BIOCHEMICAL ASPECTS OF DENITRIFICATION IN *THIOBACILLUS DENITRIFICANS*

by

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PREFACE

Some of the results presented in this thesis have been published in following scientific journals:-

1. Sulphite- and NADH-dependent nitrate reductase from *Thiobacillus denitrificans*
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   J. Gen. Microbiol. 100, 49-58 (1977).

2. Some properties of a nitrate reductase from *Thiobacillus denitrificans*.
   V. Sawhney and D.J.D. Nicholas,
   Proc. Aust. Biochem. Soc. 10, 13 (1977).

3. Sulphide-linked nitrite reductase from *Thiobacillus denitrificans* with cytochrome oxidase activity: Purification and properties.
   V. Sawhney and D.J.D. Nicholas,
   J. Gen. Microbiol. 106, 119-128 (1978).

4. Studies on sulphide-linked nitrite reductase from *Thiobacillus denitrificans*.
   V. Sawhney and D.J.D. Nicholas,
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5. Regulation of NADH-linked nitrate reductase by NAD^+ and 5'-AMP in *Thiobacillus denitrificans*.
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DECLARATION

I hereby declare that the work presented in this thesis has been carried out by myself between January 1975 to December 1977. To the best of my knowledge no material given herein has been submitted in any form for any degree in any university.

The abbreviations for chemicals and symbols in general follow either the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochem. J.* (1966) *101*, 1-7) or the Instructions to Authors for the *Biochemical Journal* (*Biochem J.* (1973) *131*, 1-20).

Chemicals

5'-AMP	adenosine 5'-phosphate
3'-AMP	adenosine 3'-phosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
5'-CMP	cytidine 5'-phosphate
5'-GMP	guanosine 5'-phosphate
5'-TMP	thymidine 5'-phosphate
5'-UMP	uridine 5'-phosphate
CTP	cytidine 5'-triphosphate
GTP	guanosine 5'-triphosphate
UTP	uridine 5'-triphosphate
BVH	benzyl viologen (reduced)
DEAE-cellulose	diethylaminoethyl cellulose
DNase	deoxyribonuclease
DCIP	2,6-dichlorophenol indophenol
FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
GSH	glutathione (reduced)
HQNO	2-heptyl-4-hydroxyquinoline-N-oxide
NAD^+	nicotinamide-adenine dinucleotide, oxidized

NADH	nicotinamide-adenine dinucleotide, reduced
$NADP^+$	nicotinamide-adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
NEM	N-ethylmaleimide
p-CMB	p-chloromercuribenzoic acid
PMS	phenazine methosulphate
SDS	sodium dodecyl sulphate
-SH	sulphydryl
TMPD	N,N,N',N'-tetramethylparaphenylenediamine

Symbols and Units

A	absorbance
A^o	angström
ΔA	change in absorbance
cm	centimetre
E	extinction
E'_o	standard electrode potential at a given pH
g	unit of gravitational field
HCl	hydrochloric acid
hr	hour(s)
kc	kilocycle
kcal	kilocalories
K_m	Michaelis constant
ℓ	litre
M	Molar
mg	milligram
min	minute(s)

ml	millilitre
mmole	millimole
mM	millimolar
mV	millivolts
MVH	methyl viologen (reduced)
N	normal
nm	nanometer
nmole	nanomole
%	per cent
$K_3Fe(CN)_6$	potassium ferricyanide
p.s.i.	pound per square inch
sec	second(s)
µl	microlitre
µmole	micromole
µM	micromolar
U.V.	ultraviolet
V	volts
S10	supernatant fraction left after centrifuging broken cells at 10,000x g for 30 min
S18	supernatant fraction obtained after centrifuging disrupted cells at 18,000x g for 45 min
S100 and P100	supernatant and pellet fractions, respectively, obtained after centrifuging S10 or S18 at 100,000x g for 90 or 120 min

Temperatures are expressed as degrees Centigrade (^{o})

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SUMMARY

1. This thesis embodies results of an investigation on some biochemical aspects of denitrification in a chemolithotrophic bacterium, *Thiobacillus denitrificans*, grown under anaerobic conditions.

2. In this bacterium nitrate reductase, nitrite reductase and O_2 uptake were all associated with membrane fractions and their activities were coupled to the oxidation of inorganic sulphur compounds.

3. In the pellet fraction (P100), nitrate reductase utilized either sulphite or NADH as a reductant. Both the sulphite- and NADH-linked nitrate reductase activities were affected by inhibitors of the respiratory chain. Reducing equivalents from either sulphite or NADH were transferred to nitrate *via* an electron transfer system consisting of flavins, quinones and cytochromes *b* and c_{554}.

4. A sulphite-dependent nitrate reductase was solubilized with sodium deoxycholate. The NADH-dependent activity which was lost after this treatment was restored on adding menadione.

5. A requirement for cytochrome c_{554} for nitrate reduction was established from the spectral changes for this cytochrome in the P100 fraction on reduction with either sulphite or NADH and its reoxidation with nitrate. A cytochrome c_{554} and FAD were isolated from the solubilized nitrate reductase. This cytochrome had a molecular

weight of 13,000 daltons, a redox-potential of +.223 V at pH 7.0 and it functioned as an effective electron donor for nitrate reduction.

6. Data are presented for the involvement of a common respiratory chain for the reduction of nitrate and the uptake of O_2. A scheme is presented for the transfer of electrons from sulphite and NADH to both nitrate and O_2.

7. The NADH-linked nitrate reductase was inhibited by NAD^+ and 5'-AMP and their effects were reversed with menadione. The inhibition of NADH-linked nitrate reductase by NAD^+ was dependent on the NAD^+/NADH ratio. The positive cooperative influence of NADH on enzyme activity was increasingly suppressed by higher concentrations of NAD^+. It is proposed that NAD^+ and 5'-AMP regulate the NADH-linked nitrate reductase activity to enable the bacterium to conserve NADH for various anabolic reactions.

8. Nitrite reductase activity in this bacterium is linked to the oxidation of sulphide. The nitrite reductase was dissociated from cell membranes in acetone-treated cells. The solubilized enzyme was purified twenty-fold. Sulphide-linked O_2 uptake was detected in the P100 fraction as well as in the purified enzyme.

9. Spectral studies of the P100 fraction and the purified enzyme confirmed the involvement of cytochromes *c* and *d* for the reduction of nitrite and for O_2 uptake. Neither the reduction of nitrite nor the uptake of O_2 was affected by the inhibitors of the electron transport chain.

10. In the purified nitrite reductase, cytochromes *c* and *d* were present in a molar ratio of 1:1. The sequence of electron transfer from sulphide to nitrite *via* the cytochrome *cd* complex was established. The redox-potential of cytochrome *d* was +22 mV more positive than that of cytochrome *c*.

11. Cytochromes c_{551} and c_{554} were isolated from this bacterium. Cytochrome c_{551} stimulated both nitrite reductase and O_2 uptake by the purified enzyme whereas either cytochrome c_{554} from *T. denitrificans* or mammalian cytochrome *c* was without effect. Reduced cytochrome c_{551} functioned as an electron donor for the reduction of nitrite and O_2 in the purified enzyme. It is proposed that this cytochrome functions in the sequence of electron transfer from sulphide to nitrite and O_2.

12. NO and N_2O were identified as the products of the purified nitrite reductase.

13. An absorption band at 573 nm which appeared during the oxidation of the sulphide-linked nitrite reductase, was identified as a complex between NO and the enzyme. The effects of NO on the spectra of the purified enzyme were also determined.

14. Cytochromes *c* and haem *d* were dissociated from the purified nitrite reductase by SDS treatment. These components were separated by chromatography on a Sephadex G-100 column. Spectral studies were made on the dissociated cytochrome *c* and haem *d*.

15. On the basis of these investigations an integrated scheme of electron transfer for the reduction of nitrate and nitrite, coupled to the oxidation of sulphite and sulphide respectively, is presented.

1. INTRODUCTION

The generation of metabolically utilizable energy, required for the diverse anabolic reactions, is of fundamental importance for the survival and growth of every living organism. Different groups of bacteria, the simplest prokaryotic microorganisms, fulfil their energy requirements through a number of diverse and varied mechanisms. Depending on their primary source of energy and their mode of life, bacteria have been classified as chemolithotrophs, chemoorganotrophs, photolithotrophs and photoorganotrophs (Rittenberg, 1969; Kelly, 1971; Doelle, 1975). Among these various groups, chemolithotrophs and photolithotrophs are autotrophic organisms because they are capable of fixing CO_2 and thus do not rely on any organic source of carbon. In contrast, chemoorganotrophs and photoorganotrophs are heterotrophs and depend on an exogenous supply of carbon compounds, which are more reduced than CO_2, for their growth and development.

1.1 Chemolithotrophic bacteria

Chemolithotrophs are unique since they derive their energy needs via the oxidation of simple inorganic compounds. The occurrence of chemolithotrophic bacteria was first reported by Winogradsky in 1890. Since then several microorganisms belonging to this group have been identified. These autotrophic bacteria incorporate CO_2 into their cellular constituents and are able to thrive on a simple inorganic salt medium containing an appropriate oxidizable substrate, e.g. reduced sulphur compounds for *Thiobacilli* (Kelly, 1968; Hutchinson *et al.*, 1969; Roy and Trudinger, 1970), ammonium for *Nitrosomonas* (Peck, 1968; Wallace and Nicholas, 1969; Aleem, 1970) and ferrous iron

for *Ferrobacillus ferrooxidans* (Schlegel, 1960) and *Thiobacillus ferrooxidans* (Peck, 1968). The physiology and energy metabolism of these bacteria have been extensively reviewed by Peck (1968), Rittenberg (1969), Kelly (1971) and Aleem (1970, 1977).

1.1.1 Sulphur oxidizing bacteria - *Thiobacilli*

Thiobacilli, a group of sulphur oxidizing bacteria, were first discovered by Nathansohn (1902). These rod-shaped, motile, non-sporulating, gram-negative bacteria are found in soil, sea and fresh water. The genus thiobacillus includes *T. novellus* (Vishniac and Santer, 1957), *T. perometabolis* (London and Rittenberg, 1967), *T. ferrooxidans* (Tabita and Lundgren, 1971; Shafia *et al.*, 1972) and *T. denitrificans* (Taylor *et al.*, 1971; Taylor and Hoare, 1971). The physiology and various biochemical aspects of thiobacilli have been reviewed by Vishniac and Santer (1957), Lees (1960, 1962), Peck (1962) and Kelly (1968, 1971).

Thiobacilli are capable of oxidizing a number of reduced sulphur compounds including elemental sulphur, sulphide, sulphite, thiosulphate and tetrathionate into sulphate (Roy and Trudinger, 1970). The mechanisms involved in the oxidation of inorganic sulphur compounds have been reviewed by Peck (1968), Trudinger (1967, 1969), Roy and Trudinger (1970) and Aleem (1975).

Under natural conditions the usual substrate for their growth is either elemental sulphur or thiosulphate, the latter being preferred. Two pathways have been proposed for the oxidation of thiosulphate to sulphate in thiobacilli. Thiosulphate is either oxidized to tetrathionate which is converted, *via* polythionate, to sulphate (Nathansohn, 1902; Vishniac, 1952; London and Rittenberg, 1964;

Pankhurst, 1964) or as proposed by Peck (1962, 1968), it is first cleaved into two one-sulphur moieties which are then oxidized to sulphate. Cell-free extracts of *T. thiooxidans* (Tano *et al.*, 1968) and *T. concretivorus* (Moriarty, 1969) were reported to oxidize thiosulphate to trithionate and tetrathionate. However, in *T. denitrificans*, thiosulphate was metabolized to sulphide, polysulphide, tetrathionate and sulphite. In the presence of GSH, thiosulphate was mainly oxidized to sulphite. It was also shown that on replacing GSH with nitrate, sulphide and sulphite were the initial products of thiosulphate oxidation and these were converted into tetrathionate and sulphate (Aminuddin, 1974). Peeters and Aleem (1970) reported that thiosulphate oxidation in *T. denitrificans* was linked to nitrate reduction. They showed that under anaerobic conditions, cytochromes *b*, *c*, *a* and *o*, reduced by thiosulphate were reoxidized by nitrate.

The oxidation of sulphide to sulphate, which involves a release of eight electrons, is linked to phosphorylation through the electron transport chain. In *T. thioparus* (Vishniac and Santer, 1957; London and Rittenberg, 1964) and *T. thiooxidans* (London and Rittenberg, 1964), apart from sulphate, thiosulphate and polythionates were identified as the other products of sulphide oxidation. It is proposed that the initial product during the oxidation of sulphide is a polysulphide (Trudinger, 1967, 1969; Aminuddin and Nicholas, 1973; Saxena and Aleem, 1973). Aminuddin and Nicholas (1973) also showed that under anaerobic conditions, in the presence of nitrate, sulphide was oxidized to sulphate with concomitant reduction of nitrate to nitrite. However, in the presence of nitrite, the final product was only polysulphide.

Sulphite, the other product of thiosulphate cleavage, is oxidized *via* either an AMP-dependent APS reductase (Peck, 1961; Peck *et al.*, 1965; Bowen *et al.*, 1966; Hemfling *et al.*, 1967; Lyric and Suzuki, 1970B; Aminuddin and Nicholas, 1974A) or by an AMP-independent sulphite oxidase (Adair, 1966; Charles and Suzuki, 1966; Kodama and Mori, 1968; Moriarty and Nicholas, 1969; Lyric and Suzuki, 1970A). APS reductase catalyses the production of adenosine-5'-sulphatophosphate (APS) from sulphite and AMP. APS produced in this reaction subsequently generates ATP through the combined action of ADP-sulphurylase and adenylate kinase (Peck, 1960, 1962). In *T. denitrificans*, the purified enzyme utilized ferricyanide or O_2 as an electron acceptor (Bowen *et al.*, 1965). The other sulphite oxidizing enzyme, viz. sulphite oxidase does not require AMP for its activity. In *T. novellus* (Charles and Suzuki, 1966) and in *T. thioparus* (Lyric and Suzuki, 1970A), sulphite oxidase, which functioned with either ferricyanide or cytochrome *c* as an electron acceptor, is a soluble enzyme. However, sulphite oxidase from *T. thiooxidans* (Adair, 1966; Kodama and Mori, 1968), *T. concretivorus* (Moriarty, 1969) and *T. denitrificans* (Aminuddin and Nicholas, 1974A) was shown to be associated with cell-membranes. This particulate sulphite oxidase from *T. denitrificans* used either nitrate, O_2 or ferricyanide as an electron acceptor but the purified enzyme functioned only with ferricyanide (Aminuddin and Nicholas, 1974A).

Thiobacilli are mainly strict aerobic bacteria requiring O_2 as a terminal oxidant for cell respiration. The notable exception is *T. denitrificans* which can respire even in the absence of air by using nitrate as an alternate terminal electron acceptor. Several other

obligate or facultative anaerobes are known to thrive in the absence of O_2 by coupling their respiration to the reduction of inorganic compounds. The various compounds that can substitute for O_2 are: sulphate, as in *Desulphovibrio* and *Desulphotomaculum* (Postgate, 1965), nitrate as in the denitrifying bacteria (Payne, 1973) and CO_2 as in *Methanobacterium* (Stadtman, 1967). The mean energy yields for the transfer of a molar equivalent of electrons from organic compounds to these terminal electron acceptors (Payne, 1973), are presented in Table 1.

It is evident that the energy yield with nitrate as the oxidant is significantly higher as compared to the other inorganic compounds and its evolutionary advantage is reflected by the relatively larger number of nitrate reducers than those which utilize either sulphate or CO_2 as the terminal oxidant (Payne, 1973).

Among thiobacilli, only *T. denitrificans* can grow under anaerobic conditions with nitrate as the alternate terminal electron acceptor. Since the products of this nitrate-dependent respiration are nitrogenous gases, it has been classified as a denitrifying bacterium (Beijernick, 1904; Baalsrud and Baalsrud, 1954; Ishaque and Aleem, 1973). Although the denitrification process results in a net loss of nitrogen from the soil, it plays an important role in the nitrogen cycle in nature by maintaining the dinitrogen level in the biosphere at a steady level (Alexander, 1971).

1.2 Denitrification

Nitrate is utilized by microorganisms in two distinct ways: (a) assimilation, whereby nitrate is reduced to ammonia and incorporated

Table 1. Free-energy production with various compounds as terminal electron acceptors in bacteria (adapted from Payne, 1973).

Terminal electron acceptor	ΔF (kCal/mole)	Reference
O_2	-26.5	Payne (1970)
NO_3^-	-18.0	McCarty (1972)
SO_4^{2-}	-3.4	McCarty (1972)
CO_2	-2.4	McCarty (1972)

into cellular nitrogen compounds and (b) dissimilation, whereby nitrate serves as an alternative electron acceptor to O_2 and is reduced to nitrite. The term denitrification is used when nitrogenous gases are produced. Nitrate may be dissimilated either to nitrite only, as in *Escherichia coli* (Taniguchi *et al.*, 1956) and *Hemophilus parainfluenzae* (White, 1962) or reduced further to N_2 gas through a series of reactions as in *T. denitrificans* (Baalsrud and Baalsrud, 1954; Ishaque and Aleem, 1973). The microbial, physiological and biochemical aspects of nitrate respiration have been reviewed by Najjar and Chung (1956), Kluyver (1959), Nason (1962), Payne (1973), Gracia (1975), Delwiche and Bryan (1976), Payne (1976), Stouthamer (1976), Haddock and Jones (1977) and Thauer *et al.* (1977).

The reduction of nitrate to N_2 gas during denitrification is associated with the following changes in the free energy (Thauer *et al.*, 1977).

$$NO_3^- + H_2 \longrightarrow NO_2^- + H_2O \qquad -39Kcal/mol \qquad (1)$$

$$2NO_2^- + 3[H_2] + 2H^+ \longrightarrow N_2 + 4H_2O \qquad -190Kcal/mol \qquad (2)$$

The energy released during the reduction of nitrate to nitrite and nitrite to dinitrogen, is utilized for the production of ATP (Thauer *et al.*, 1977).

It is established that the reduction of nitrate to nitrite is mediated by nitrate reductase. The further reduction of nitrite to nitrogen gas proceeds in a sequence of reactions (3-5) with nitric oxide and nitrous oxide as the intermediates (Payne, 1973).

$$2NO_2^- + H_2 + 2[H^+] \longrightarrow 2NO + 2H_2O \quad -35.1Kcal/mol \quad (3)$$

$$2NO + H_2 \longrightarrow N_2O + H_2O \quad -73.2Kcal/mol \quad (4)$$

$$N_2O + H_2 \longrightarrow N_2 + H_2O \quad -81.6Kcal/mol \quad (5)$$

(Thauer *et al.*, 1977).

Only a limited number of denitrifying bacteria reduce nitrate through to dinitrogen. *Alcaligenes odorans* reduces nitrite but not nitrate to N_2 gas (Chatelain, 1969) while in *Pseudomonas fluorescens* (Greenberg and Becker, 1972) and *Corynebacterium nephridii* (Hart *et al.*, 1965; Renner and Becker, 1970) the final product of the denitrification process is nitrous oxide.

Most of the denitrifiers are non-fermentative bacteria which grow anaerobically only when supplied with nitrate as an alternative electron acceptor to O_2. The only obligate anaerobic bacterium which exhibits denitrification is *Propionibacterium pentosaceum* (Gent-Ruijters *et al.*, 1975).

1.2.1 Reduction of nitrate to nitrite

Nitrate reductase is the first enzyme involved in both assimilatory and dissimilatory nitrate reduction. The assimilatory nitrate reductase is usually a substrate inducible enzyme and is generally located in the soluble fraction of cell (Payne, 1973). The synthesis and activity of this enzyme is repressed by ammonia. Its activity is also inhibited by chlorate and it has been designated a B-type nitrate reductase by Pichinoty (1970). The reduction of nitrate to ammonia, which involves 8-electron transfer, in the assimilatory

pathway is not coupled to phosphorylation (Hadjipetrou and Stouthamer, 1965).

In contrast, the dissimilatory nitrate reductase functions as a respiratory enzyme in bacteria growing under anaerobic conditions. The synthesis and the functioning of this enzyme is regulated by the partial pressure of O_2 and the concentration of nitrate in the environment (Pichinoty and D'Ornano, 1961; Van't Riet *et al.*, 1968). Reduced nitrogenous compounds like ammonia and amino acids do not affect either the enzyme activity or its synthesis. The enzyme reduces chlorate, bromate but not iodate and has been classified by Pichinoty (1964) as type A. Dissimilatory nitrate reductases are generally associated with cell membranes, with the exception of those in *Spirillum itersonii* (Gauthier *et al.*, 1970) and *Clostridium perfringens* (Chiba and Ishimoto, 1973).

Some properties of dissimilatory nitrate reductases from various bacteria are summarized in Table 2. Dissimilatory reduction of nitrate to nitrite is linked to a particulate electron-transport chain consisting of dehydrogenases and electron carriers with nitrate reductase as the terminal enzyme. In many bacteria, NADH serves as an electron donor for nitrate reductase, e.g. *E. coli* (Medina and Heredia, 1958; Iida and Taniguchi, 1959; Itagaki *et al.*, 1962; Cole and Wimpenny, 1968), *Pseudomonas aeruginosa* (Fewson and Nicholas, 1961B), *Pseudomonas denitrificans* (Radcliffe and Nicholas, 1970), *Bacillus stearothermophilus* (Downey, 1966; Kiszkiss and Downey, 1972A), *Aerobacter aerogenes* (Knook *et al.*, 1973), *Proteus mirabilis* (DeGroot and Stouthamer, 1970) and *Micrococcus denitrificans* (Lam and Nicholas, 1969B). However, depending upon the type of organism and also on growth conditions, the reduction of nitrate may also be linked to various

Table 2. Electron donors, electron carriers and products of dissimilatory nitrate reductase in various organisms (Taken from Thauer *et al.*, 1977).

