimum around 8 (Fig. 29), in agreement with the data for the enzyme from other microorganisms (Meers *et al.*, 1970; Wang and Nicholas, 1985).

In the present study, the fungal enzyme had a specific requirement for NADPH as the electron donor, for α-ketoglutarate as the amino acceptor and for glutamine as the amino donor (Table 22), in agreement with the results for glutamate synthase from *Tb. thioparus*, *A. aerogenes* and *Sac. cerevisiae* (Tempest *et al.*, 1973; Roon *et al.*, 1974; Adachi and Suzuki, 1977; Masters and Rowe, 1979). However, in *Sac. cerevisiae* and *N. crassa*, NADH was required for maximum activity (Roon *et al.*, 1974; Hummelt and Mora, 1980b). Asparagine and ammonia did not substitute for glutamine in *S. sclerotiorum* as well as in *Tb. thioparus*.

The kinetic results presented herein indicate that the K_m values for glutamine (2.6 mM), α-ketoglutarate (0.35 mM) and NADPH (35 μM) are comparable with that for the enzyme from a range of bacteria (Tempest *et al.*, 1970). However, the results are higher than those for the enzyme

from *Tb. thioparus* (1100, 50 and 3 µM, respectively), *Sac. cerevisiae* (1000, 140 and 26 µM) and *Cb. vibrioforme* f. *thiosulphatophilum* (769, 270 and 13.5 µM) (Roon *et al.*, 1973; Adachi and Suzuki, 1977; Khanna and Nicholas, 1983b).

In the present study, glutamate synthase from *S. sclerotiorum* was inhibited by various amino acids (Table 23) in agreement with the results for a range of bacteria (Adachi and Suzuki, 1977; Khanna and Nicholas, 1983b; Wang and Nicholas, 1985). The enzyme from *S. sclerotiorum* was markedly inhibited by phenylalanine whereas methionine and serine restricted the enzyme from *Cb. vibrioforme* f. *thiosulphatophilum* and *Tb. thioparus* (Adachi and Suzuki, 1977; Khanna and Nicholas, 1983b).

Various metabolites including organic acids and nucleotides also inhibited glutamate synthase activity from *S. sclerotiorum* (Table 24), in agreement with the enzyme from other bacteria (Adachi and Suzuki, 1977; Khanna and Nicholas, 1983b). Thus ATP (among adenine nucleotides) was the strongest inhibitor for the enzyme from *S. sclerotiorum* as well as the enzyme from *Cb. vibrioforme* f. *thiosulphatophilum* and *Tb. thioparus*.

Glutamate synthase from *S. sclerotiorum* is inhibited by several compounds (Tables 25 and 26). Azaserine, an analogue of glutamine, markedly inhibited the enzyme from *S. sclerotiorum*, in agreement with the results for the enzyme from *Rh. japonicum* (Vairinhos *et al.*, 1983), *D. gummosa* (Wang and Nicholas, 1985) and spinach (Hirasawa and Tamura, 1984). The metal chelating agents, O-phenanthroline and α,α'-dipyridyl also restricted enzyme activity as reported by Adachi and Suzuki (1977) for the enzyme from *Tb. thioparus*.

The enzyme from *S. sclerotiorum* was completely inhibited by p-CMB; this effect was reversed by the addition of cysteine. The results indicating the importance of thiol groups for the enzyme activity confirm the results of Adachi and Suzuki (1977) and Hirasawa and Tamura (1984) for the enzyme from *Tb. thioparus* and spinach, respectively.

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