Organism	Electron donor	Electron carrier	Reference
(A) OBLIGATE ANAEROBES			
Clostridium perfringens ($NO_3^- \rightarrow NO_2^-$)	Pyruvate	Ferredoxin	Chiba and Ishimoto (1973, 1977)
Propionibacterium pentosaceum ($NO_3^- \rightarrow N_2$)	NADH, lactate, glycerol-phosphate	Cytochrome *b*	Schwartz (1973), Sone (1974), Gent-Ruijters *et al.* (1975)
Staphylococcus aureus ($NO_3^- \rightarrow NO_2^-$)	Lactate	MK, cytochrome *b*	Chang and Lascelles (1963), Sasarman *et al.* (1974), Burke and Lascelles (1975)
(B) FACULTATIVE ANAEROBES			
Aerobacter aerogenes ($NO_3^- \rightarrow NO_2^-$)	NADH	UQ, cytochrome *b*	Forget and Pichinoty (1964), Knook and Planta (1971B), Van't Riet and Planta (1969, 1975)
Bacillus stearothermophilus ($NO_3^- \rightarrow NO_2^-$)	NADH, succinate	MK, cytochrome *b*	Downey (1962), Schulp and Stouthamer (1970), Kiszkiss and Downey (1972A, B)
Escherichia coli ($NO_3^- \rightarrow NO_2^-$)	NADH, lactate, formate, glycerol-phosphate	UQ, cytochrome *b*	Itagaki *et al.* (1962), Forget (1974) MacGregor *et al.* (1974), Haddock and Kendall-Tobias (1975), Clegg (1976)
Micrococcus denitrificans ($NO_3^- \rightarrow N_2$)	NADH, succinate	UQ, cytochrome *b*	Scholes and Smith (1968), Lam and Nicholas (1969A, B, C), Forget (1971), Sapshead and Wimpenny (1972)
Psuedomonas aeruginosa ($NO_3^- \rightarrow N_2$)	NADH	Cytochrome *b* and *c*	Fewson and Nicholas (1961)
Pseudomonas denitrificans ($NO_3^- \rightarrow N_2$)	Formate, NADH	Cytochrome *b*	Radcliffe and Nicholas (1970)
Proteus mirabilis ($NO_3^- \rightarrow NO_2^-$)	NADH, formate	UQ cytochrome *b*	DeGroot and Stouthamer (1970)
Spirillum itersonii ($NO_3^- \rightarrow NO_2^-$)		Cytochrome *b*	Gauthier *et al.* (1970)
Thiobacillus denitrificans ($NO_3^- \rightarrow N_2$)	Sulphite, sulphide, NADH	Cytochrome *b* and *c*	Baalsrud and Baalsrud (1954), Peeters and Aleem (1970), Adams *et al.* (1971), Aminuddin and Nicholas (1973, 1974B)

Abbreviations : MK, Menaquinone-6 (vitamin K_2); UQ, ubiquinone.

organic acids. Thus, in *E. coli* (Taniguchi and Itagaki, 1960), *P. mirabilis* (DeGroot and Stouthamer, 1970) and *P. denitrificans* (Radcliffe and Nicholas, 1970) formate is an effective electron donor (Table 2). The enzyme from *T. denitrificans* is unique since its activity is coupled to the oxidation of inorganic sulphur compounds (Aubert *et al.*, 1959; Baalsrud and Baalsrud, 1954; Peeters and Aleem, 1970; Adams *et al.*, 1971; Aminuddin and Nicholas, 1974B). Several workers have found that the purified preparations of dissimilatory nitrate reductases from various bacteria do not utilize either NADH or formate but either reduced flavins or dyes are effective electron donors for nitrate reduction (Fewson and Nicholas, 1961B; Lam and Nicholas, 1969B; Radcliffe and Nicholas, 1970; Kiszkiss and Downey, 1972A; Rosso *et al.*, 1973).

In several of the early studies, FAD and FMN were shown to be electron carriers for nitrate reduction in bacteria (Nason, 1962). Recently, however, ubiquinones have also been implicated in nitrate respiration in gram-negative bacteria (Table 2). Extraction of ubiquinones from the membrane fractions of *E. coli* (Itagaki *et al.*, 1962; Itagaki, 1964; Enoch and Lester, 1974) and *A. aerogenes* (Knook and Planta, 1971A, B), resulted in a loss of nitrate reductase activity with the physiological electron donors. In gram-positive bacteria, however, naphthoquinones are probably involved in the functioning of dissimilatory nitrate reductases (Downey, 1962). A requirement of quinone for nitrate respiration was also demonstrated in the menaquinone-deficient mutants of *Staphylococcus aureus* (Sasarman *et al.*, 1974).

All the nitrate respiring bacteria studied so far contain at least one *b*-type cytochrome which participates in nitrate reduction (Najjar and Chung, 1956; Itagaki *et al.*, 1961; Nason, 1963; Downey, 1966; Lam and Nicholas, 1969B; Ruiz-Herrera and DeMoss, 1969;

DeGroot and Stouthamer, 1970; Radcliffe and Nicholas, 1970; Van't Riet *et al.*, 1972; Knook *et al.*, 1973; Stouthamer, 1976). Under anaerobic conditions, the electrons from the respiratory chain are diverted to nitrate at the level of cytochrome *b*. The enhanced production of cytochrome *c* in the anaerobic cultures of nitrate grown *E. coli* and *A. aerogenes* indicated its role in nitrate respiration (Gray *et al.*, 1963; Fujita and Sato, 1966). Further evidence for the participation of cytochrome *c* in nitrate reduction has been obtained in the cell-free preparations of *E. coli* (Gray *et al.*, 1963), *M. denitrificans* (Fewson and Nicholas, 1961A), *P. aeruginosa* (Fewson and Nicholas, 1961B), and *T. denitrificans* (Aubert *et al.*, 1959; Peeters and Aleem, 1970; Aminuddin and Nicholas, 1974B) where nitrate was shown to re-oxidize reduced cytochrome *c*. It has been suggested that in some bacteria different forms of *c*-type cytochrome mediate the transfer of electrons to nitrate and O_2 (Payne, 1973).

Besides the above-mentioned components of the electron transport chain, molybdenum and iron have also been shown to be the constituents of purified nitrate reductase from *E. coli* (Taniguchi and Itagaki, 1960; Forget, 1974; MacGregor *et al.*, 1974), *M. denitrificans* (Forget, 1971) and *M. halodenitrificans* (Rosso *et al.*, 1973). Lam and Nicholas (1969B), using radioactive molybdate had shown earlier that molybdenum was a component of 118-fold purified nitrate reductase from *M. denitrificans*. Labile sulphide has been found in purified enzyme preparations from *E. coli* (Forget, 1974), *M. halodenitrificans* (Rosso *et al.*, 1973) and *M. denitrificans* (Forget, 1971). Forget and Dervartanian (1972) observed that electron spin resonance (ESR) signals due to Mo(V) and Fe(III) were eliminated on reduction of the enzyme and this was accompanied by the appearance of the signals due to Fe(II)-S. Addition of nitrate to the reduced enzyme restored the ESR signals attributed to Mo(V) and Fe(III). These

investigations indicated that molybdenum and iron function as electron carriers in the reduction of nitrate by nitrate reductase.

1.2.2 Regulation of nitrate reductase

Both the activity and synthesis of dissimilatory nitrate reductase is controlled by nitrate and the concentration of O_2 in the growth medium. Although the enzyme is generally nitrate-inducible, anoxia is also important for the onset of the synthesis of the enzyme as reported for *A. aerogenes* (Pichinoty and D'Ornano, 1961), *E. coli* (Showe and DeMoss, 1968), *P. mirabilis* (DeGroot and Stouthamer, 1970), and *Pseudomonas perfectomarinus* (Payne *et al.*, 1971). In accordance with its respiratory function under anaerobic conditions, the reduction of nitrate by dissimilatory nitrate reductase is coupled to the electron transport-dependent oxidative phosphorylation of the cell. A free energy change of -38.5 Kcal per pair of electrons has been calculated for the reduction of nitrate to nitrite (Decker *et al.*, 1970). Thus due to a relatively high redox-potential of NO_3^-/NO_2^- couple (E_o' = +421 mV), the reduction of nitrate with most of the reductants, except succinate, is sufficiently exergonic to allow for the formation of 2 moles of ATP per mole of nitrate reduced (Kröger, 1977). ATP formed in this dissimilatory pathway is utilized for biosynthetic reactions of the cell. Since the principal function of the respiratory process is to furnish metabolic energy, it is conceivable that the cellular respiration, whether linked to O_2 or nitrate, be regulated by the energy status of the organism. Thus in *Mycobacterium tuberculosis*, NADH-oxidase which is linked to oxidative phosphorylation, was stimulated by AMP and this effect was reversed by NAD^+ (Worcel *et al.*, 1965). In *Mycobacterium phlei*, the activity of the respiratory NADH-oxidase was controlled by the levels of NAD^+, AMP and inorganic phosphate (Bogin *et al.*, 1969).

NADH-dehydrogenase from *E. coli* was competitively inhibited by NAD^+ and the extent of inhibition was dependent on the NAD^+/NADH ratio (Dancey and Shapiro, 1976). These investigations indicate that in bacteria the aerobic respiration may be controlled through the energy charge and redox state of the cell. However, no information is available about the factors which might control the rate of nitrate respiration.

In all the chemolithotrophic bacteria, CO_2 fixation supplies their carbon requirements and ATP needed for the assimilation of CO_2 is generated during the oxidation of inorganic substrates *via* the electron transport chain. It has been suggested that the rate of energy generation through the electron transport is controlled by CO_2 and its reductive assimilation (Aleem, 1977). The mechanism for the generation of NADH, the reductant essential for CO_2 fixation in chemolithotrophic bacteria, was elucidated by the pioneering work of Aleem *et al.* (1963). These workers demonstrated that in *Nitrobacter*, *Nitrosomonas* and *Ferrobacillus*, NADH was produced via an ATP-driven reverse flow of electrons through the respiratory chain. Subsequent work with *T. novellus* (Aleem, 1966A, C), *Nitrosomonas europaea* (Aleem, 1966B), *Nitrobacter agilis* (Sewell and Aleem, 1969), *T. denitrificans* (Peeters and Aleem, 1970) and *T. neapolitanus* (Aleem, 1969; Saxena and Aleem, 1972; Roth *et al.*, 1973) has established that this phenomenon is widespread in the chemolithotrophic bacteria. Although the reverse-electron flow for the generation of NADH operates in lithotrophic bacteria, according to Kelly (1971) it is not reasonable to assume that the oxidation of NADH by the normal mode of electron flow does not occur in obligate lithotrophs. In contrast, Smith *et al.* (1967) have proposed that failure to oxidize NADH was the prime cause of obligate lithotrophy. However, several workers have noted the presence of NADH-oxidase activity

in various obligate chemolithotrophs including *T. neopolitanus* (Trudinger and Kelly, 1968), *T. thiooxidans* (Tano and Imai, 1968) and *Nitrobacter winogradskyi* (Gool and Laudelout, 1967).

To summarise, in chemolithotrophic bacteria, NADH is largely produced *via* an ATP-driven reverse electron flow and NADH can also be oxidized by the same electron transport chain. To enable NADH to be conserved for biosynthetic reactions, it is essential that some mechanism should operate to regulate its oxidation *via* the electron transport chain.

1.2.3 Dissimilatory nitrite reductase

The synthesis of dissimilatory nitrite reductase is also controlled by the concentrations of O_2 and nitrate in the growth medium (Delwiche and Bryan, 1976). No exceptional substrates have been reported for the dissimilation of nitrite. Several oxidizable substrates are utilized in culture but *in vitro* this enzyme is active with either NADH or reduced flavins (Najjar and Chung, 1956; Fewson and Nicholas, 1961A; Walker and Nicholas, 1961; Nason, 1962; Lam and Nicholas, 1969C). Nitrite reductase from *P. aeruginosa*, reduced nitrite with reduced forms of either flavin, pyocyanine, methylene blue or 1,4-naphthoquinone but not with NAD(P)H or reduced cytochrome *c* (Walker and Nicholas, 1961). Nitrite reductase from *P. perfectomarinus* utilized either NADH or NADPH for its activity (Payne, 1973). The enzyme from *T. denitrificans* differs from the other dissimilatory nitrite reductases because it functions with sulphide as the reductant (Aminuddin and Nicholas, 1974B).

Nitrite reductase from denitrifying bacteria such as *P. aeruginosa* (Horio *et al.*, 1961A; Walker and Nicholas, 1961; Kuronen and Ellfolk, 1972;

Gudat *et al.*, 1973), *Alcaligenes faecalis* (Iwasaki and Matsubara, 1971) and *M. denitrificans* (Lam and Nicholas, 1969C; Newton, 1969) have been purified and their properties examined. The enzyme from these sources contained two different haem compounds, namely cytochromes *c* and *d*.

A crystalline preparation of nitrite reductase from *P. aeruginosa*, which was previously referred to as cytochrome oxidase (Yamanaka and Okunuki, 1974), has been studied extensively. This enzyme, besides accepting electrons from several reductants, functioned with either reduced cytochrome c_{551} or the reduced copper-containing protein, azurin, as a donor. Both the reductants were isolated from *P. aeruginosa* (Horio, 1958; Horio *et al.*, 1958, 1961A). Nitrite reductase from *M. denitrificans* was also shown to function with reduced cytochrome c_{552} which was isolated from the same organism (Newton, 1969). As in *P. aeruginosa*, the purified nitrite reductase from *M. denitrificans* (Lam and Nicholas, 1969C; Newton, 1969) and *A. faecalis* (Iwasaki and Matsubara, 1971) also exhibited cytochrome oxidase activity. Both the nitrite reductase and cytochrome oxidase activities of the purified enzyme preparations from these organisms were inhibited by cyanide but CO affected only the reaction with O_2. The molecular weight of nitrite reductase from *P. aeruginosa* (Gudat *et al.*, 1973) and *M. denitrificans* (Newton, 1969) was 120,000. Nagata *et al.* (1970) calculated the molecular weight of purified nitrite reductase from *P. aeruginosa* to be 65,000 on the basis of its amino acid composition. The molecular weight of 90,000 was reported for the crystalline enzyme from *A. faecalis* (Iwasaki and Matsubara, 1971).

Investigations by Radcliffe and Nicholas (1968) and Matsubara

and Mori (1968) indicate that nitrite reductase from *P. denitrificans*, which had a molecular weight of 70,000, was distinctly different in structural properties from the enzyme from *P. aeruginosa*. Thus, unlike the enzyme from the latter organism, which has cytochromes *c* and *d* as the prosthetic groups, nitrite reductase from *P. denitrificans* contained cytochrome c_{552} or c_{553} and copper as its prosthetic groups. The enzyme from *Achromobacter cycloclastes*, with a molecular weight of 65,000 was, however, reported to be a copper protein and did not contain either flavins or cytochromes (Iwasaki and Matsubara, 1972). There is no evidence for siroheme as a prosthetic group of dissimilatory nitrite reductases as is usually the case with the assimilatory nitrite reductases (Murphy *et al.*, 1974; Vega *et al.*, 1975).

With most of the purified dissimilatory nitrite reductases studied thus far, nitric oxide has been identified as the product of the reaction (Renner and Becker, 1970; Barbaree and Payne, 1967).

1.2.4 Nitric oxide reductase and nitrous oxide reductase

The further reduction of nitric oxide to nitrous oxide is catalyzed by nitric oxide reductase (Payne *et al.*, 1971; Matsubara and Iwasaki, 1971; Miyata, 1971; Cox and Payne, 1973). Only a limited information is available about the properties of this enzyme. In *P. perfectomarinus* (Cox and Payne, 1973) nitric oxide reductase was reported to be a soluble enzyme whereas in *A. faecalis* (Matsubara and Iwasaki, 1971) and *P. denitrificans* (Miyata *et al.*, 1969), the enzyme was shown to be membrane bound. The purified nitrite reductase from *A. faecalis* exhibited nitric oxide reductase activity as well. However its suggested physiological role was for the reduction of nitrite because of the presence of a separate particulate nitric oxide reductase which had much lower K_m for nitric oxide (Matsubara and Iwasaki, 1972).

Nitrous oxide, the product of nitric oxide reductase, is finally reduced to dinitrogen by nitrous oxide reductase (Matsubara and Iwasaki, 1971; Cox and Payne, 1973; Matsubara, 1970, 1971, 1975). This particulate enzyme is very labile and has not been characterized. *In vitro*, the enzyme functions with reduced form of either viologen dyes or dichlorophenol indophenol but the physiological reductant has not been identified. A possible role of transition metals in this step of denitrification has been indicated by the inhibition of nitrous oxide reductase by carbon monoxide (Matsubara, 1971), cyanide and azide (Matsubara, 1975). In addition, Matsubara (1971, 1975) have suggested the involvement of cytochromes *b* and *c* in the reduction of nitrous oxide. Payne *et al.* (1971) have also observed that in *P. perfectomarinus*, a *c*-type cytochrome was tightly associated with membrane fractions containing nitric oxide reductase.

1.3 Aim of thesis

The objective of these investigations was to examine some biochemical aspects of denitrification in the chemolithotrophic bacterium, *Thiobacillus denitrificans*. Work on the following lines was carried out:-

(1) To compare the properties and determine the relationship between the sulphite- and NADH-linked nitrate reductase activities.

(2) To explore the mechanism by which the utilization of NADH for nitrate reductase is regulated.

(3) To identify the components of electron-transport chain involved in the reduction of nitrate, nitrite and O_2.

(4) To study the properties of the sulphide-linked nitrite reductase.

2. MATERIALS AND METHODS

2.1 Preparation of solutions and buffers

Double glass-distilled water was used to dispense all the reagents used in this work. Sodium sulphite ($Na_2SO_3.9H_2O$), sodium sulphide ($Na_2S.9H_2O$) and NADH were dispensed in 0.05M Tris-HCl buffer (pH 7.5) just prior to use. Crystals of sodium sulphide were washed with distilled water and carefully dried several times with filter paper before weighing.

The stock solutions of 0.1M of Tris-HCl, potassium phosphate and Tris-maleate buffers were prepared by the method of Gomori (1955) and stored at 2°. The pH of all the buffers were adjusted at room temperature ($20-25^{\circ}$) using a Beckman H5 pH meter which was standardised frequently. The pH of Tris-HCl buffer was adjusted at the temperature at which it was to be used subsequently (Sigma Technical Bulletin 106B, 1967).

2.2 Biological materials

2.2.1 Bacterium

Thiobacillus denitrificans (9547, NClB strain AB5) was purchased from the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland.

2.2.2 Enzymes

Alcohol dehydrogenase, snake venom pyrophosphatase and DNase were supplied by Sigma Chemical Co. (St. Louis, Missouri, U.S.A.) while inorganic pyrophosphatase was from Calbiochem (Los Angeles, California, U.S.A.).

2.3 Chemicals and other materials

2.3.1 Nucleotides and marker proteins

ATP, ADP, AMP, NADH, NADPH, NAD^+, $NADP^+$, FMN and FAD were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.) as were the following marker proteins: cytochrome *c* (horse heart, type VI), bovine serum albumin (fraction V), ovalbumin (grade V), γ-globulin and myoglobin.

2.3.2 Other chemicals

Rotenone, amytal, 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), antimycin A, p-chloromercuribenzoic acid (p-CMB), phenazine methosulphate (PMS), glutathione (reduced) and Tris[hydroxymethyl]aminomethane (Tris) were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.); sodium diethyldithiocarbamate (DIECA) was from Merck (Darmstadt, Germany). Bathocuproin and blue dextran were from Fluka (Buchs, Switzerland) and Pharmacia Fine Chemicals (Uppsala, Sweden) respectively. Atabrine was from Imperial Chemical Industries (Marlston, South Australia). ^{15}N-nitrite (27 atom % excess) was obtained from Office Industrial De L'Azote, France.

All the other chemicals were of the highest purity available.

2.3.3 Chromatographic materials

Whatman DEAE-cellulose (type 32) and Whatman 3MM paper were supplied by H. Reeve Angel, London EC4 and Sephadex G-25, G-100, G-150 and Sepharose 6B were from Pharmacia Fine Chemicals (Uppsala, Sweden).

2.4 Culture of the bacterium

2.4.1 Growth of the organism

Thiobacillus denitrificans was grown anaerobically in a medium described by Adams *et al.* (1971). The composition of the medium used is given in Table 3. Batch cultures of the organism were grown at 28° in 55ℓ carboys. The pH of the culture medium during growth was maintained at 7.0 by an automatic titration with sterile 20% (w/v) K_2CO_3 by means of a pH stat unit (Radiometer, Titrator 11 pH meter 8, Copenhagen, Denmark). Optimum growth was obtained within 48-60 hr.

2.4.2 Maintenance of the culture

T. denitrificans, maintained in a liquid culture described in Table 3, was sub-cultured three times a week. Microscopic examination and plating of the culture were carried out periodically to check that there were no contaminants present.

2.4.3 Harvesting

The bacteria were harvested in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous flow rotor (SS-34). The cells were washed twice with cold 0.05M Tris-HCl buffer (pH 7.5) and when not used immediately, were stored at -15°.

2.5 Enzyme techniques

2.5.1 Preparation of acetone-dried cells

The washed cells, suspended in 0.025M Tris-HCl buffer (pH 7.5) (500 mg wet weight/ml), were added slowly to ten volumes of cold acetone at -15° with continuous stirring. After 10 min, the mixture was quickly filtered through a Büchner funnel, fitted with Whatman No. 41

Table 3. Composition of the culture solution for *T. denitrificans*.

The solution of KH_2PO_4 (200 gm/l) was adjusted to pH 6.5 with KOH, autoclaved at 17 lb/in^2 for 30 min, cooled and then added at the rate of 10 ml/l of the culture medium. The following salts: $Na_2S_2O_3.5H_2O$, KNO_3, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ and NH_4Cl were dissolved and autoclaved together; $FeSO_4$ and $KHCO_3$ were sterilized separately. Appropriate amounts of the stock solutions of these three fractions were combined after cooling and made up to the required volume with sterile double-distilled water just before inoculation.

100 ml of fresh cell-suspension (0.5%, w/v) was used for inoculating each litre of the medium.

Chemical	Concentration (g/l of culture solution)
$Na_2S_2O_3.5H_2O$	5.0
KOH	0.2
$KHCO_3$	1.0
KNO_3	2.0
NH_4Cl	0.5
$MgSO_4.7H_2O$	0.5
$FeSO_4$	0.01
$CaCl_2.2H_2O$	0.1
KH_2PO_4	2.0

filter paper. The temperature during filtration was maintained below 0^{o} by packing an ice-salt mixture around the Büchner funnel. The cells, after washing twice with three volumes of cold acetone, were dried under a stream of N_2 gas at 4^{o}. They were then dried completely under vacuum in a dessicator over 36N H_2SO_4 and stored under vacuum at 4^{o} over NaOH pellets.

2.5.2 Preparation of homogenates and cell-free extracts

All the operations described in this Section were carried out at 4^{o}.

2.5.2.1 Cell-free extracts of fresh cells

The washed cells, suspended (25%, w/v) in 0.05M Tris-HCl buffer (pH 7.5) were disrupted by passing them through a chilled Aminco French pressure cell (American Instrument Co., Maryland, U.S.A.) twice at 20,000 p.s.i. (Hughes *et al.*, 1971). The homogenate was then centrifuged either at 10,000x g for 30 min or at 18,000x g for 45 min in Sorvall-SS3 centrifuge (SS-34 rotor) to sediment the intact cells. The supernatant fraction (S10 or S18 respectively), referred to as the crude extract was used either as such or centrifuged further as described in Section 2.5.2.3.

2.5.2.2 Cell-free extracts of acetone-dried cells

The acetone-dried cells were ground to a fine powder in a cold pestle and mortar and then suspended (10%, w/v) in 0.025M Tris-HCl buffer (pH 7.5). After adding DNase (0.1 mg/10 ml cell suspension), the preparation was stirred with a magnetic stirrer for 3-4 hr at 4^{o} in an ice bath. It was then filtered through two layers of cheese-cloth and the filtrate was passed through a chilled Aminco French pressure

cell twice at 20,000 p.s.i. This homogenate was centrifuged at 18,000x g for 45 min as described in Section 2.5.2.1. The resultant supernatant fraction (S18), which is referred to as the crude extract, was the source of the sulphide-linked nitrite reductase.

2.5.2.3 Preparation of S100 and P100 fractions

The crude extracts (S10 and S18 of the fresh cells or acetone-dried cells, respectively) were centrifuged at 100,000x g for 90-120 min in a Spinco Ultracentrifuge, Model L (rotor type 30) to obtain a supernatant (S100) and a pellet or membrane fraction (P100). The pellet fraction (P100) was suspended in 0.05M Tris-HCl buffer (pH 7.5) and dispersed uniformly by grinding in a glass homogenizer (Kontas Glass Co., New Jersey, U.S.A.).

2.5.3 Solubilization of nitrate reductase

The following procedure was employed to dissociate nitrate reductase from the cell membranes:-

The pellet fraction (P100), suspended in 0.05M Tris-HCl buffer (pH 7.5), was diluted with the same buffer to a final protein concentration of 30 mg/ml. It was subjected to an ultrasonic treatment (MSE Ultrasonicator; 60W output) for 30 sec and then incubated with sodium deoxycholate (0.06 mg/mg protein) at 30^{o} in a reciprocating waterbath (100 oscillations/min). After 30 min, this preparation was centrifuged at 100,000x g for 60 min and the supernatant fraction thus obtained, was passed through a Sephadex G-25 column (1.25 x 15 cm) which had been equilibrated with 0.05M Tris-HCl buffer (pH 7.5). The resulting preparation is referred to as the solubilized enzyme.

2.5.4 Enzyme assays

2.5.4.1 Nitrate reductase

Nitrate reductase activity with either sulphite or NADH as the electron donor, was determined by measuring the rate of nitrite production.

The enzyme activity, with sulphite as a donor, was assayed in open test tubes (1.3 x 10 cm). The reaction mixture contained: Tris-HCl buffer (pH 7.5), 90 µmoles; KNO_3, 1 µmole; Na_2SO_3, 1 µmole and 0.05 ml of enzyme in a total volume of 2 ml. Tubes which contained either the boiled enzyme or those without sulphite served as the control. After equilibrating the tubes in a reciprocating water bath (100 oscillations/min) at 30° for 5 min, the reaction was started by adding a freshly prepared solution of Na_2SO_3. After 15 min incubation, nitrite produced was determined as described in Section 2.7.2.

The reaction with NADH as the electron donor was also conducted in open test tubes (1.3 x 10 cm). The reaction mixture contained the following: Tris-HCl buffer (pH 7.5), 80 µmoles; KNO_3, 1 µmole; NADH, 1 µmole and 0.05 ml enzyme preparation. Control tubes contained the boiled enzyme preparation. The reaction was initiated by adding NADH and after incubating for 15 min at 30°, it was stopped by adding 0.1 ml of crystalline alcohol dehydrogenase (0.3 mg protein/ml 0.05M Tris-HCl buffer, pH 7.5) and 0.1 ml of N acetaldehyde to oxidize the residual NADH which interferes in the diazotization reaction (Medina and Nicholas, 1957; Stulen, 1970). The amount of nitrite produced was determined as described in Section 2.7.2.

Specific activity of nitrate reductase is expressed in nmoles NO_2^- produced/15 min/mg protein.

2.5.4.2 Nitrite reductase

Nitrite reductase activity was determined by following the rate of nitrite utilization. The enzyme activity in the P100 fraction was assayed in test tubes (1 x 7.5 cm) stoppered with rubber subaseal caps (Size 17, Wm Freeman, Barnsley, York, U.K.). Nitrite reductase activity of the purified enzyme preparation and also of the various fractions obtained during the purification of the enzyme was, however, assayed in Warburg flasks fitted with stopcocks and with their side-arms stoppered with subaseal caps.

For determining the enzyme activity with sulphide as an electron donor, the reaction mixture contained the following: Tris-HCl buffer (pH 7.5), 35 µmoles; $NaNO_2$, 1 µmole; Na_2S, 2 µmoles and 0.1 ml of enzyme, in a final volume of 1 ml. The test tubes or the Warburg flasks were thoroughly evacuated and filled with a high purity N_2 gas. After 5 min preincubation at 30°, the reaction was started by injecting, with an air-tight glass microsyringe (Scientific Glass Engineering Co., Melbourne, Australia), a freshly prepared solution of sulphide through the subaseal caps. The reaction was terminated after 15 min by removing the subaseal caps and adding 1 ml of 10% (w/v) zinc acetate in 10% (v/v) ethanol to the reaction mixture. This procedure precipitated sulphide which would otherwise interfere with the diazotization reaction. The contents of the Warburg flasks were then transferred to test tubes and centrifuged at 2,000x g for 15 min in a bench centrifuge (M.S.E.). Nitrite, in an appropriate aliquot of the supernatant fraction, was determined as described in Section 2.7.2.

Except for the reductant, the composition of the reaction mixture and the experimental details for determining nitrite reductase activity with NADH plus menadione as the electron donating system, were the same as described above for the assay with sulphide as the reductant. The reaction mixture, which in addition contained 2 µmoles menadione, was dispensed into either test tubes or Warburg flasks. These were then evacuated and filled with pure N_2 gas. The reaction was started by injecting 2 µmoles of NADH through the subaseal caps. After incubating at 30^{o} for 15 min, the reaction was terminated with 1 ml of 10% (w/v) zinc acetate in 10% (v/v) ethanol. After centrifuging, an appropriate aliquot of the supernatant was taken for determining the residual nitrite.

For ascorbate plus PMS as the electron donor system, the reaction mixture and the assay procedure were as described for sulphide except that ascorbate plus PMS replaced sulphide as the reductant. The reaction mixture in addition contained 1 µmole of PMS. The reaction was initiated by injecting 2 µmoles of neutralized solution of ascorbate *via* the subaseal into the reaction mixture. The reaction was terminated after 15 min as described previously and after centrifuging, nitrite was determined in an appropriate aliquot of the supernatant by the procedure described in Section 2.7.2.

Specific activity of nitrite reductase is expressed in nmoles NO_2^- utilized/15 min/mg protein.

2.5.4.3 Cytochrome oxidase

The activity of cytochrome oxidase was determined from the rate of oxygen uptake measured by Rank Oxygen Electrode as described in Section 2.6.1. After adding 1.8 ml of 0.05M Tris-HCl buffer

(pH 7.5) to the reaction vessel, the contents were equilibrated for 5 min at 30°. Then 0.1 ml of the enzyme preparation was injected into the reaction vessel with a Hamilton gas-tight microsyringe. After a further equilibration for 2 min, the reaction was started by injecting 2 µmoles of freshly prepared solution of the electron donor.

The specific activity is expressed as nmoles O_2 utilized/min/mg protein.

2.5.5 Determination of Michaelis - Menten constants (K_m)

For determining the K_m values, the enzyme activity was measured at varying concentrations of the 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 the substrate concentration as described by Lineweaver and Burk (1934).

2.5.6 Determination of molecular weights

Molecular weights of nitrite reductase and the cytochrome c_{554}, isolated from *T. denitrificans* (Section 2.5.7), were estimated by gel filtration through Sephadex G-150 or G-75 columns by the procedure of Andrews (1964, 1965).

2.5.6.1 Nitrite reductase

Sephadex G-150 column (2.5 x 50 cm), prepared as described in Section 2.6.8.2, was equilibrated for 2 days with 0.05M Tris-HCl buffer (pH 7.5) containing 0.1M KCl. Void volume of the column was determined from the elution of blue dextran. The column was calibrated

with a mixture of the following marker proteins of known molecular weights: γ-globulin (160,000), bovine serum albumin (68,000), ovalbumin (45,000) and myoglobin (17,800). Fractions, 3 ml each, were collected at 4° using L.K.B. Ultrasac fraction collector. The elution of these proteins from the column was followed by absorbance at 280 nm and at 410 nm for myoglobin. The purified nitrite reductase was then loaded onto the column either alone or along with the marker proteins. The elution of nitrite reductase from the column was monitored by assaying the enzyme activity in the various fractions with sulphide as an electron donor, as described in Section 2.5.4.2. Molecular weight of nitrite reductase was calculated according to the following equation of Squire (1964):

$$M^{1/3} = 151[1.47 - (V_e/V_o)^{1/3}]$$

where V_e = elution volume of the protein

V_o = void volume of the column.

2.5.6.2 Cytochrome c_{554}

Experimental details were as described for the estimation of molecular weight of nitrite reductase except that a Sephadex G-75 column (2 x 35 cm) was used and all the operations were carried out at room temperature.

2.5.7 Isolation of cytochrome c_{554} and flavin from nitrate reductase

Nitrate reductase was dissociated from the cell membranes (P100 fraction) by the procedure described in Section 2.5.3. The solubilized enzyme was placed in a boiling water bath for 15 min. After cooling, the precipitated proteins were removed by centrifuging at 100,000x g for 30 min. The supernatant fraction thus obtained, was loaded onto a DEAE-cellulose column (DE 32; 3 x 12 cm) which had been prepared and

equilibrated with 0.05M Tris-HCl buffer (pH 7.5) as described in Section 2.6.8.1. The column was eluted sequentially at room temperature with 100 ml each of 0.05M Tris-HCl buffer (pH 7.5) containing 0.1 and 0.2M NaCl respectively and 3 ml fractions were collected using L.K.B. Ultrasac fraction collector. Red-coloured fractions containing cytochrome *c* were recovered with the buffer containing 0.1M NaCl whereas yellow-coloured fractions were obtained on subsequent elution of the column with the buffer containing 0.2M NaCl. The red and yellow fractions were pooled separately, desalted by passing through a Sephadex G-25 column (1.25 x 15 cm) and concentrated in a rotatory vacuum evaporator at 30^{o}.

2.5.8 Dissociation of nitrite reductase into cytochrome *c* and haem *d* components

The purified nitrite reductase was dissociated into cytochrome *c* and haem *d* components by incubating it with 1 - 1.5 mg sodium dodecyl sulphate (SDS) per mg protein at 30^{o} for 30 min in an oscillating water bath. The detergent-treated enzyme was loaded onto a Sephadex G-100 column (2.5 x 45 cm) which had previously been equilibrated with 0.025M Tris-HCl buffer (pH 7.5) as described in Section 2.6.8.2. The sample was eluted, at room temperature, with the equilibrating buffer and 3 ml fractions were collected in an L.K.B. Ultrasac fraction collector. The absorbance at 280 nm and 405 nm of each fraction was monitored.

2.6 General techniques

2.6.1 Oxygen electrode

Oxygen uptake was measured polarographically with a Rank oxygen electrode (Bollisham, Cambridge, U.K.), connected to a Rikadenki recorder (Model B181).

The electrode was standardized in the presence of 0.05M Tris-HCl buffer (pH 7.5) by the method of Dixon and Kleppe (1966). The full span of the chart paper corresponded to 0.25 µmoles O_2/ml buffer.

The reaction vessel was maintained at 30^{o} by circulating water, from a temperature-controlled water bath, through the outer jacket. The reaction mixture in a final volume of 2 ml, contained either 0.05M or 0.025M Tris-HCl buffer (pH 7.5), the enzyme preparation and an appropriate reductant. The reaction was started by injecting the reductant from an air-tight glass micro-syringe, through an inlet port in the reaction vessel. The contents were continuously stirred by a small magnetic bar. The decrease in oxygen concentration in the reaction mixture was recorded.

2.6.2 Irradiation of the samples with U.V. light

The enzyme preparation (1 ml) dispensed in 2 ml of 0.05M Tris-HCl buffer (pH 7.5), was poured into a small Petri-dish (5 cm diameter) and placed on a layer of aluminium foil in an ice bucket. The sample was exposed to U.V. light (HPW 125W, Phillips) from above at a distance of 15 cm. The control sample, also kept under identical conditions was, however, protected from the U.V. light by covering the Petri-dish completely with an aluminium foil.

2.6.3 Absorption spectra

For spectral studies, a Shimadzu Multipurpose Recording Spectrophotometer, Model MPS-50L (Shimadzu Seisakusho Ltd, Kyoto, Japan) was used. The instrument was operated with photomultiplier at 400 volts

and automatic slit control. The spectra were recorded at room temperature in 3.5 ml quartz cuvettes (1 cm light path) in the absorbance range of either 0-1 or -0.1 to +0.1.

For absolute spectra, the sample cuvette contained 0.05M Tris-HCl buffer (pH 7.5) and the enzyme preparation in a final volume of 2.5 ml. Its spectrum was recorded against the buffer in the reference cuvette.

For difference spectra, both the sample and the reference cuvettes contained the enzyme preparation and 0.05M Tris-HCl buffer (pH 7.5) in a final volume of 2.5 ml. After adding a crystal of $K_3Fe(CN)_6$ to the reference cuvette and an appropriate reductant to the sample cuvette, the reduced *versus* oxidized spectra of the sample was recorded.

Difference spectra of the enzyme preparations under anaerobic conditions were recorded in a special Thunberg cuvette fitted with a side-arm. In all these studies, an ordinary quartz cuvette was used for the reference solution. The enzyme preparation and 0.05M Tris-HCl buffer (pH 7.5), in a final volume of 2.5 ml, were dispensed into both the sample and the reference cuvettes. The reductant was placed in the side-arm of the sample cuvette. After thoroughly evacuating the sample cuvette, with a water pump, it was filled with high purity, O_2-free N_2 gas. This procedure was repeated 3 times. The reductant was then tipped into the cuvette from the side-arm and the contents were mixed thoroughly. A crystal of $K_3Fe(CN)_6$ was added to the reference cuvette and the difference spectra recorded.

2.6.4 Redox-potential of cytochromes

Mid-point potential of cytochrome c_{554}, isolated from nitrate reductase (Section 2.5.7) was determined by the procedure of Davenport and Hill (1952), except that 1 cm open glass cuvettes were used.

Ferro-ferricyanide couple, E'_o of +0.423V (O'Reilly, 1973), was used for the reduction and oxidation of cytochrome c_{554}. The redox-potential was calculated from the Nernst equation:-

$$E'_o = 0.423 - 0.059 \log \frac{K_4Fe(CN)_6}{K_3Fe(CN)_6} \times \frac{\text{Ferricytochrome}}{\text{Ferrocytochrome}}$$

The difference in the mid-point potential between cytochromes *c* and *d* of *cd* complex of nitrite reductase was determined from the following equation, according to the procedure of Shimada and Orii (1976):-

$$E_m(\text{cyt. } d) - E_m(\text{cyt. } c) = 0.059 \log x \text{ K.}$$

where K = equilibrium constant and is calculated from the following relationship:

$$K = \frac{(c^{3+})(d^{2+})}{(c^{2+})(d^{3+})}$$

The equilibrium constant (K) was determined experimentally by measuring the % of cytochromes *c* and *d* in their oxidized and reduced forms at various time intervals after reducing the purified nitrite reductase with sulphide (see Section 2.6.3). The % of the reduced cytochromes *c* and *d* were calculated on the basis of the maximum reduction of these cytochromes obtained after the complete reduction of the enzyme with sulphide.

2.6.5 Paper chromatography of flavins

Aliquots (50 µl) of the flavin isolated from the solubilized nitrate reductase (Section 2.5.7) as well as of the authentic samples of FAD (2 mg/ml) and FMN (2 mg/ml) were spotted 7.5 cm apart on a

Whatman 3MM paper (45 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 U.V. light.

2.6.6 High voltage electrophoresis

The flavin, isolated from the solubilized nitrate reductase, was pre-incubated at 30° with either 20 mg snake venom pyrophosphatase for 30 min or with 20 mg of inorganic pyrophosphatase for 30 and 60 min respectively. Then 50 µl of each of the separated flavin as well as of those treated with the hydrolytic enzymes were spotted 2.5 cm apart onto a 3MM Whatman chromatographic paper (15 x 55 cm). Authentic standards of FAD and FMN were also spotted onto the paper which was then subjected to a high-voltage electrophoresis, using 0.1M sodium citrate buffer (pH 5.0), at 1500 V for 1 hr in an apparatus designed by Tate (1968). The electrophoretic mobilities of the flavins were determined under U.V. light, after drying the paper.

2.6.7 Mass spectrometry

The products of nitrite reduction were identified in a mass spectrometer. The reaction was carried out in Warburg flasks, fitted with glass stopcocks and with their side-arms stoppered with subaseals. The reaction mixture in a final volume of 1 ml contained: 0.6 ml of 0.05M Tris-HCl buffer (pH 7.5), 2 µmoles of $^{15}NO_2^-$ (27 atom % excess) and 0.2 ml of the enzyme preparation. The flasks were rigorously evacuated with a two-stage oil pump and filled with pure helium gas. This procedure was repeated three times. After pre-incubating for 5 min at 30° in a reciprocating water bath (100 oscillations/min),

5 µmoles of a freshly prepared sulphide solution was injected from a gas-tight syringe *via* the subaseals into the side-arms of the flasks. The reaction was started by mixing the contents. After various time intervals, the reaction was terminated by injecting 250 µl of 10M KOH solution into the flasks through the subaseals. The gases formed were then identified in a MS-2 Mass Spectrometer (Associated Electrical Industries, Manchester, U.K.).

2.6.8 Preparation of columns

2.6.8.1 DEAE-cellulose

Whatman DE-32 was prepared according to the instructions given in the Whatman Technical Bulletin IE2. The cellulose was treated with 0.5N HCl and washed with double-distilled water until the pH of the filtrate was 4.0. It was then treated with 0.5N NaOH and washed several times with double-distilled water until the pH of the filtrate was 8.0. The cellulose was finally equilibrated with 0.05M Tris-HCl buffer (pH 7.5). The column was packed at 2^{o} by pouring the homogenous slurry of the ion exchanger into a glass column. After the cellulose had settled, 0.05M Tris-HCl buffer (pH 7.5) was passed through the column until the pH of the effluent was the same as that of the equilibrating buffer. The sample was loaded onto the column and eluted with an appropriate buffer. For every experiment, DE-32 was regenerated afresh as described above. When not in use, the cellulose was stored at 2^{o} in 0.05M Tris-HCl buffer (pH 7.5) containing 0.1% (w/v) sodium azide.

2.6.8.2 Sephadex and Sepharose 6B

The columns for gel chromatography on Sephadex G-25, G-100, G-150 and Sepharose 6B were prepared according to the instructions given by the manufacturers (Pharmacia Fine Chemicals, Uppsala, Sweden). The gel beads were suspended in 0.05M Tris-HCl buffer (pH 7.5) and stirred

gently with a glass rod. After standing for 30 min, the fine particles were removed by decantation. This procedure was repeated a few times. The gel was then allowed to swell at room temperature in the same buffer for an appropriate time depending on the type of Sephadex used. After degassing 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. An appropriate pressure was maintained during packing and running of the column. The column was equilibrated with 0.05M Tris-HCl buffer (pH 7.5). When not in use, the column was stored at 2^{o} in the presence of 0.1% (w/v) sodium azide.

The void volume of the column was determined from the elution volume of blue dextran.

2.6.9 Membrane ultrafiltration

Enzyme preparations were concentrated using a Diaflo PM-10 membrane, in an Amicon ultrafiltration unit (Amicon Corp, U.S.A.). The ultrafiltration unit, which was kept in ice during filtration, was operated under N_2 gas at 20 lb/in^2. The PM-10 membrane retains proteins of molecular weight greater than 10,000.

2.7 Chemical determinations

2.7.1 Protein

Protein was determined by the micro-biuret method of Itzhaki and Gill (1964), using bovine serum albumin as a standard. The optical density was recorded at 310 nm. Absorbance of the fractions, eluted from the columns during enzyme purification, was monitored at 280 and 260 nm in order to assess their protein contents. The protein concentration was calculated from the following equation as described

by Kalckar (1947):-

$$\text{Protein (mg/ml)} = 1.45[E_{280}] - 0.74[E_{260}].$$

2.7.2 Nitrite

Nitrite was determined by the procedure of Hewitt and Nicholas (1964). An aliquot containing 20-100 nmoles of nitrite was diluted to 2 ml with double-distilled water. The red azo dye was developed by adding 1 ml of 1% (w/v) sulphanilamide in N HCl, followed by 1 ml of 0.01% (w/v) N-1-naphthylethylenediamine dihydrochloride. After 15 min, these were read at 540 nm in a Shimadzu (QV-50) spectrophotometer. The concentration of nitrite was determined from a standard curve.

2.7.3 Copper and iron

Copper was estimated according to the procedure described by Nambiar (1976). Purified nitrite reductase (containing 12.5 mg protein) was dried in acid-washed pyrex test tubes and digested for 20 min with 5 ml of an acid mixture of HNO_3, $HClO_4$ and H_2SO_4 in the proportion of 40 : 4 : 1. After cooling, the volume was made to 20 ml with deionized water. Copper in the digested sample was precipitated with 4 ml of 0.25% (w/v) ammonium pyrrolidine dithiocarbamate and then extracted by phase separation into 4 ml of methyl isobutylketone. The upper organic phase was injected directly into Varian Atomic Absorption Spectrophotometer (Varian Techtron Model AA4) and read at 3250A$^{\circ}$.

For determining the iron content, the samples were first digested with the acid mixture as described for copper. The digested samples were diluted to 25 ml with deionized water and their iron contents were determined at 2485A$^{\circ}$ in a Varian Atomic Absorption

Spectrophotometer.

2.7.4 Haems *c* and *d*

A spectrophotometric procedure of Newton (1969), based on the absorption at 618 nm of the pyridine haemochromogen of green haem, was used to estimate the haem *d* content of nitrite reductase. The sample was made 25% (v/v) with respect to pyridine. This was followed by the addition of NaOH to a final concentration of 0.1M. The mixture was divided equally and dispensed into two cuvettes (1 cm light path). A crystal of $K_3Fe(CN)_6$ was then added to the reference cuvette to oxidize the green haem which is otherwise autoreducible in the presence of pyridine and alkali. The difference in the A (618-650 nm) between sample and reference cuvettes was recorded and the haem *d* content was calculated from the extinction coefficient value of 19.6 mM^{-1} cm^{-1}.

The haem *c* content of nitrite reductase was determined from the absorption spectra of the alkaline pyridine haemochromogen, prepared as described above for haem *d*. After adding pyridine and alkali, a few crystals of $Na_2S_2O_4$ were added to the sample cuvette. The difference in the absorbance at 550 nm between the sample and the reference cuvettes was measured. An extinction coefficient value of 30.2 mM^{-1} cm^{-1} (Horio *et al.*, 1961A) was used to calculate the amount of haem *c* in the sample.

3. RESULTS

3.1 Nitrate reductase

3.1.1 Distribution of sulphite-linked nitrate reductase in various cell fractions

The results in Table 4 show that most of the enzyme activity was located in the pellet fraction (P100), obtained after centrifuging the crude extract (S10) at 100,000x g for 2 hr. The specific activity of the enzyme in this fraction was 2.5-fold greater than that of the crude extract. Sulphite-linked nitrate reductase activity was low in the high speed supernatant fraction (S100).

3.1.2 Enzyme activity with various electron donors

The effectiveness of various reductants and organic acids for the reduction of nitrate in the P100 fraction are presented in Table 5. The results show that besides sulphite, which is the physiological reductant for the enzyme, NADH was also equally effective for nitrate reduction. A relatively low activity was obtained with ascorbate plus TMPD whereas other metabolites including NADPH, α-ketoglutarate, lactate, pyruvate and succinate were ineffective.

3.1.3 Comparison of the properties of the sulphite- and NADH-linked nitrate reductase

3.1.3.1 Nitrate reductase activity with either sulphite or sulphite plus NADH as the electron donor

Since both sulphite and NADH were effective electron donors for nitrate reduction, the effect of various concentrations of sulphite

Table 4. Distribution of sulphite-linked nitrate reductase in various cell fractions.

The various cell-fractions (S10, S100 and P100) were prepared in 0.05M Tris-HCl buffer (pH 7.5) as described in Sections 2.5.2.1 and 2.5.2.3. Nitrate reductase activity with sulphite as an electron donor was determined as described in Section 2.5.4.1.

Fraction	Procedure	Specific activity (nmoles NO_2^- produced/15 min/mg protein)
I	Homogenate prepared by passing the washed cells through a French pressure cell, twice at 20,000 p.s.i.	152
II	Supernatant (S10) left after centrifuging I at 10,000x g for 30 min.	151
III	Supernatant fraction (S100) obtained by centrifuging II at 100,000x g for 90 min.	15
IV	Pellet fraction (P100) from III dispensed in 0.05M Tris-HCl buffer (pH 7.5)	385

Table 5. Nitrate reductase activity with various electron donors.

The reaction mixture in a total volume of 2 ml, contained the following in µmoles: Tris-HCl buffer (pH 7.5), 85; $NaNO_3$, 1; electron donor, 1; and 0.3 ml of cell-free extract (S10 containing 42 mg protein/ml). The reaction was carried out at 30^{o} as described in Section 2.5.4.1.

Electron donor	Specific activity (nmoles NO_2^- produced/15 min/mg protein)
Sodium sulphite	90
NADH	95
NADPH	4
α-Ketoglutarate	9
Sodium ascorbate	7
Ascorbate + N,N,N',N'-tetramethylparaphenylenediamine (TMPD)	22
Sodium pyruvate	8
Sodium succinate	12
Sodium lactate	9

alone as well as in the presence of 0.5 and 1.0 mM NADH was studied. The results in Table 6 indicate that the amount of nitrite produced with sulphite plus NADH was almost equivalent to the sum of nitrite formed by these reductants individually. This effect was observed even when both sulphite and NADH were used at optimal concentrations in the reaction mixture.

3.1.3.2 Distribution

The results in Table 7 show that both the sulphite- and NADH-linked nitrate reductase activities had a similar pattern of distribution in the various cellular fractions. Enzyme activity, with either of these reductants, was almost exclusively associated with the membrane fraction (P100). Only negligible sulphite- and NADH-dependent nitrate reductase activities were found in the high speed supernatant fraction (S100). Specific activities of the enzyme, with either of these reductants, were about 2.5-fold higher in the pellet fraction (P100) than in the crude extract (S10).

3.1.3.3 Incubation time, stability and pH optima

Incubation time. Nitrate reductase activity, with either sulphite or NADH as the reductant, was linear up to 15 min (Figure 1). Thus, an incubation time of 15 min was routinely used for assaying the enzyme activity.

Stability during storage. The NADH-linked nitrate reductase in the P100 fraction was more unstable during storage than the sulphite-linked activity. Thus, the enzyme activity with sulphite as a donor was not affected after storing the P100 fraction at 0° for 5 days whereas more than a half of the NADH-dependent activity was lost. However,

Table 6. Nitrate reductase activity with either sulphite, NADH or sulphite plus NADH as the electron donors.

The details of the assay procedures as described in Section 2.5.4.1 except that the concentrations of sulphite or NADH were varied as indicated in the Table. The enzyme activity in the P100 fraction (0.5 mg protein) was determined with either sulphite, NADH or sulphite plus NADH as electron donors.

Concentration of sulphite (mM)	Specific activity (nmoles NO_2^- produced/15 min/mg protein)		
	without NADH	with 0.5mM NADH	with 1.0mM NADH
0	0	317	330
0.125	121	417	434
0.250	152	434	445
0.500	178	478	460
0.750	171	439	456
1.000	169	434	456
1.250	165	417	445
1.500	160	417	408

Table 7. Distribution of sulphite - and NADH-linked nitrate reductase in various cell fractions.

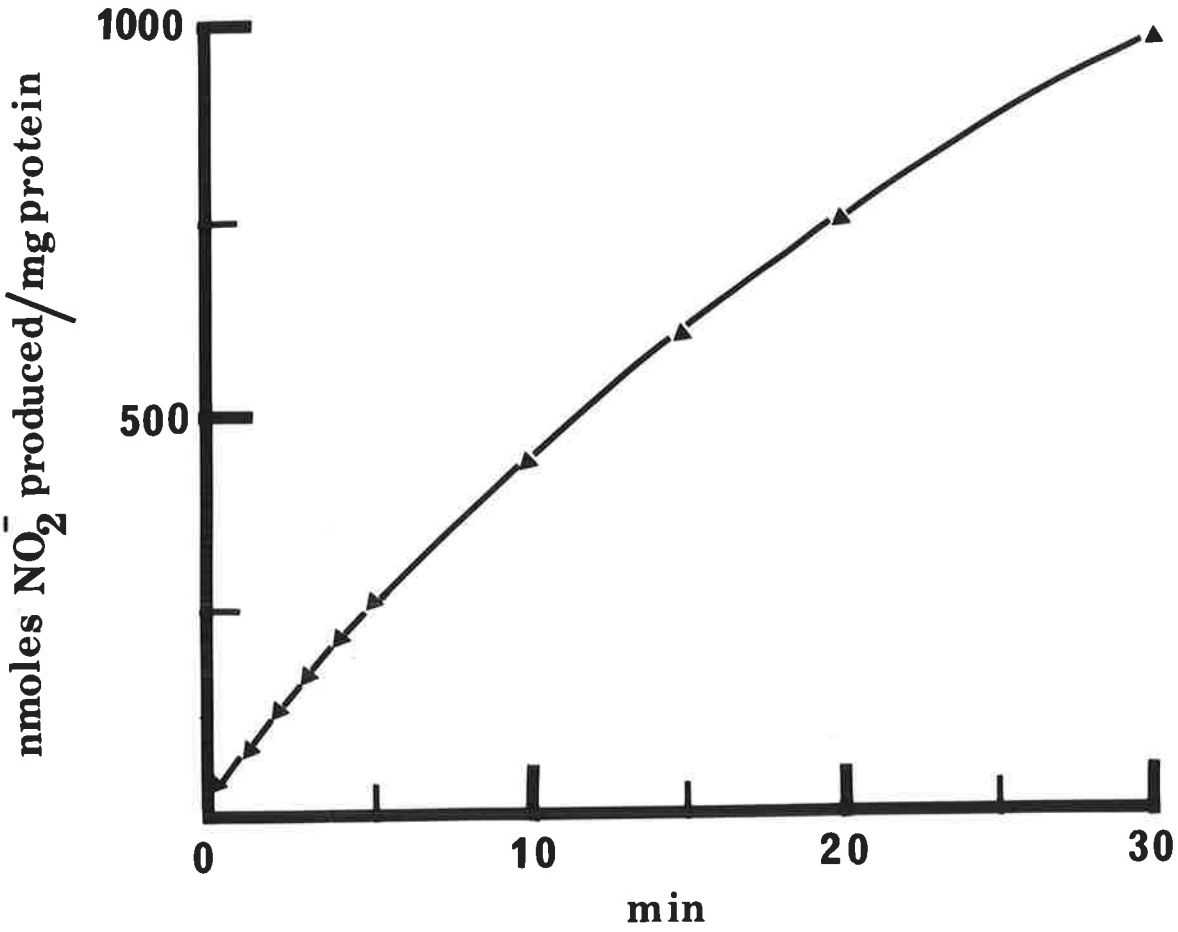
The various cell fractions (S10, S100 and P100) were prepared in 0.05M Tris-HCl buffer (pH 7.5) as described in Sections 2.5.2.1 and 2.5.2.3. Enzyme activity was determined with either sulphite or NADH as the electron donor as described in Section 2.5.4.1.

Fraction	Specific activity (nmoles NO_2^- produced/15min/mg protein)	
	Electron donor	
	Sulphite	NADH
Cell homogenate	128	126
S10	117	117
S100	23	9
P100	300	340

Figure 1. Effect of incubation time on nitrate reduction.

Nitrate reductase activity was assayed in P100 fraction with either SO_3^{2-} or NADH as the electron donor as described in Section 2.5.4.1, except that the period of incubation has varied as shown in the Figure.

Figure 1



the activity with NADH as a donor was completely restored on preincubating the stored pellet (P100) with 5-10 mM GSH at 4° for 15 min (Table 8). The sulphite-linked nitrate reductase was not affected by GSH.

Stability during heat treatment. Incubation of the P100 fraction at 40° for 30 min had no effect on the sulphite-linked nitrate reductase activity. However, the heat treatment at 45° for 30 min or at 50° for 10 min lowered its activity by 38 and 88%, respectively (Figure 2A).

The NADH-linked nitrate reductase activity was diminished by about 85% after 10 min at 50°. Incubation at 45° or 40° for 30 min decreased the enzyme activity by 55 and 40% respectively (Figure 2B). Thus both the sulphite- and NADH-linked activities were equally sensitive to heat treatment at 50° but the sulphite-linked activity was relatively more stable during incubations at 40° and 45°.

pH optima. The pH optima of the sulphite- and NADH-linked nitrate reductase activities were 8.0 and 7.0 respectively (Figure 3A and B). A significantly lower sulphite-linked activity was recorded in phosphate buffer. Thus at pH 8.0, in the presence of phosphate buffer, only 30% of the activity with either Tris-HCl or Tris-maleate buffers was obtained. Nitrate reductase activity with NADH as an electron donor was, however, almost the same in either of these buffers.

3.1.3.4 K_m values

Effect of nitrate concentration. The enzyme activity at varying concentrations of nitrate, with either sulphite or NADH as an

Table 8. Stability of sulphite - and NADH-linked nitrate reductase during storage and the restoration of NADH-dependent activity with GSH.

P100 fraction, prepared as described in Section 2.5.2.3, was stored at 0^{o}. After 5 days, nitrate reductase activity with either sulphite or NADH as an electron donor was assayed as described in Section 2.5.4.1 except that the enzyme preparation, in 0.05M Tris-HCl buffer (pH 7.5), was pre-incubated at 0^{o} for 15 min with the specified concentrations of GSH prior to adding the other components of the reaction mixture. The enzyme activity before storage was 300 and 340 nmoles NO_2^- produced/15 min/mg protein with sulphite and NADH, respectively, as the electron donors.

Final concentration of GSH (mM)	% of activity before storage	
	Electron donor	
	Sulphite	NADH
0	100	48
0.25	100	79
0.50	100	81
1.00	100	85
2.50	100	93
5.00	98	100
10.00	84	98
15.00	82	88

Figure 2. Thermal stability of nitrate reductase.

A : SO_3^{2-} as an electron donor.
B : NADH as an electron donor.

P100 fraction (containing 0.5 mg protein), taken in 0.05 M Tris-HCl buffer (pH 7.5), was pre-incubated at either 40°, 45° or 50° for the prescribed times. The enzyme activity, with either SO_3^{2-} or NADH as the reductant, was determined as described in Section 2.5.4.1, after having equilibrated the enzyme preparation at 30° for 5 min. The results are expressed as % activity of the control samples (without heat treatment) which were 200 and 240 nmoles NO_2^- produced/15 min/mg protein with SO_3^{2-} and NADH respectively, as the electron donors.

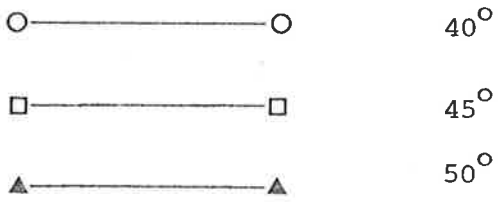


Figure 2A

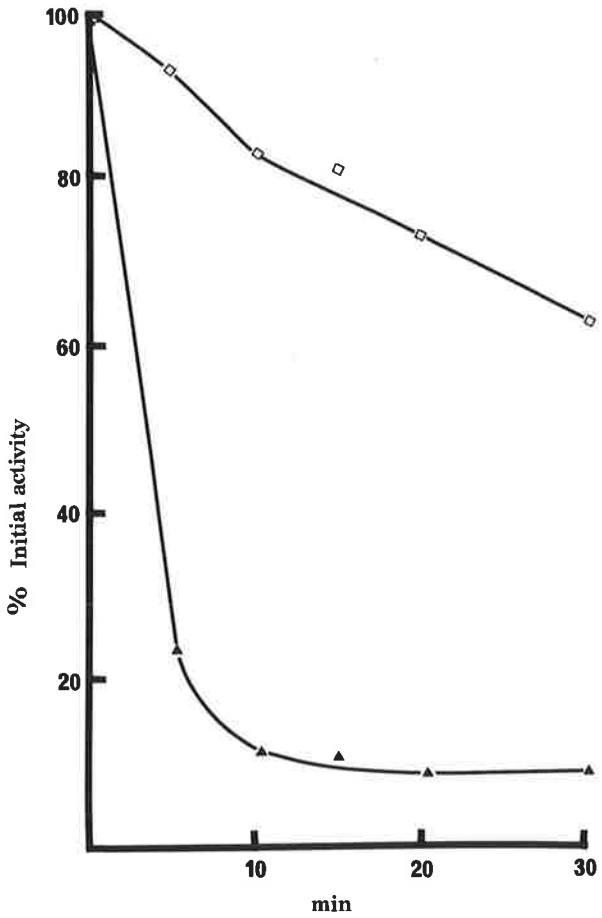


Figure 2B

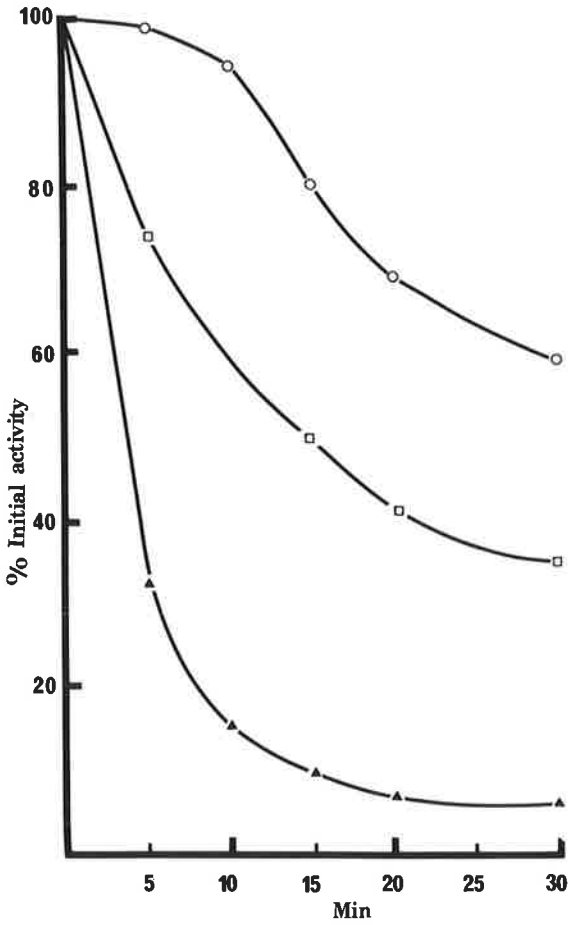
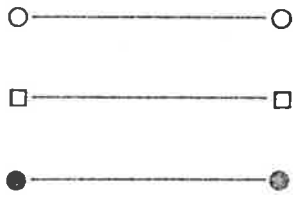


Figure 3. Effect of pH on nitrate reductase activity.

A : SO_3^{2-} as an electron donor.

B : NADH as an electron donor.

Nitrate reductase activity, with either SO_3^{2-} or NADH as the electron donor, was determined according to the procedure described in Section 2.5.4.1 except that 0.05 M of either phosphate, Tris-HCl or Tris-maleate buffer of indicated pH were used. The pH values given in the Figure correspond to the final pH of the reaction mixture.



Phosphate

Tris-HCl

Tris-maleate

Figure 3A

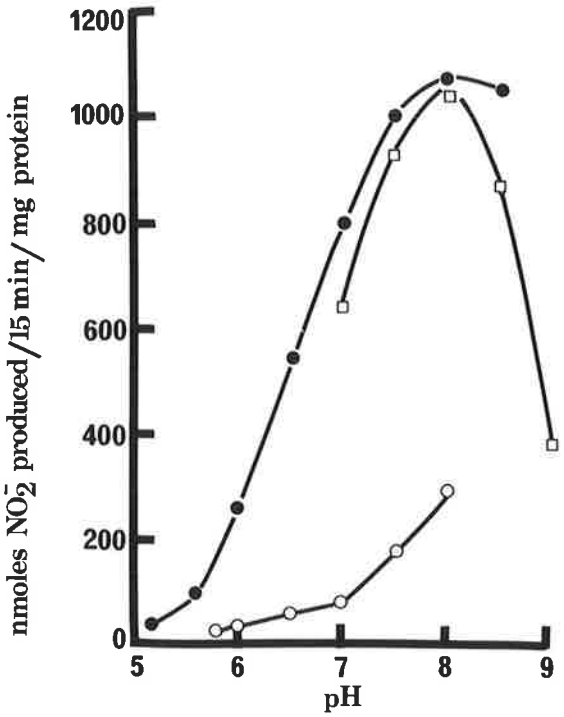
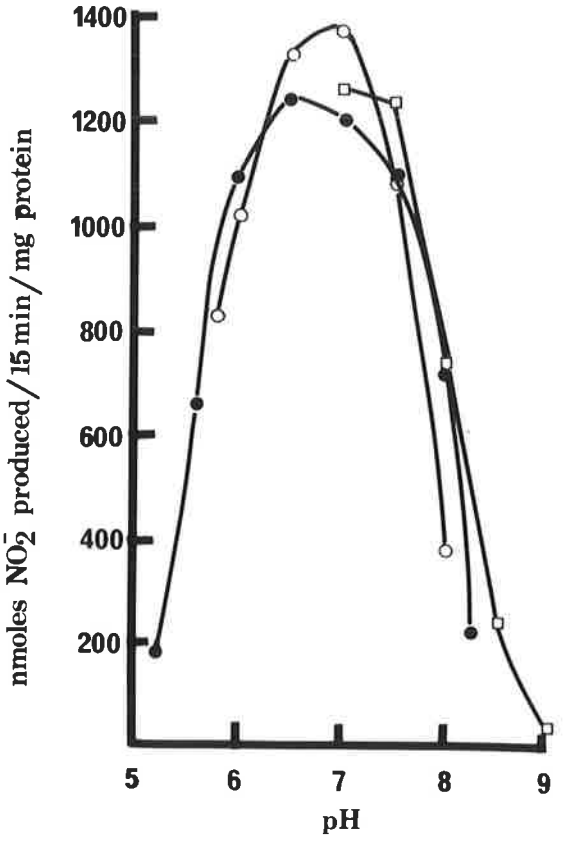


Figure 3B



electron donor, exhibited a normal Michaelis-Menten kinetics. The K_m values for nitrate with either sulphite or NADH as the reductant, were 41.6 and 83.3 μM respectively (Figure 4A and B).

Effect of sulphite or NADH concentrations. Nitrate reductase activity was determined at varying concentrations of either sulphite or NADH. The results in Figure 5A and B show that the rate of nitrate reduction increased proportionally with increasing concentrations of the reductant. The K_m values of 0.1 and 0.33 mM were obtained for sulphite and NADH respectively.

3.1.4 Characterization of the electron transport chain involved in nitrate reduction

3.1.4.1 Effects of inhibitors

Since sulphite and NADH were effective electron donors for nitrate reduction, the effects of various inhibitors on the enzyme activity in P100 fraction with both reductants were compared.

Sulphydryl reagents. The data in Table 9 show that the thiol-binding agents were potent inhibitors of nitrate reductase activity when either of the electron donors was used. The sulphite-linked nitrate reductase was strongly inhibited by N-ethylmaleimide (NEM) and to a lesser extent by 0.1 M p-CMB and by 5 mM iodoacetamide. The inhibitory effect of 1 mM NEM was almost completely reversed by 2 mM GSH. The NADH-linked nitrate reductase activity was much more sensitive than the sulphite-linked activity to p-CMB and iodoacetamide. The inhibition of the NADH-linked enzyme activity by 0.01 mM p-CMB and by 1 mM NEM was reversed by GSH.

Figure 4. Double-reciprocal plot of the effect of nitrate concentration on enzyme activity.

A : SO_3^{2-} as the reductant.
B : NADH as the reductant.

Nitrate reductase activity in P100 fraction was determined as described in Section 2.5.4.1 except that the concentration of nitrate in the reaction mixture was varied as indicated in the Figure. 1/V represents the reciprocal of nmoles NO_2^- produced/15 min.

Figure 4A

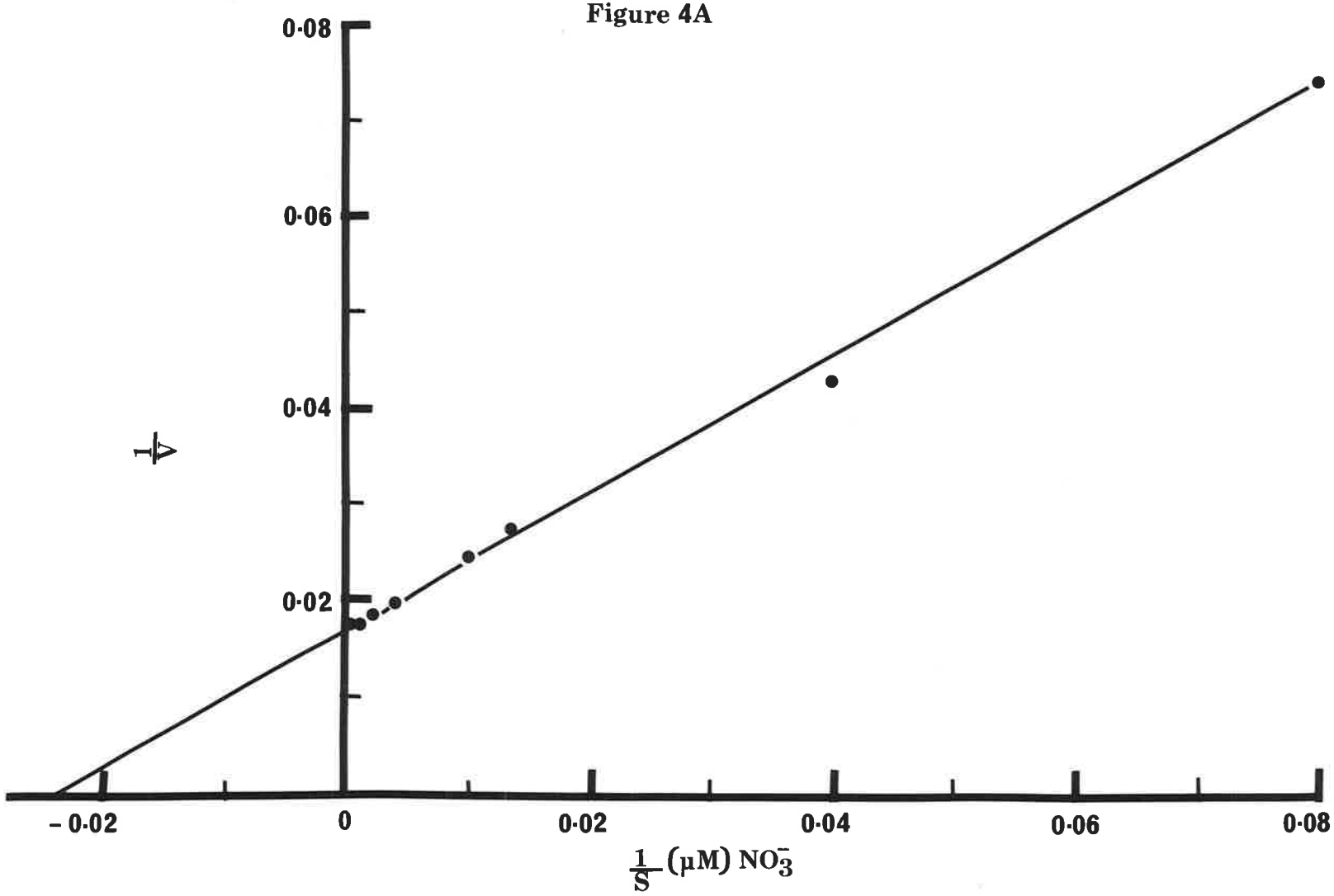


Figure 4B

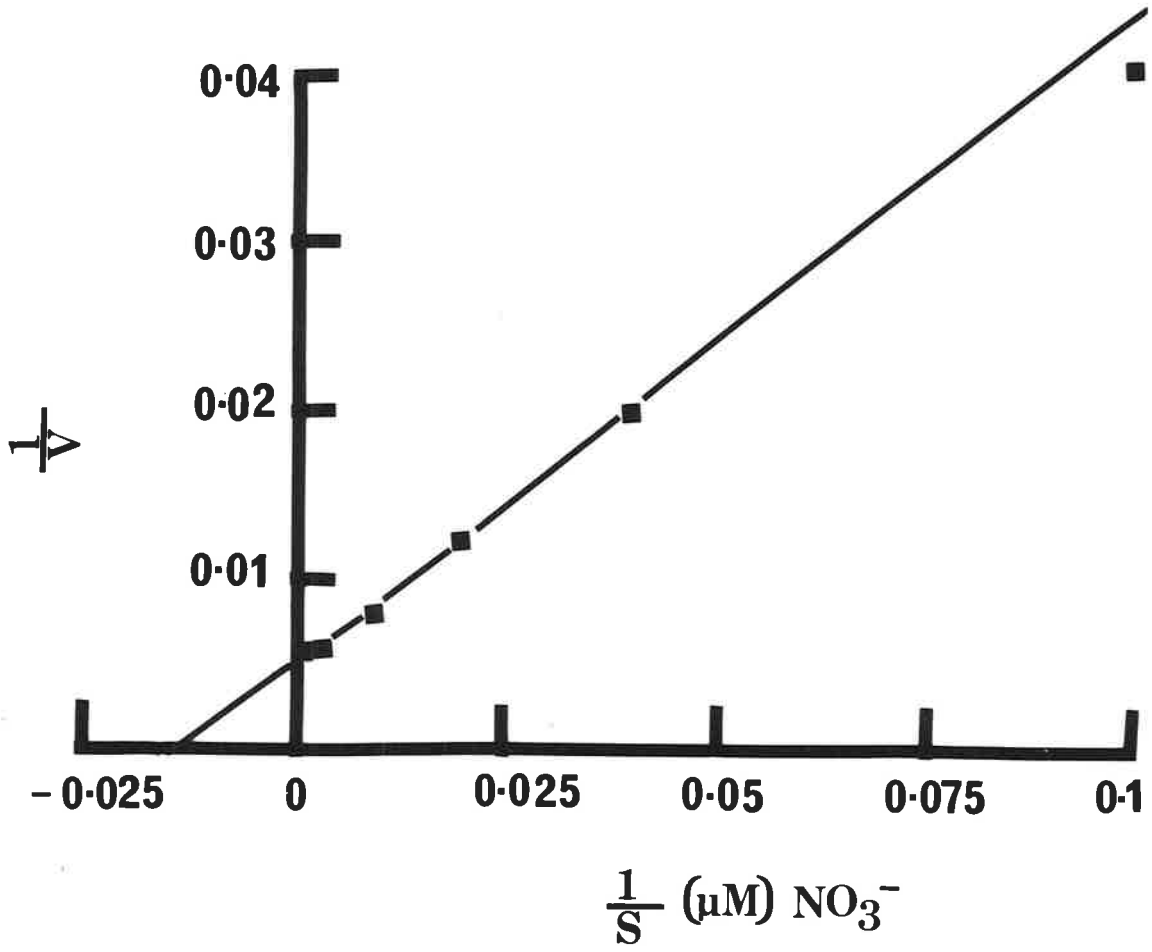


Figure 5. A Lineweaver - Burk's plot of the effect of various concentrations of electron donor on nitrate reductase activity.

A : SO_3^{2-} as an electron donor.
B : NADH as an electron donor.

Nitrate reductase activity, in the P100 fraction containing 0.5 mg protein, was determined as described in Section 2.5.4.1 except that the reaction mixture contained various concentrations of either SO_3^{2-} (Figure 5A) or NADH (Figure 5B). $\frac{1}{V}$ represents nmoles NO_2^- produced/15 min.

Figure 5A

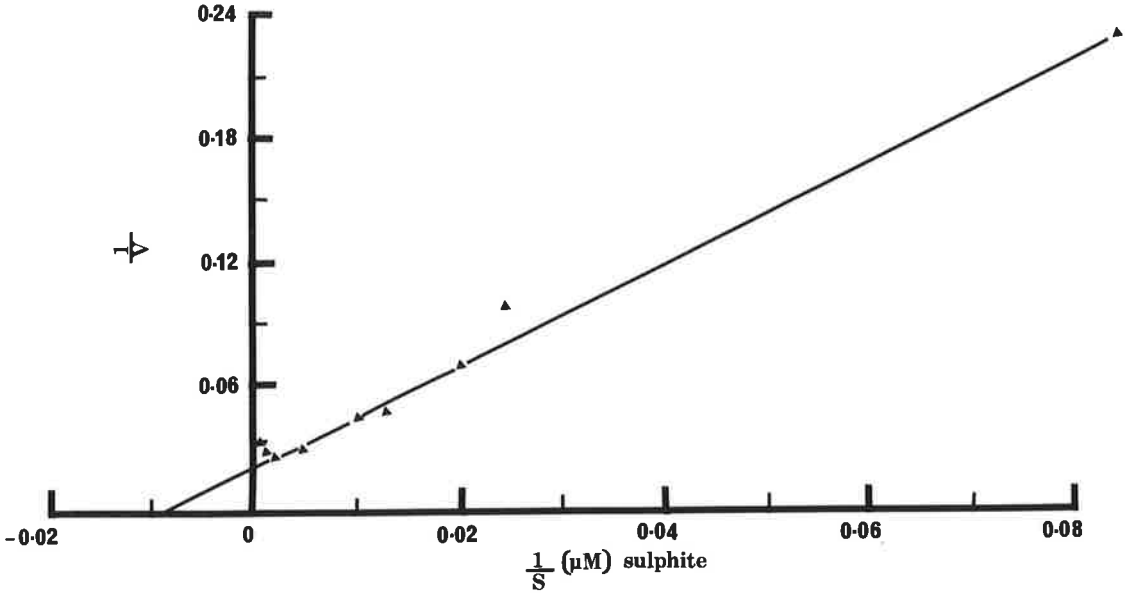


Figure 5B

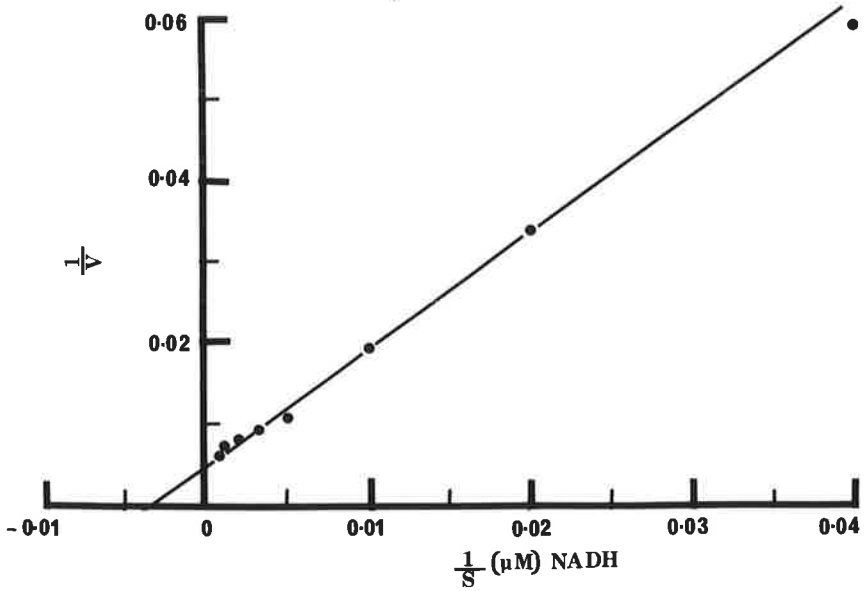


Table 9. Effect of inhibitors of -SH groups on nitrate reductase activity with either sulphite or NADH as the electron donor.

P100 fraction (0.6 mg protein) in 0.05M Tris-HCl buffer (pH 7.5) was incubated at 30° for 15 min with the inhibitor (final concentrations as indicated) before addition of other components of the reaction mixture. GSH was added, where indicated, to the enzyme preparation 5 min prior to starting the reaction with the appropriate electron donor.

Results are expressed as % inhibition with respect to the control sample (without inhibitor). The specific activity of the control sample with sulphite and NADH as the electron donors was 390 and 330 nmoles NO_2^- produced/15 min/mg protein respectively.

Addition to the reaction mixture	Final concentration (mM)	% inhibition	
		Electron donor	
		Sulphite	NADH
p-Chloromercuribenzoate	0.01	10	100
p-Chloromercuribenzoate + GSH	0.01 + 2.0	10	37
p-Chloromercuribenzoate	0.1	58	100
p-Chloromercuribenzoate + GSH	0.1 + 2.0	68	84
Iodoacetamide	5.0	49	84
Iodoacetamide + GSH	5.0 + 5.0	49	84
N-Ethylmaleimide	1.0	88	62
N-Ethylmaleimide + GSH	1.0 + 2.0	10	20
N-Ethylmaleimide	5.0	95	75
N-Ethylmaleimide + GSH	5.0 + 2.0	93	69

Flavoprotein inhibitors. Amytal (5 mM) and rotenone (1 mM) inhibited the sulphite-linked nitrate reductase activity by about 70% whereas atabrine was not inhibitory (Table 10). The inhibition by either amytal or rotenone was not relieved by FMN or menadione.

The NADH-linked nitrate reductase on the other hand, was not affected by amytal but atabrine (2 mM) inhibited its activity by 76%. Nitrate reductase activity with either of the reductants, was inhibited by rotenone to almost the same extent. The inhibition of the NADH-dependent enzyme activity by atabrine was reversed completely by menadione whereas the effect of rotenone was relieved only partially.

Inhibitors of electron transport chain. HQNO, antimycin A and dicumarol were potent inhibitors of the sulphite-linked enzyme activity (Table 11). Menadione or coenzyme Q_{10} were not effective in reversing the inhibitory effects of any of these compounds.

The NADH-linked nitrate reductase was less sensitive to HQNO and antimycin A than was the sulphite-linked enzyme. The enzyme activity with NADH as an electron donor was markedly inhibited by 1 mM dicumarol. Menadione, but not coenzyme Q_{10}, reversed the inhibitory effects of HQNO and antimycin A on the NADH-linked nitrate reductase activity.

Metal chelators. Sodium diethyldithiocarbamate (5 mM), bathocuproin (5 mM), azide (0.05 mM), cyanide (0.5 mM), KSCN (0.5 mM) and dithiol (10 mM) inhibited both the sulphite- and NADH-linked nitrate reductase activities to approximately the same extent (Table 12). Other chelating agents like 2,2'-bipyridyl, 8-hydroxyquinoline and urethane

Table 10. Effects of flavin inhibitors on nitrate reductase activity with either sulphite or NADH as the electron donor.

The composition of the reaction mixture was as described in Section 2.5.4.1. The enzyme preparation (P100 fraction), taken in 0.05M Tris-HCl buffer (pH 7.5), was pre-incubated at 30^{o} for 10 min with various concentrations of the inhibitor prior to the addition of substrates. Menadione was added just before starting the reaction with the electron donor.

Results are expressed as % inhibition with respect to the control (without inhibitor). The specific activity of the control was 380 and 342 nmoles NO_2^- produced/15 min/mg protein with sulphite and NADH, respectively, as the electron donors.

Addition to the reaction mixture	Final concentration (mM)	% inhibition	
		Electron donor	
		Sulphite	NADH
Amytal	2.0	38	11
	5.0	69	22
Amytal + menadione	5.0 + 5.0	69	22
Atabrine	0.5	0	59
	2.0	23	76
Atabrine + menadione	2.0 + 5.0	23	2
Rotenone	0.5	45	60
	1.0	76	84
Rotenone + menadione	0.5 + 1.0	45	37

Table 11. Effects of inhibitors of the electron transport chain on nitrate reductase activity with either sulphite or NADH as the electron donor.

P100 fraction (0.5 mg protein), in 0.05M Tris-HCl buffer (pH 7.5), was pre-incubated with the inhibitor for 10 min before adding the substrates. Menadione or coenzyme Q_{10} were included in the reaction mixture as indicated. The specific activity of the control sample (without inhibitor) was 260 and 230 nmoles NO_2^- produced/15 min/mg protein with sulphite and NADH respectively, as the electron donors.

Inhibitor	Final concentration	% inhibition Electron donor	
		Sulphite	NADH
2-n-Heptyl-4-hydroxyquinoline-N-oxide (HQNO)	0.1 µM	42	0
HQNO	0.5 µM	69	0
HQNO + coenzyme Q_{10}	0.5 µM + 0.1 mM	52	-
HQNO	5.0 µM	97	29
HQNO + menadione	5.0 µM + 1 mM	97	3
HQNO + coenzyme Q_{10}	5.0 µM + 0.1 mM	97	17
Antimycin A	0.05 mM	60	33
	0.1 mM	70	45
Antimycin A + menadione	0.1 mM + 1.0 mM	70	2
+ coenzyme Q_{10}	0.1 mM + 0.25 mM	60	36
Dicumarol	1.0 mM	72	86

Table 12. Effects of metal chelators and other inhibitors on nitrate reductase activity with either sulphite or NADH as the electron donor.

Nitrate reductase activity was determined as described in Section 2.5.4.1. P100 fraction (0.5 mg protein) in 0.05 M Tris-HCl buffer (pH 7.5) was pre-incubated with the inhibitor for 10 min prior to adding the substrates. The specific activity of the control sample (without inhibitor) was 260 and 230 nmoles NO_2^- produced/15 min/mg protein with sulphite and NADH respectively, as the electron donors.

Inhibitor	Final concentration (mM)	% inhibition Electron donor	
		Sulphite	NADH
2,2'-Bipyridyl	5.0	17	3
Sodium diethyldithiocarbamate	5.0	51	49
8-Hydroxyquinoline	5.0	0	4
Bathocuproin	5.0	38	59
Urethane	5.0	16	11
Sodium azide	0.01	56	41
	0.05	85	71
Potassium cyanide	0.5	58	34
Potassium thiocyanate	0.5	64	46
Dithiol	10.0	98	70

did not affect either of these activities.

Chlorate. Both the sulphite- and NADH-linked nitrate reductase activities were inhibited by chlorate. Double-reciprocal plots of the effect of chlorate on the enzyme activity at various concentrations of nitrate are presented in Figure 6A and B. Chlorate inhibited the NADH-dependent activity competitively with respect to nitrate while it showed a mixed type inhibition on the sulphite-linked nitrate reductase.

3.1.4.2 Effect of U.V. irradiation

Irradiation of the S10 and P100 fractions with ultra-violet light resulted in a profound inhibition of the sulphite- and the NADH-linked nitrate reductase activities (Figure 7A and B). An exposure of an enzyme preparation to U.V. light for 90 min, as described in Section 2.6.2, resulted in a loss of 45 and 68% of the sulphite-linked activity in the S10 and P100 fractions respectively. The NADH-linked nitrate reductase activity in the S10 and P100 fractions were also affected to a similar extent.

Inclusion of coenzyme Q_{10} (0.1 mM) in the assay mixture failed to reverse the effect of U.V. irradiation for 60 min, on nitrate reductase activity in P100 fraction with either sulphite or NADH as the reductant.

Menadione had no effect on the sulphite-linked enzyme activity in the P100 fraction which had been irradiated with U.V. light for 60 min. The effects of menadione on the NADH-linked nitrate reductase activity of an untreated and U.V.-irradiated P100 fractions are shown in Figure 8A and B. At lower concentrations, menadione stimulated

Figure 6. Double-reciprocal plot of the effect of potassium chlorate at varying concentrations of nitrate on nitrate reductase activity.

A : SO_3^{2-} as the reductant.
B : NADH as the reductant.

Nitrate reductase activity was determined with either SO_3^{2-} or NADH as the reductant as described in Section 2.5.4.1. P100 fraction (0.6 mg protein), in 0.05 M Tris-HCl buffer (pH 7.5), was pre-incubated for 10 min with either 0.5 mM or 1.0 mM $KClO_3$ prior to the addition of the specified concentrations of nitrate. The reaction was started by adding the respective electron donor.

●——————● Without ClO_3^-

△——————△ 0.5 mM ClO_3^-

○——————○ 1.0 mM ClO_3^-

Figure 6A

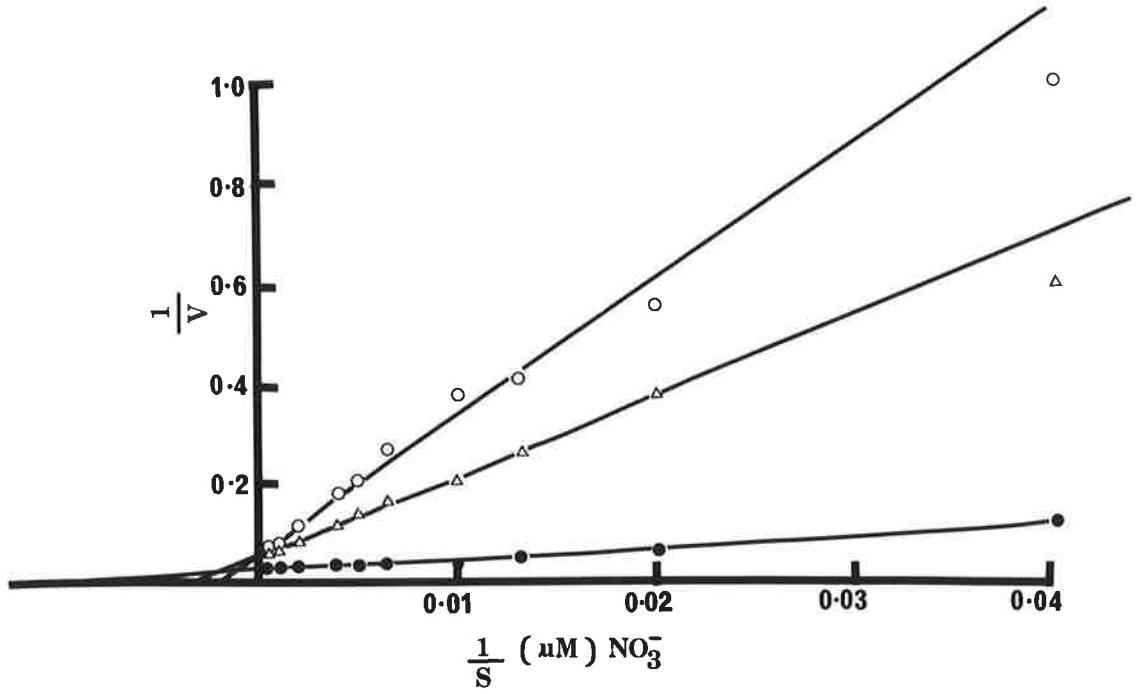


Figure 6B

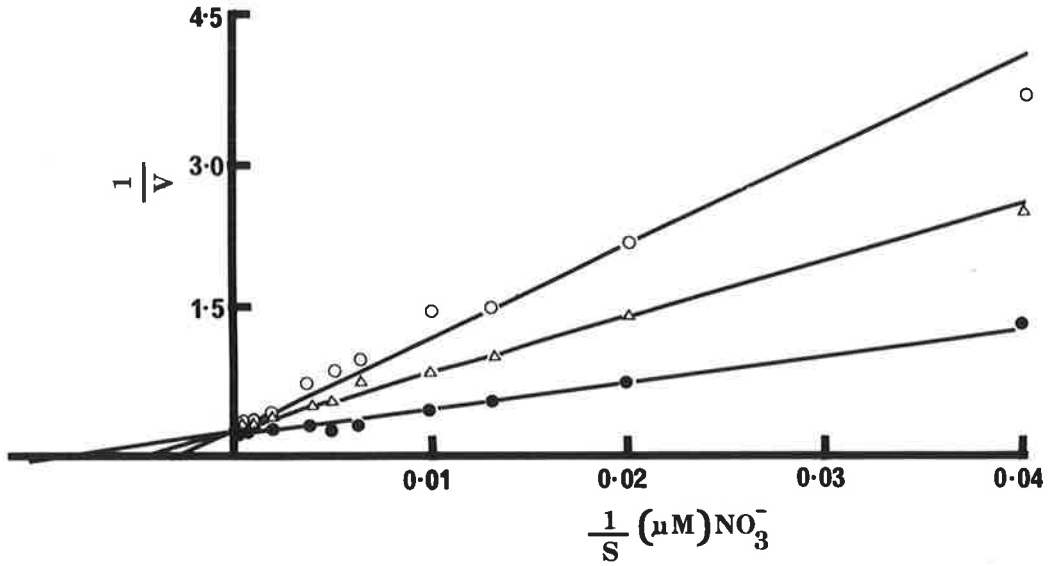


Figure 7. Effect of U.V. irradiation on nitrate reductase activity in S10 and P100 fractions.

A : SO_3^{2-} as an electron donor.
B : NADH as an electron donor.

S10 and P100 fractions were prepared as described in Sections 2.5.2.1 and 2.5.2.3 respectively. 1 ml aliquot of S10 fraction (34 mg protein/ml) or P100 fraction (25 mg protein/ml), was dispensed in 2 ml of 0.05 M Tris-HCl buffer (pH 7.5) and exposed to U.V. light as described in Section 2.6.2. At the times indicated, 100 µl aliquots were withdrawn and assayed for nitrate reductase activity with either SO_3^{2-} or NADH as the electron donor as described in Section 2.5.4.1. The results are presented as % of the initial activity (prior to irradiation), which for SO_3^{2-} and NADH dependent activities were 123.7 and 123 nmoles NO_2^- produced/15 min/mg protein (S10 fraction) and 182.4 and 182 nmoles NO_2^- produced/15 min/mg protein (P100 fraction) respectively.

△——————△ S10 fraction

○——————○ P100 fraction

Figure 7A

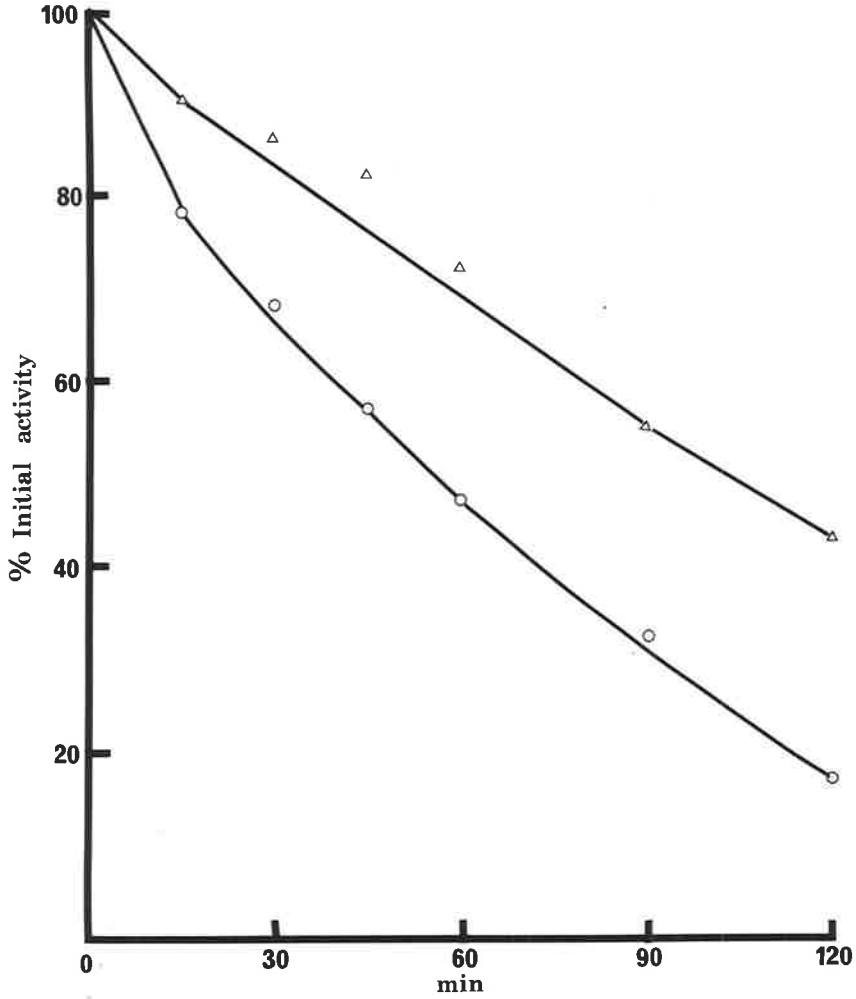


Figure 7B

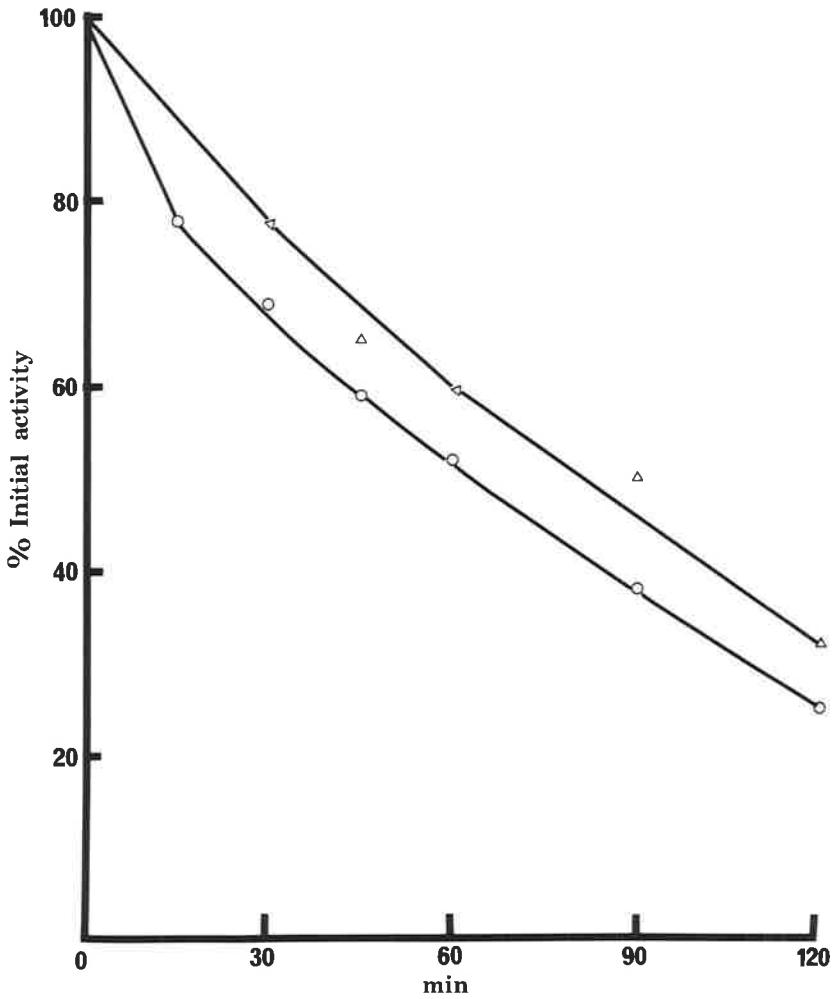
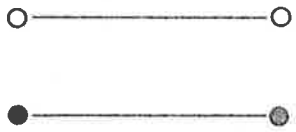


Figure 8. Effects of various concentrations of menadione on nitrate reductase activity in the U.V.-irradiated P100 fraction.

A : SO_3^{2-} as the reductant.
B : NADH as the reductant.

P100 fraction was irradiated with U.V. light for 60 min as described in Section 2.6.2. Nitrate reductase activity in the presence of menadione, both in the control and the U.V.-irradiated samples, was determined with either SO_3^{2-} or NADH as the reductant as described in Section 2.5.4.1.



P100 fraction

P100 fraction, irradiated with U.V. light for 60 min

Figure 8A

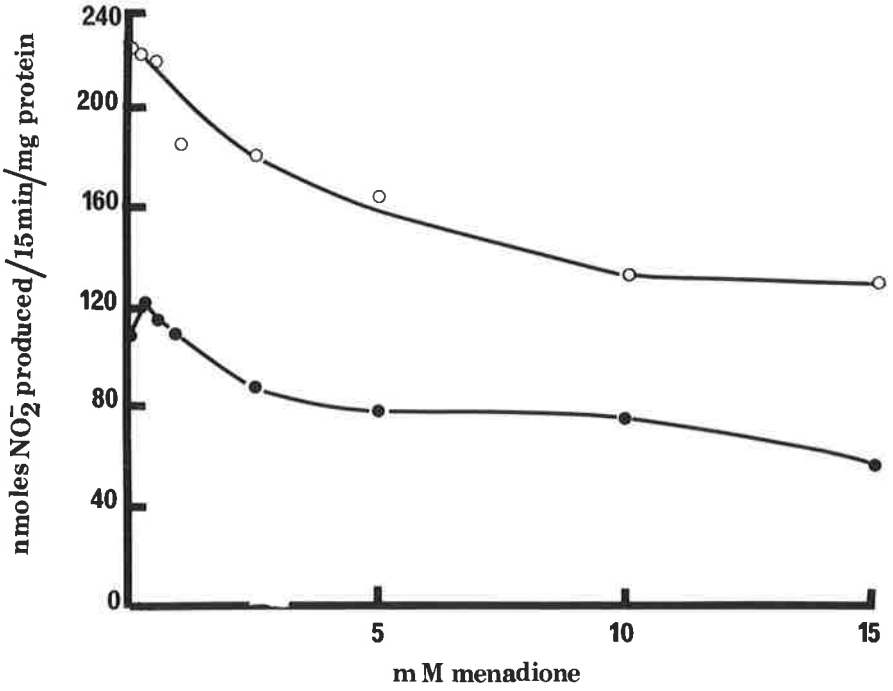
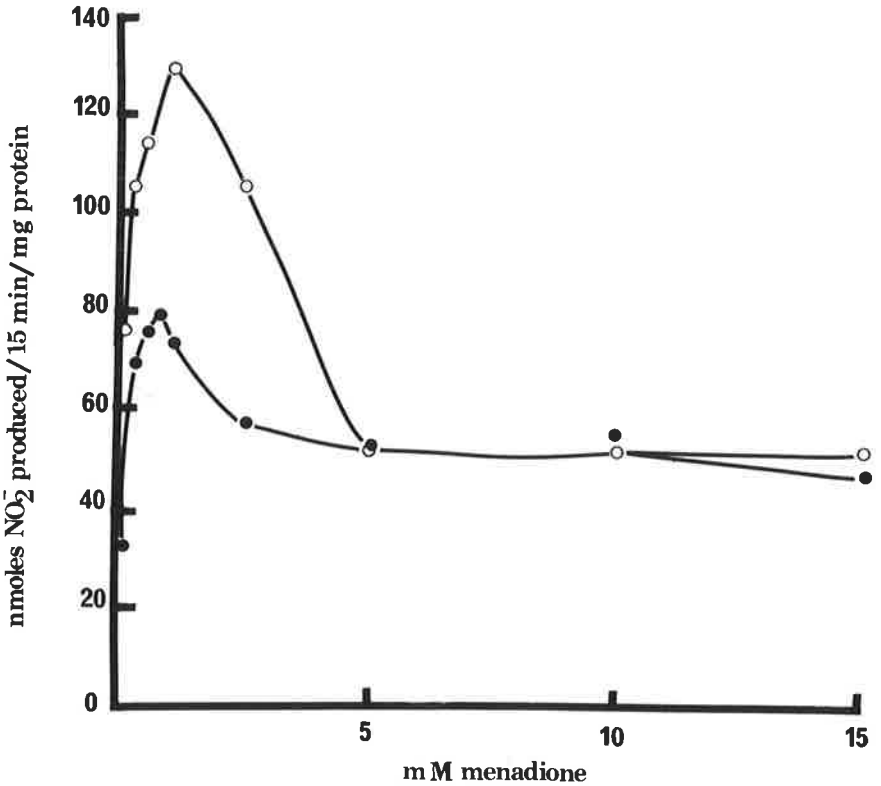


Figure 8B



the enzyme activity of the untreated as well as of the U.V.-irradiated P100 fractions. However, 0.5 mM menadione reversed the inactivation caused by U.V. irradiation by about 25%.

3.1.5 Spectral properties of sulphite- and NADH-linked nitrate reductase

The difference spectra of the P100 fraction, reduced with either sulphite or NADH, showed absorption maxima at 424, 524 and 554 nm. These absorption bands, which corresponded to those of reduced cytochrome *c*, disappeared on adding nitrate to either sulphite- or NADH-reduced enzyme (Figure 9). Nitrite had no effect on the spectra of the reduced enzyme. These results indicate that cytochrome *c* is involved in the reduction of nitrate.

3.1.6 Characterization of the electron transport chain linked to oxygen

3.1.6.1 Various electron donors

The suitability of various compounds as hydrogen donors for O_2 utilization was investigated in crude extract (S10). The results in Table 13 show that sulphite, NADH and, to a lesser extent, NADPH and ascorbate supported the uptake of O_2 whereas succinate, pyruvate, lactate and α-ketoglutarate were ineffective.

3.1.6.2 Comparison of oxygen uptake in various cell fractions with either sulphite or NADH as reductant

The maximum rate of O_2 uptake was observed in the pellet fraction (P100) with either of the reductants (Table 14). The supernatant fraction (S100) had low activity. As shown earlier

Figure 9. Difference spectra of P100 fraction.

P100 fraction (6 mg protein) and 0.05 M Tris-HCl buffer (pH 7.5), in a final volume of 3 ml, were dispensed into each of the sample and the reference cuvettes. A crystal of $K_3Fe(CN)_6$ was added to the reference cuvette whereas to the sample cuvette, 2 μmoles of either SO_3^{2-} or NADH was added and the difference spectra were recorded (Section 2.6.3).

A. ———————— reduced *versus* oxidized spectra

B. — — — — — — — spectra recorded after adding 4 μmoles of $NaNO_3$ to A

Figure 9

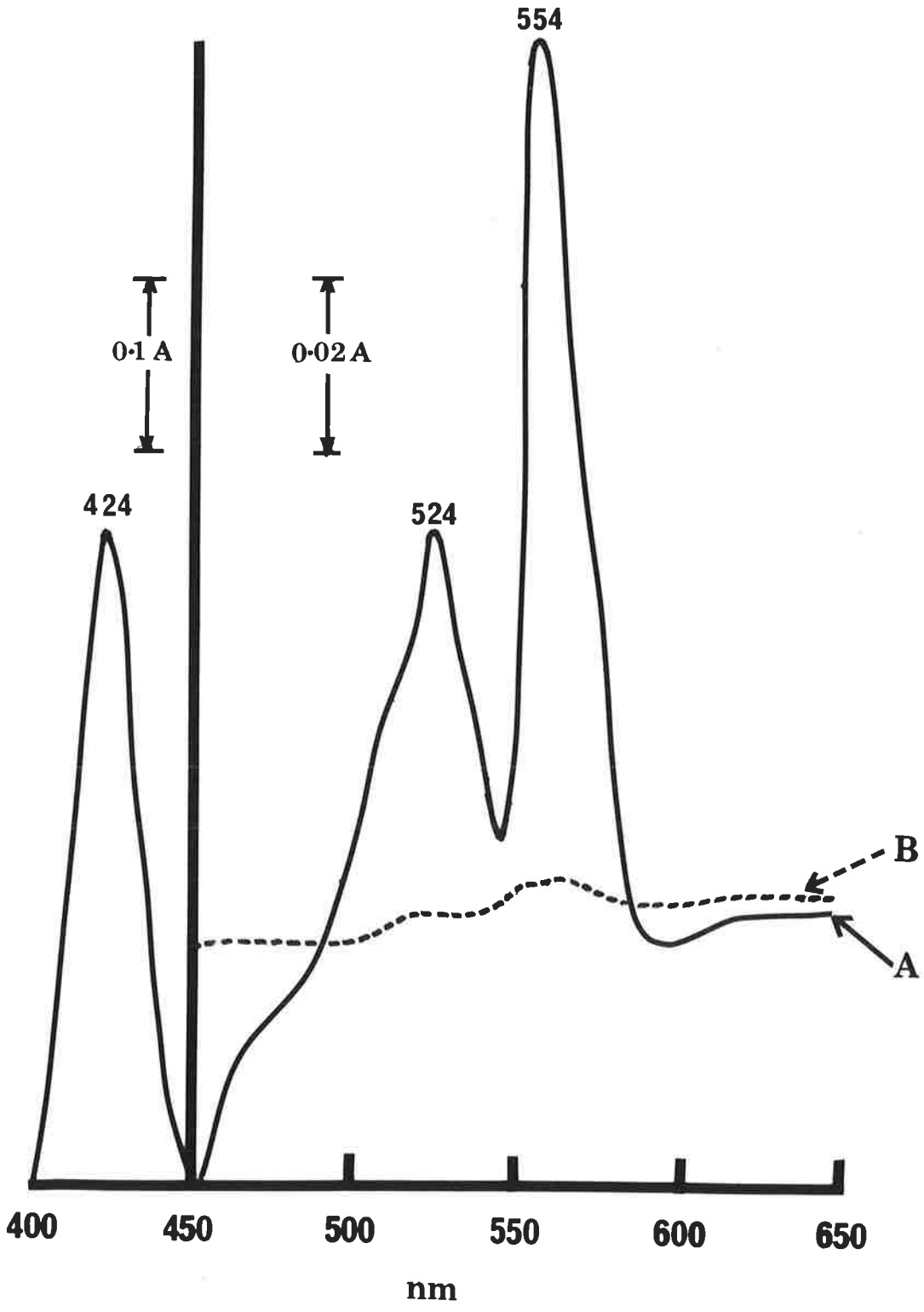


Table 13. Oxygen uptake with various electron donors.

Oxygen uptake in S10 fraction was measured as described in Section 2.6.1. The reaction mixture in a total volume of 2 ml, contained the following in μmoles: Tris-HCl buffer (pH 7.5), 85; electron donor, 2; and 0.1 ml of S10 fraction (34 mg protein/ml).

Electron donor	Specific activity (nmoles O_2 utilized/min/mg protein)
Sodium sulphite	32.3
NADH	29.6
NADPH	10.2
Sodium succinate	3.6
Sodium pyruvate	4.4
Sodium lactate	2.1
Sodium ascorbate	12.5
α-Ketoglutarate	1.5

Table 14. Oxygen uptake by various cell fractions with either sulphite or NADH as the electron donor.

The various cell fractions (S10, S100 and P100) were prepared in 0.05M Tris-HCl buffer (pH 7.5) as described in Sections 2.5.2.1 and 2.5.2.3. The rate of oxygen uptake was determined with an electrode system as described in Section 2.6.1.

Fraction	Specific activity (nmoles O_2 utilized/min/mg protein)	
	Electron donor	
	Sulphite	NADH
Disrupted cells	27.7	19.6
S10	21.2	16.0
S100	4.8	3.7
P100	22.3	19.1

(Table 7) nitrate reductase activity was also associated with this particulate fraction.

3.1.6.3 Effects of various inhibitors

Thiol-binding reagents. The effects of thiol-binding reagents on the sulphite-dependent O_2 uptake are given in Table 15. All the three inhibitors diminished the uptake of O_2. The inhibition by either p-CMB or NEM was reversed by GSH. Comparison of these results with those in Table 9 indicate that the sulphite-linked nitrate reduction in the P100 fraction was also inhibited by these compounds.

Inhibitors of the electron transport chain. The effects of various inhibitors of electron transport chain on O_2 uptake with sulphite as the reductant were also examined (Table 16). At 2 mM each, amytal, atabrine and rotenone diminished O_2 uptake by 47, 63 and 71% respectively and 0.5 mM of either antimycin A or dicumarol inhibited the utilization of O_2 by 70 and 59% respectively. At 5 µM HQNO, O_2 uptake was suppressed by 85%.

Metal chelators. Azide, cyanide and arsenite were potent inhibitors of the sulphite-linked O_2 uptake (Table 17). This activity was more sensitive to cyanide than the sulphite-linked nitrate reductase (Table 12). Bathocuproin, 8-hydroxyquinoline and 2,2'-bipyridyl, at 5 mM each, inhibited O_2 uptake by about 50% whereas urethane at the same concentration had no effect.

3.1.7 Solubilization of sulphite-linked nitrate reductase

In order to examine the effectiveness of various detergents in solubilizing the membrane-bound nitrate reductase, the P100 fraction

Table 15. Effects of sulphydryl group inhibitors on oxygen uptake with sulphite as an electron donor.

Oxygen uptake was determined as described in Section 2.6.1. P100 fraction (1.3 mg protein) in 0.05 M Tris-HCl buffer (pH 7.5) was pre-incubated with the inhibitor for 10 min at 30°. GSH was added, as indicated, just before starting the reaction with sulphite. The enzyme activity is expressed as % inhibition as compared to the activity of control sample (without inhibitor) which was 18 nmoles O_2 utilized/min/mg protein.

Inhibitor	Final concentration (mM)	% inhibition
p-Chloromercuribenzoate	0.1	26
	1.0	43
p-Chloromercuribenzoate + GSH	1.0 + 1.0	18
N-Ethylmaleimide	1.0	40
	2.0	53
N-Ethylmaleimide + GSH	1.0 + 2.0	0
Iodoacetamide	1.0	20
	2.0	24
	5.0	30
Iodoacetamide + GSH	5.0 + 5.0	31

Table 16. Effects of inhibitors of the electron transport chain on oxygen uptake with sulphite as an electron donor.

Oxygen uptake was measured as described in Section 2.6.1. P100 fraction was pre-incubated for 10 min at 30° with inhibitor prior to adding the electron donor. The specific activity of the control sample (without inhibitor) was 22 nmoles O_2 utilized/min/mg protein.

Inhibitor	Final concentration	% inhibition
Amytal	2 mM	47
	5 "	68
Atabrine	0.5 mM	30
	1.0 "	50
	2.0 "	63
Rotenone	0.5 "	31
	1.0 "	44
	2.0 "	71
Antimycin A	0.05 "	54
	0.5 "	67
2-n-Heptyl-4-hydroxyquinoline-N-oxide	0.1 µM	52
	0.5 "	81
	5.0 "	85
Dicumarol	0.5 mM	59
	1.0 "	64
	2.0 "	75

Table 17. Effects of metal chelators and other inhibitors on oxygen uptake with sulphite as an electron donor.

Experimental details as in Table 16. The specific activity of the control sample (without inhibitor) was 27 nmoles O_2 utilized/min/mg protein.

Inhibitor	Final concentration (mM)	% inhibition
Sodium azide	0.01	53
	0.1	70
Potassium cyanide	0.01	86
	0.1	92
Sodium arsenite	0.2	52
	0.1	77
Bathocuproin	5.0	53
Urethane	5.0	14
8-Hydroxyquinoline	5.0	55
2,2'-Bipyridyl	5.0	40

(30 mg protein/ml) was incubated at 30° for 30 min with either of the following detergents: Tween 80 (2%, v/v), digitonin (0.5 mg/mg protein), Triton X-100 (10%, v/v), sodium deoxycholate (0.05 mg/mg protein) and sodium dedocyl sulphate (0.05 mg/mg protein). After treatment with the detergents, the P100 fraction was centrifuged at 100,000x g for 60 min and the enzyme activity was determined in the supernatant fraction. Among the various detergents used, only sodium deoxycholate and sodium dedocyl sulphate were effective in dissociating the enzyme from the membranes. Less than 30% of the enzyme was solubilized on incubating the P100 fraction for 30 min with lipase II (0.3 mg/mg protein).

The effects of various concentrations of sodium deoxycholate on the solubilization of the sulphite-linked nitrate reductase are illustrated in Figure 10. Maximum solubilization of the enzyme was obtained on incubating the P100 fraction with 0.06 mg sodium deoxycholate per mg protein.

The effects of incubation of P100 fraction, with the optimum concentration of sodium deoxycholate at 30°, for various time intervals are illustrated in Figure 11. Maximum solubilization was achieved after 30 min incubation.

3.1.8 Inactivation of NADH-linked nitrate reductase on solubilization and restoration of its activity by PMS and menadione

Incubation of the P100 fraction with sodium deoxycholate resulted in a marked loss in the specific activity of the NADH-linked nitrate reductase and only 10% of its original activity remained in

Figure 10. Effect of various concentrations of sodium deoxycholate on the solubilization of the sulphite-linked nitrate reductase in P100 fraction.

P100 fraction, prepared as described in Section 2.5.2.3, was diluted with 0.05 M Tris-HCl buffer (pH 7.5) so that its protein concentration was 30 mg/ml. 2 ml aliquots of this P100 fraction were then incubated with the specified concentrations of sodium deoxycholate at 30° for 30 min. After centrifuging at 100,000x g for 60 min, nitrate reductase activity in the supernatant fractions was assayed by the procedure described in Section 2.5.4.1.

Figure 10

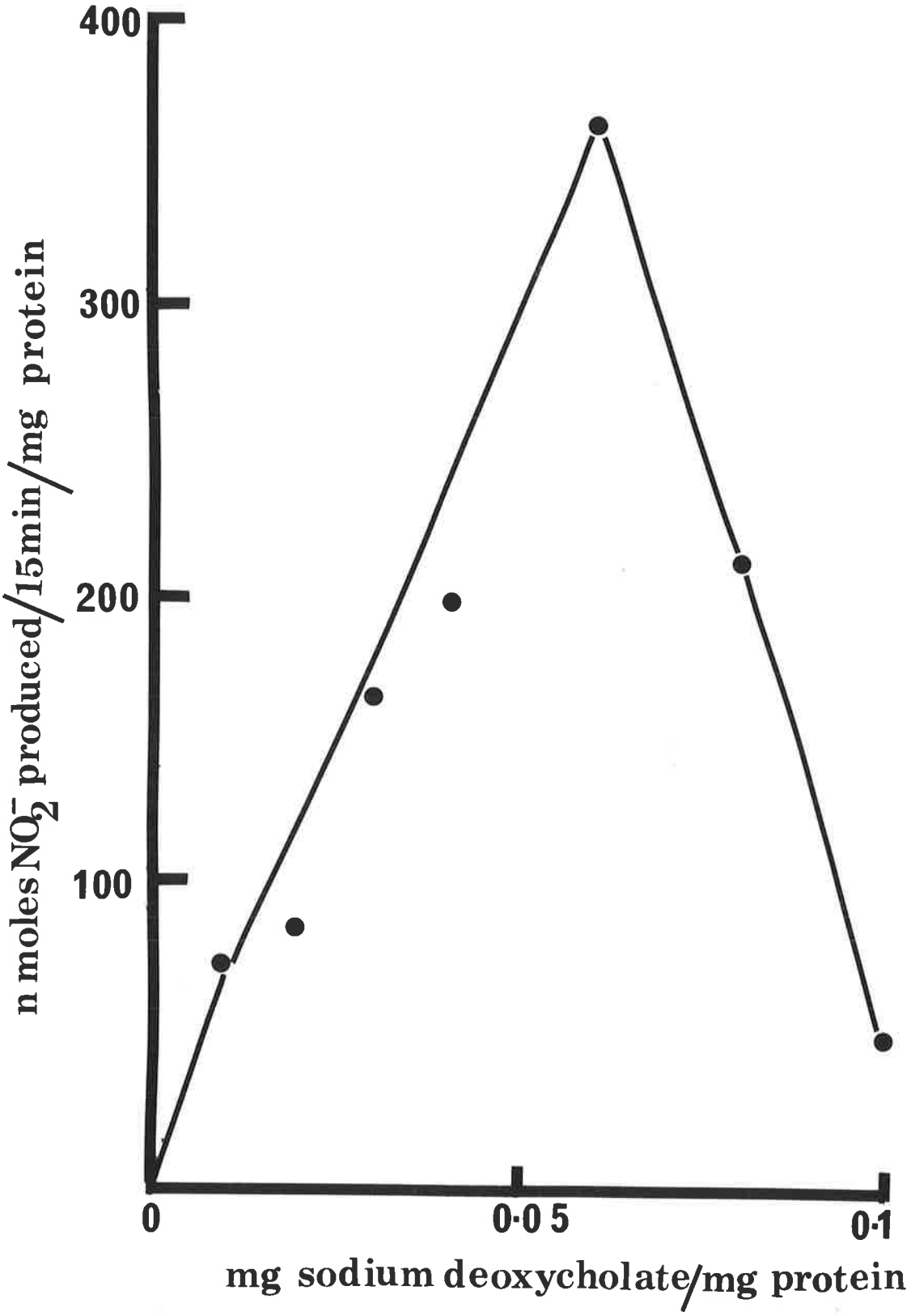
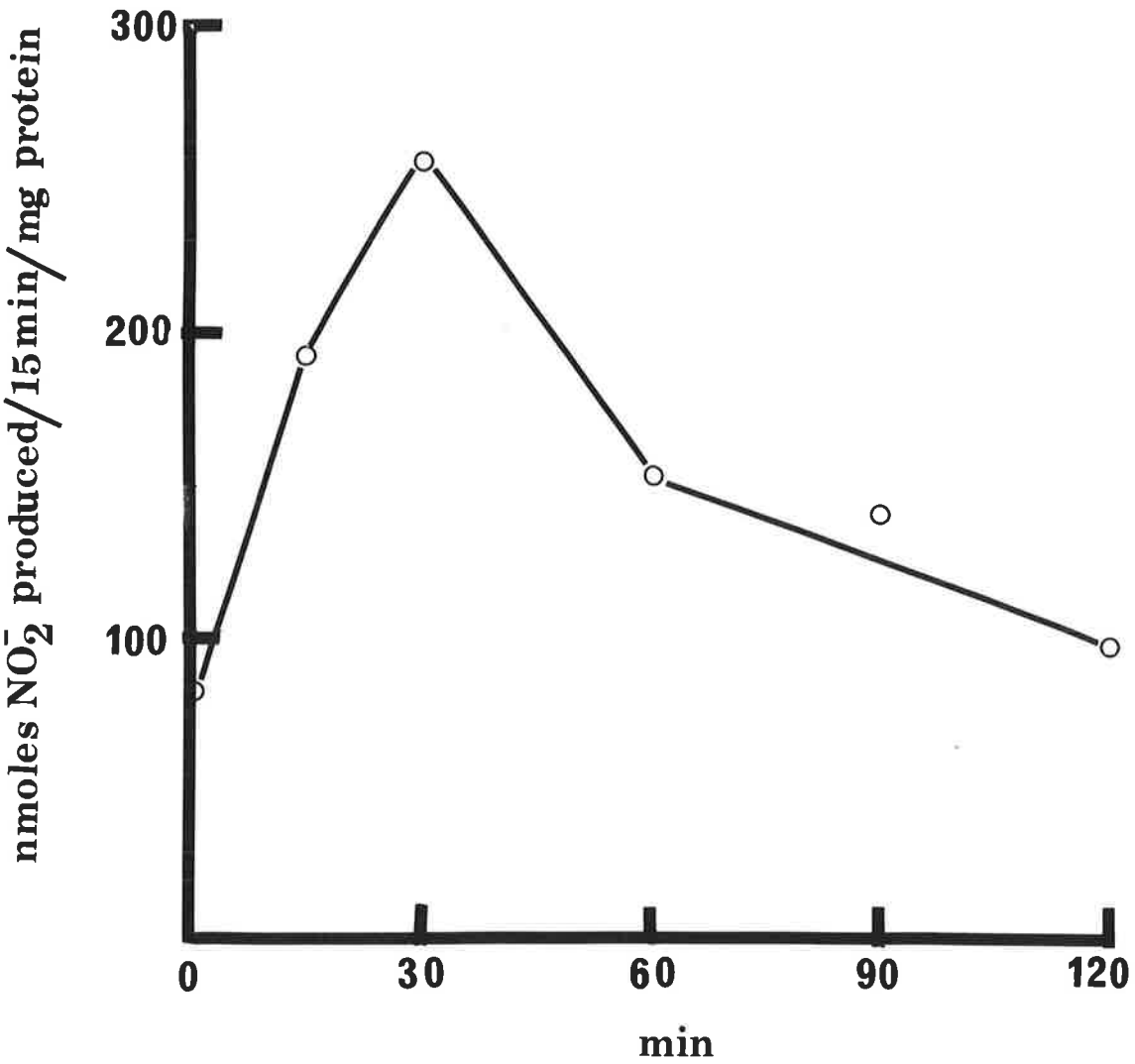

400
300
200
100
n moles NO_2^- produced/15min/mg protein
0
0·0 5
0·1
mg sodium deoxycholate/mg protein

Figure 11. Effect of incubation time on the solubilization of the sulphite-linked nitrate reductase from P100 fraction by sodium deoxycholate treatment.

Experimental details are as in Figure 10 except that 2 ml aliquots of P100 fraction (30 mg protein/ml) were incubated with 0.06 mg sodium deoxycholate/mg protein at 30° for the indicated periods. Nitrate reductase activity in the supernatant fractions, after centrifuging at 100,000x g for 60 min, was then determined according to the procedure described in Section 2.5.4.1.

Figure 11


300
200
100
nmoles NO_2^- produced/15 min/mg protein
0
30
60
90
120
min

the deoxycholate-treated fraction. This activity was not recovered even after desalting, either the treated P100 fraction or the S100 fraction obtained from it, through a Sephadex G-25 column (Table 18). On the other hand, the specific activity of the sulphite-linked nitrate reductase increased after incubation of the P100 fraction with sodium deoxycholate.

An attempt was made to restore the NADH-linked nitrate reductase activity in the deoxycholate-treated P100 fraction by supplementing the reaction mixture with various compounds, namely GSH, FMN, PMS, PMS plus mammalian cytochrome *c* or menadione (Table 19). FMN or GSH did not have any effect whereas PMS and menadione increased the NADH-linked enzyme activity of the deoxycholate treated P100 fraction by 40 and 70% respectively. The inclusion of mammalian cytochrome *c* along with PMS did not enhance the enzyme activity further.

Since menadione was the most effective in restoring the enzyme activity, the effects of graded amounts of menadione on the NADH-linked nitrate reductase activity of the deoxycholate-treated P100 fraction were examined. The results in Figure 12 show that the enzyme activity in this fraction was fully restored by 2.5 mM menadione. However, the enzyme activity of the untreated P100 fraction was progressively inhibited with increasing concentrations of menadione in the reaction mixture.

3.1.9 Purification of the sulphite-linked nitrate reductase and properties of a partially purified enzyme

3.1.9.1 Purification

All operations during the preparation of cell-free extracts

Table 18. Effect of sodium deoxycholate treatment on nitrate reductase activity with either sulphite or NADH as the electron donor.

P100 fraction, prepared as in Section 2.5.2.3, was incubated with 0.06 mg sodium deoxycholate/mg protein for 30 min at 30°. A portion of this preparation was passed through a Sephadex G-25 column (1.25 x 20 cm). The remainder was centrifuged at 100,000x g for 60 min and the supernatant fraction thus obtained was also passed through a Sephadex G-25 column as described above. Nitrate reductase activity in these preparations was determined with either sulphite or NADH as the electron donor as described in Section 2.5.4.1.

Fraction	Procedure	Specific activity (Electron donor)	
		Sulphite	NADH
I	P100 fraction.	300	340
II	P100 fraction treated with sodium deoxycholate for 30 min at 30°.	368	31.5
III	Fraction II passed through a Sephadex G-25 column.	318	22
IV	Supernatant fraction obtained after centrifuging fraction II at 100,000x g for 60 min.	453	24
V	Fraction IV passed through a Sephadex G-25 column and eluted with 0.05 M Tris-HCl buffer (pH 7.5).	428	16

Specific activity : nmoles NO_2^- produced/15 min/mg protein.

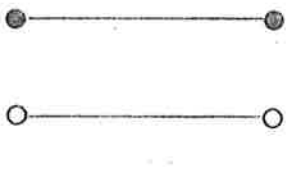
Table 19. Restoration of NADH-linked nitrate reductase in deoxycholate-treated P100 fraction.

P100 fraction was incubated with sodium deoxycholate and then passed through a Sephadex G-25 column (1.25 x 20 cm) as described in Table 18. Nitrate reductase activity in the pooled fractions from the Sephadex column was determined with NADH as the reductant. The reaction mixture was the same as described in Section 2.5.4.1 except that in addition it contained either GSH, FMN, PMS, mammalian cytochrome *c* or menadione at the concentrations given in the Table.

Addition to the basic reaction mixture	Final concentration (mM)	Specific activity (nmoles NO_2^- produced/15 min/mg protein)
None	-	22.7
GSH	2.0	25.0
FMN	0.1	23.0
Phenazine methosulphate (PMS)	0.5	127
PMS + cytochrome *c*	0.5 + 0.025	139
Menadione	1.0	235

Figure 12. Restoration by menadione of NADH-linked nitrate reductase activity in the deoxycholate-treated P100 fraction.

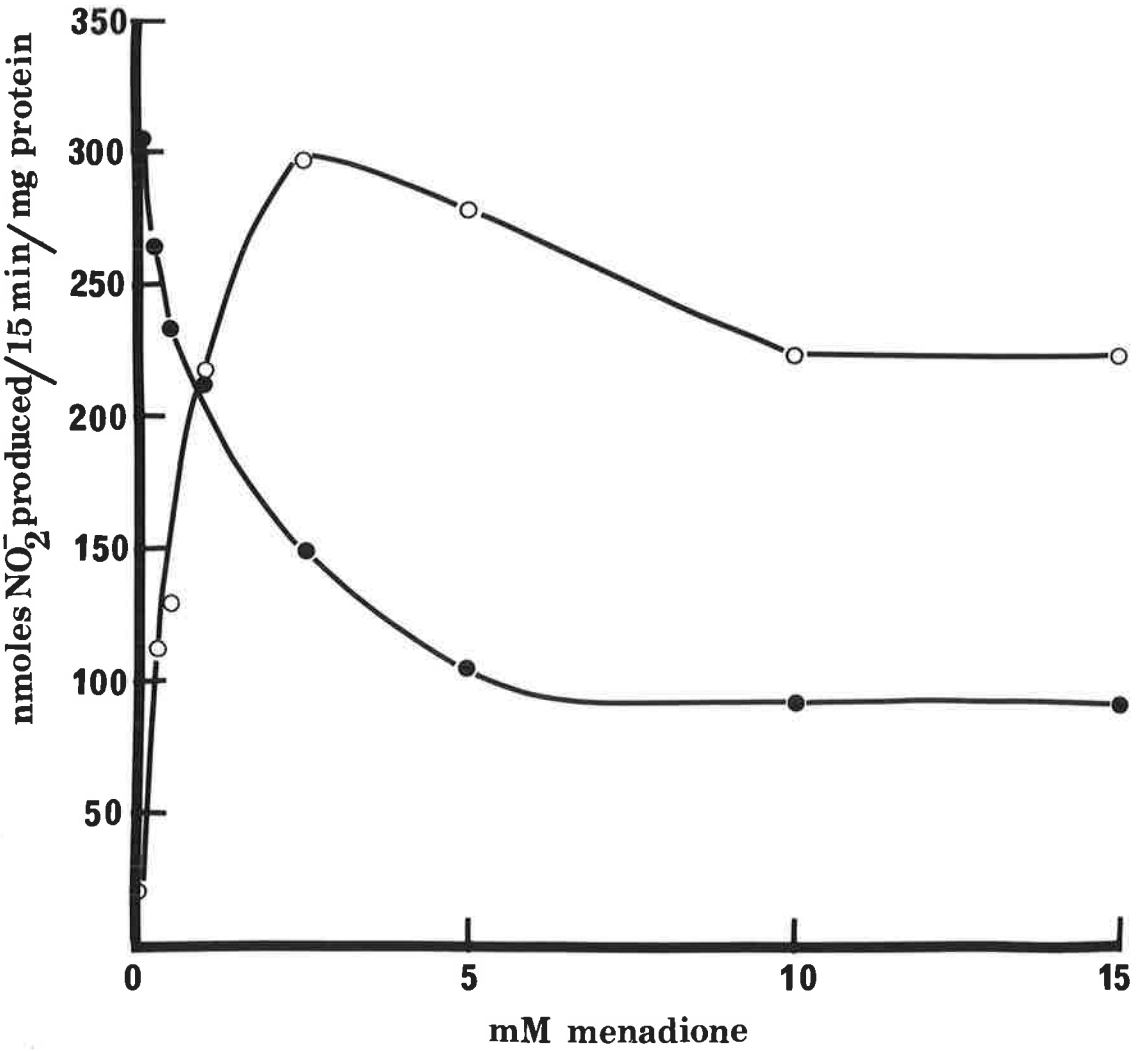
P100 fraction was treated with sodium deoxycholate at 30° for 30 min as described in Figure 10. Nitrate reductase activity in the untreated and the deoxycholate-treated P100 fractions was assayed, with NADH as the reductant, according to the procedure given in Section 2.5.4.1 except that specified concentrations of menadione were also included in the reaction mixture.



P100 fraction

deoxycholate-treated P100 fraction

Figure 12


350
300
250
200
150
100
50
nmoles NO_2^- produced/15 min/mg protein
0
5
10
15
mM menadione

and enzyme purification were carried out at 4° unless otherwise stated.

Washed cells, suspended (25%, w/v) in 0.05 M Tris-HCl buffer (pH 7.5), were disrupted in a French pressure cell as described in Section 2.5.2.1. The resultant homogenate was centrifuged at 10,000x g for 30 min and the supernatant fraction (Fraction I, Table 20) thus obtained, was further centrifuged at 100,000x g for 2 hr. The pellet fraction (P100) was suspended in 0.05 M Tris-HCl buffer (pH 7.5) using a glass homogenizer. This suspension was diluted with the same buffer until its protein concentration was 30 mg/ml. This fraction (Fraction II) was treated with an ultrasonic probe (20 Kc/sec) for 30 sec, and then incubated with sodium deoxycholate as described in Section 2.5.3. The deoxycholate-treated P100 fraction was centrifuged at 100,000x g for 60 min. The reddish-brown supernatant fraction (Fraction III, Table 20) was loaded onto a Sepharose 6B column (2.5 x 40 cm; void volume, 60 ml) which had been equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). The sample was eluted with the equilibrating buffer. The enzyme was recovered in the fractions immediately after the void volume of the column (Figure 13). The active fractions (11-18) were pooled (Fraction IV, Table 20).

The summary of this purification procedure given in Table 20 shows that a 6-fold purified enzyme, with an overall recovery of 84%, was obtained.

3.1.9.2 Properties

Effect of enzyme concentration. The enzyme activity was linear up to a protein concentration of 0.48 mg in the assay mixture (Figure 14).

Figure 13. Elution profile of nitrate reductase from a Sepharose 6B column.

Fraction III (Table 20) was loaded on to a Sepharose 6B column (2.5 x 40 cm) which had previously been equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). The sample was eluted with the same buffer at a flow rate of 20 ml/hr and 3 ml fractions were collected.

Nitrate reductase activity with SO_3^{2-} as an electron donor was determined in 1.0 ml aliquots of each fraction as described in Section 2.5.4.1. The protein concentration was determined from the absorbance at 280 nm.

O– – – – – – –O Nitrate reductase activity

●————————● E_{280}

Figure 13

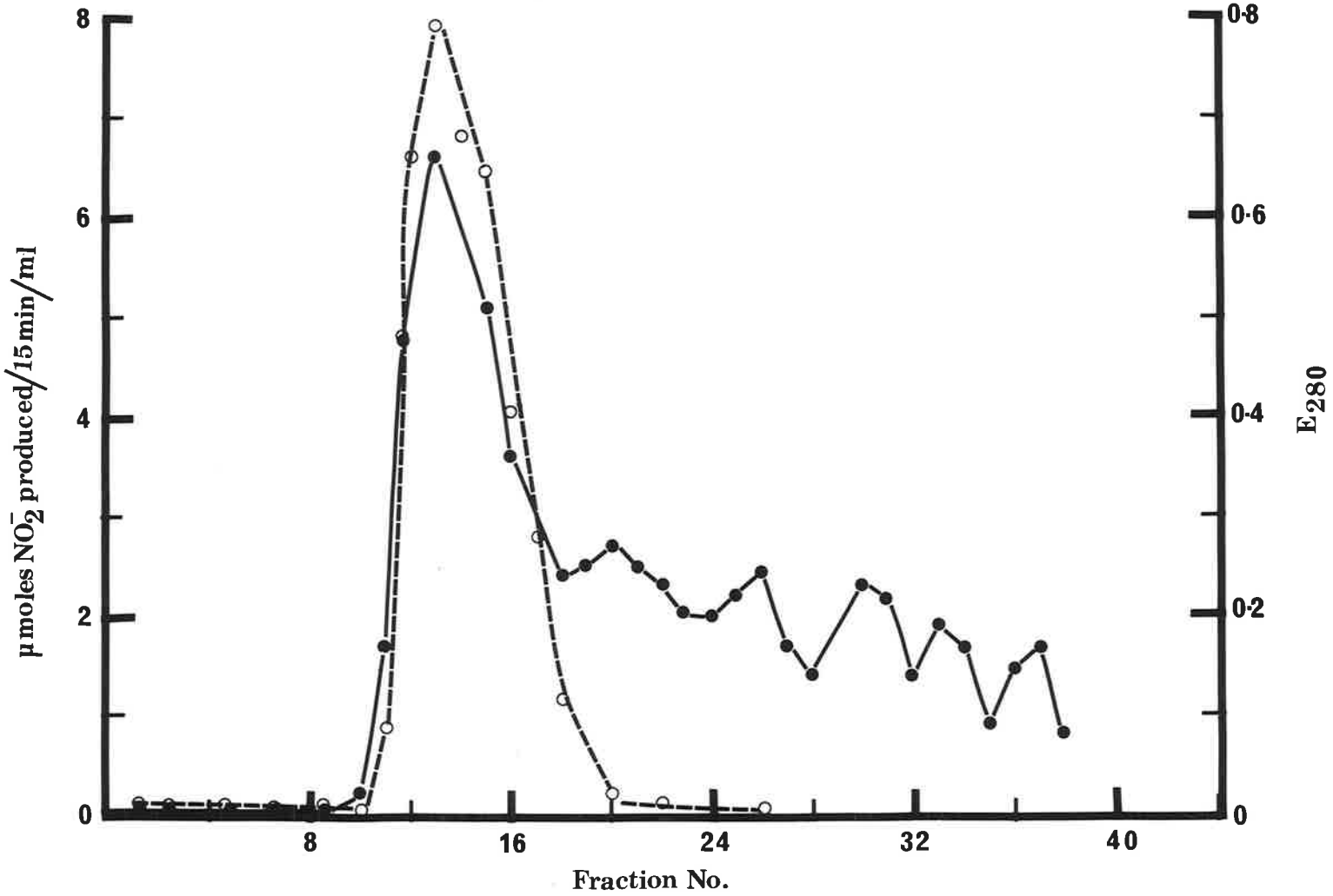

8
6
4
2
0
µmoles NO_2^- produced/15 min/ml
0·8
0·6
0·4
0·2
0
E_{280}
8
16
24
32
40
Fraction No.

Table 20. Purification of sulphite-linked nitrate reductase.

Enzyme activity was determined by measuring the rate of nitrite production as described in Section 2.5.4.1. Washed cells, suspended in 0.05M Tris-HCl buffer (pH 7.5), were disrupted in French pressure cell (Section 2.5.2.1). The supernatant fraction (S10), obtained after centrifuging the broken cells at 10,000x g for 30 min, was used as the starting material for the purification of nitrate reductase.

Total activity : µmoles NO_2^- produced/15 min.

Specific activity : µmoles NO_2^- produced/15 min/mg protein.

Table 20. Purification of sulphite-linked nitrate reductase.

Fraction	Procedure	Total activity	Total protein (mg)	Specific activity	Fold purification	% recovery
I	Supernatant fraction (S10) after centrifuging the disrupted cells at 10,000x g for 30 min	330.6	1827	0.18	1.0	
II	Particulate fraction (P100) left after centrifuging I at 100,000x g for 2 hr and suspended in 0.05 M Tris-HCl buffer (pH 7.5)	273.4	690	0.40	2.2	83
III	P100 fraction ultra-sonicated for 30 sec and treated with sodium deoxycholate (0.06 mg/mg protein) for 30 min at 30°C; supernatant fraction after centrifuging at 100,000x g for 60 min	254.0	370	0.69	3.8	77
IV	Fraction III eluted from a Sepharose-6B column with 0.05 M Tris-HCl buffer (pH 7.5)	276	260	1.1	5.9	84

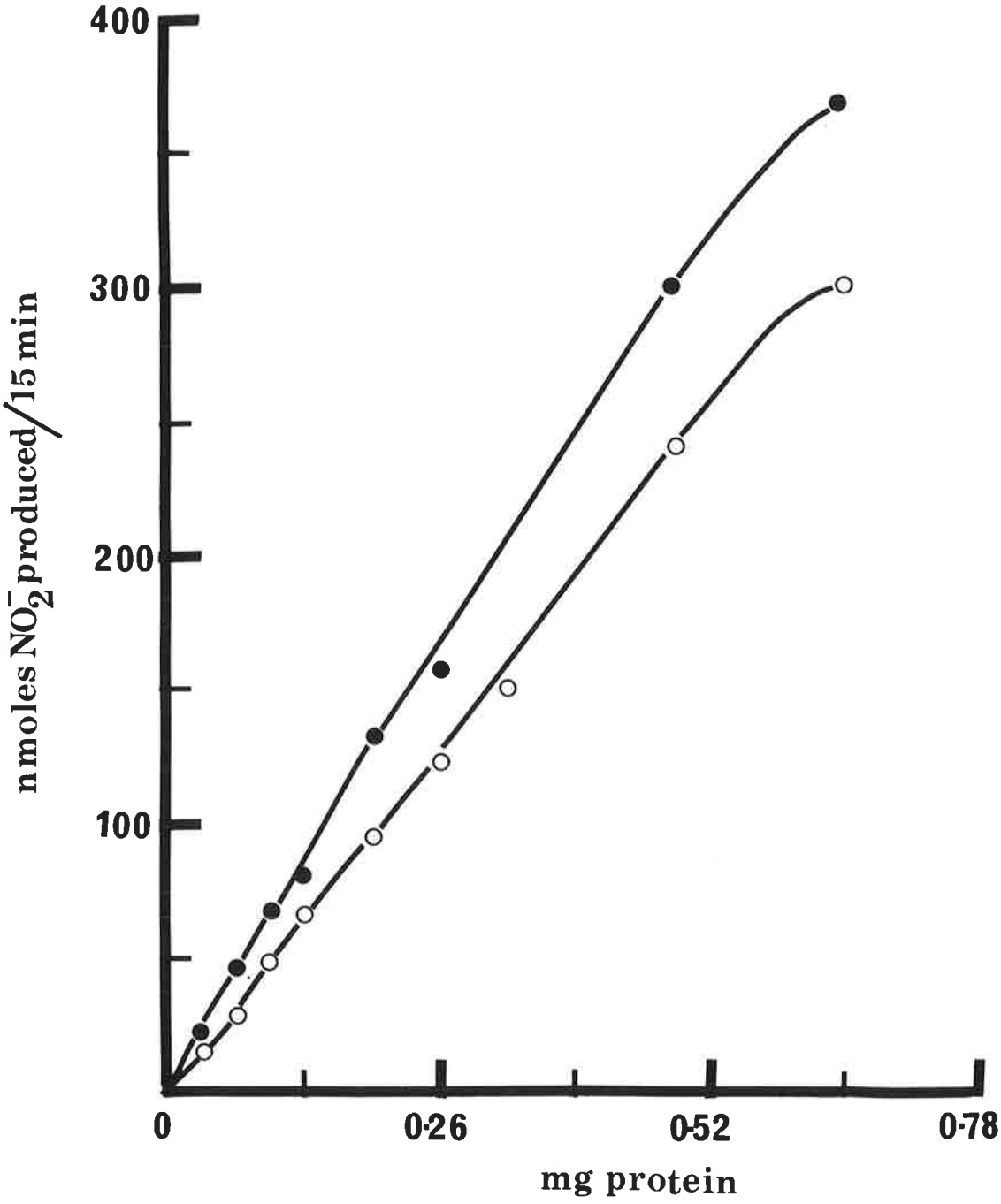
Figure 14. Effect of protein concentration on the sulphite-linked nitrate reductase activity.

Nitrate reductase activity, with SO_3^{2-} as the reductant, was determined as described in Section 2.5.4.1 except that the amount of the partially purified enzyme preparation (Fraction IV, Table 20) in the reaction mixture was varied over the range of 0-650 µg protein. The assay was done at the following concentrations of SO_3^{2-} and NO_3^-.

○——————○ 2 µmoles each of SO_3^{2-} and NO_3^-

●——————● 5 µmoles each of SO_3^{2-} and NO_3^-

Figure 14


400
300
200
100
n moles NO_2^- produced/15 min
0
0·26
0·52
0·78
mg protein

Stability of the enzyme during storage. The partially purified enzyme (Fraction IV, Table 20) was more stable at 0^{o} than at either 5^{o} or -15^{o}. Storing the enzyme preparation at 0^{o} and -15^{o} for 36 hr resulted in a loss of 59 and 92% of the enzyme activity respectively (Table 21). As compared to the partially purified enzyme, nitrate reductase activity in the P100 fraction was more stable since 80% of the initial activity was retained even after storing it at 0^{o} for one month.

Heat treatment. The partially purified enzyme was also more sensitive to heat treatment than the enzyme in P100 fraction. Incubation of the partially purified enzyme preparation at 50^{o} for 5 min resulted in complete loss of nitrate reductase activity and its activity was lowered by 80 and 30% after 15 min at 45 and 40^{o} respectively (Figure 15).

3.1.10 Characterization of cytochrome *c* and flavin isolated from nitrate reductase

3.1.10.1 Cytochrome *c*

The red-coloured fraction isolated from the solubilized nitrate reductase (Section 2.5.7) was identified as cytochrome *c* from its spectral and physical properties.

On reduction with sodium dithionite, it showed absorption bands at 417, 524 and 554 nm (Figure 16). This spectra corresponded to that of cytochrome *c* in the P100 fraction. The evidence for the participation of cytochrome *c* in nitrate reductase, as deduced from the spectral investigations, has already been presented (Figure 9). This isolated cytochrome *c*, in addition, was reduced enzymically by sulphite in the presence of nitrate reductase. The redox-potential of the isolated cytochrome *c*, determined as described in Section 2.6.4,

Table 21. Stability of the partially purified nitrate reductase on storage for 36 hr at 5^{o}, 0^{o} and -15^{o}.

Aliquots of the partially purified enzyme (Fraction IV, Table 20) were stored at the temperatures given in the Table. After 36 hr, the enzyme activity was assayed as described in Section 2.5.4.1. The results are expressed as % of the initial activity (before storage) which was 1060 nmoles NO_2^- produced/15 min/mg protein.

Storage temperature	% Initial activity
5^{o}	34
0^{o}	41
-15^{o}	8

Figure 15. Thermal stability of partially purified sulphite-linked nitrate reductase.

Aliquots of a partially purified nitrate reductase (Fraction IV, Table 20) were kept at either 40, 45 or 50°. Samples were withdrawn at the indicated times and after equilibrating at 30° for 5 min, were assayed for nitrate reductase activity according to the procedure given in Section 2.5.4.1.

The results are presented as % activity of the control sample (prior to heat treatment). Activity of the control was 1000 nmoles NO_2^- produced/15 min/mg protein.

□——————□ Pre-incubated at 40°

○——————○ Pre-incubated at 45°

●——————● Pre-incubated at 50°

Figure 15

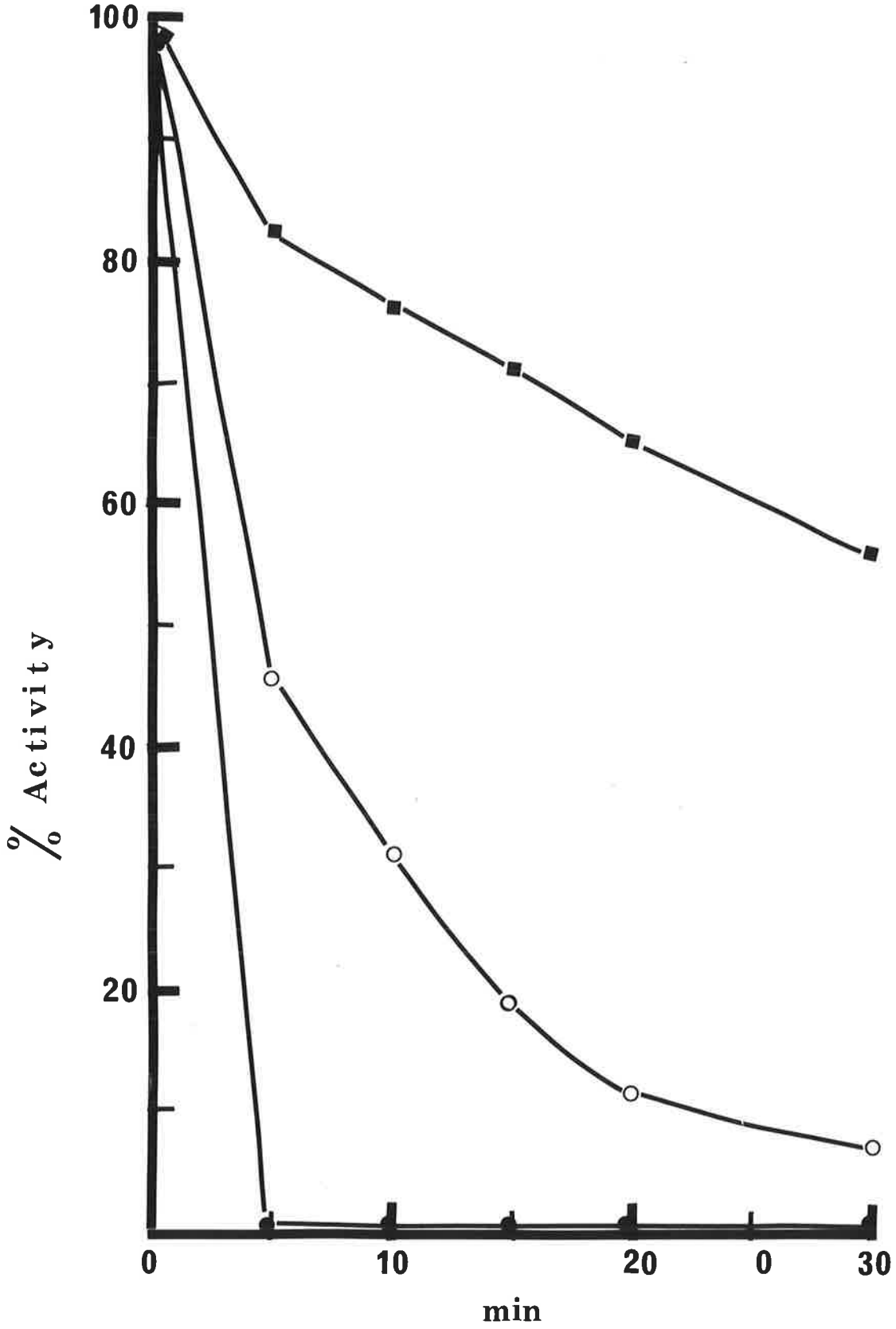
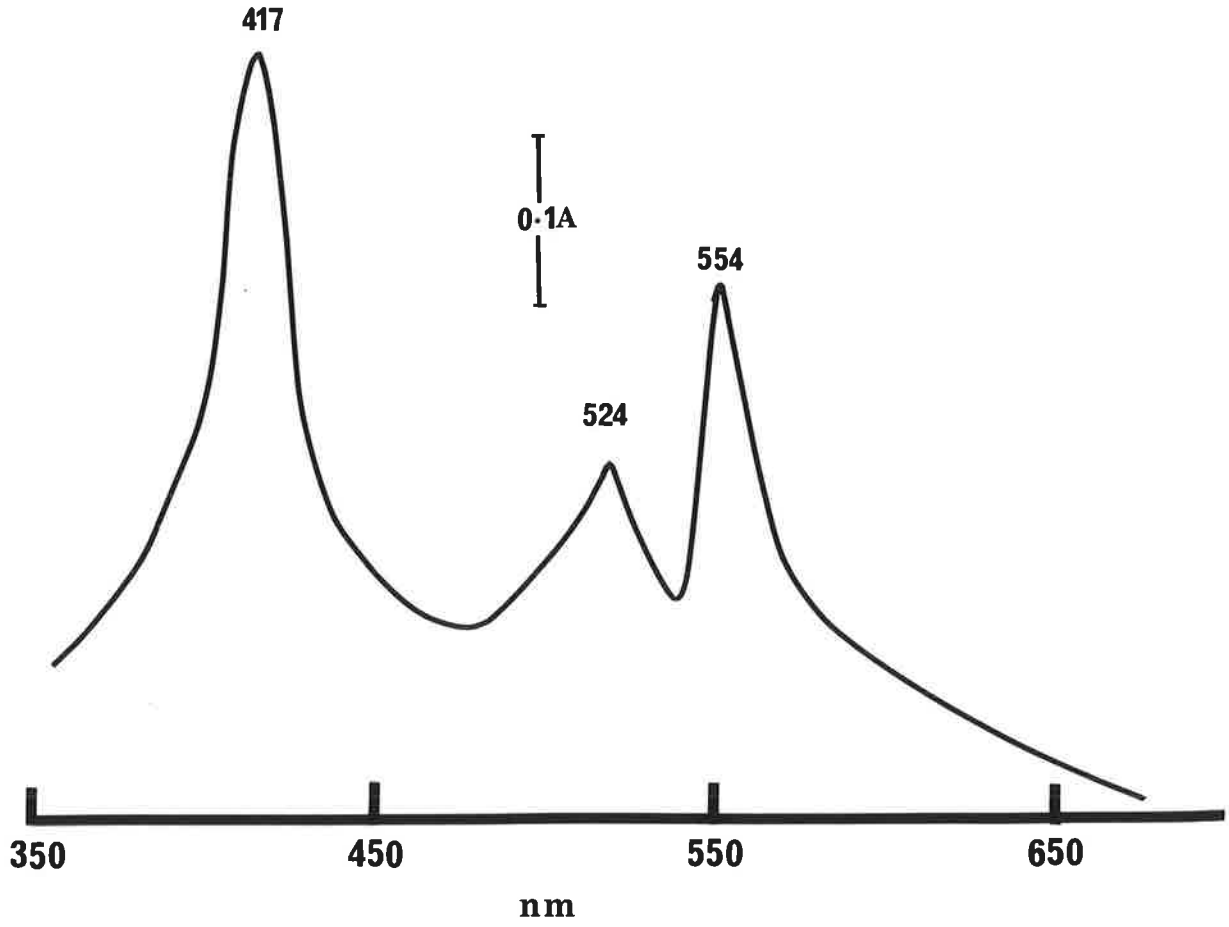


Figure 16. Spectra of sodium dithionite-reduced cytochrome c_{554}, separated from solubilized nitrate reductase.

Cytochrome c_{554} was separated from the solubilized nitrate reductase (Fraction III, Table 20) as described in Section 2.5.7. Its spectrum was recorded, against 0.05 M Tris-HCl buffer (pH 7.5), after reducing it with a crystal of $Na_2S_2O_4$.

Figure 16



was +0.223V. Its molecular weight, estimated by gel-filtration through Sephadex G-75, was 13,000. Thus, both its redox-potential and molecular weight were similar to those of mammalian cytochrome *c*.

This bacterial cytochrome *c*, in its reduced state, served as an effective electron donor for the reduction of nitrate by the solubilized enzyme (Fraction IV, Table 20). A plot of the enzyme activity against various concentrations of the reduced cytochrome *c* exhibited a typical Michaelis - Menten kinetics (Figure 17). The K_m value for the reduced cytochrome *c* was 1.53 μM which reflects a very high affinity of the enzyme for the cytochrome. However, the ability to reduce nitrate enzymically was not specific to the bacterial cytochrome *c* since, as shown in Figure 18, reduced mammalian cytochrome *c* was equally effective. The apparent K_m value for the mammalian cytochrome *c* was 1.33 μM.

3.1.10.2 Flavin

The yellow-coloured fraction isolated from nitrate reductase (Section 2.5.7) showed an absorption band at 450 nm which disappeared on adding sodium dithionite. These spectral properties indicated that it was a flavin compound.

The isolated flavin, along with authentic samples of FMN and FAD, were chromatographed on 3MM Whatman paper using t-butanol : water (60 : 40) as the solvent as described in Section 2.6.5. On examining the dried chromatograms under U.V. light, the flavin isolated from nitrate reductase was found to have co-migrated with standard FAD (Figure 19).

Figure 17. Effect of various concentrations of cytochrome c_{554} on nitrate reductase activity.

Cytochrome c_{554}, separated from solubilized nitrate reductase (Fraction III, Table 20) as described in Section 2.5.7, was reduced with $Na_2S_2O_4$ and the excess reductant was removed by passing it through a Sephadex G-25 column (1.2 x 15 cm). The reduced cytochrome c_{554} was used, in place of SO_3^{2-}, as an electron donor for the activity of solubilized nitrate reductase (Fraction III, Table 20) as described in Section 2.5.4.1.

A Lineweaver - Burk's plot of the effect of various concentrations of cytochrome c_{554} on the rate of nitrate reduction is presented in the inset.

Figure 17

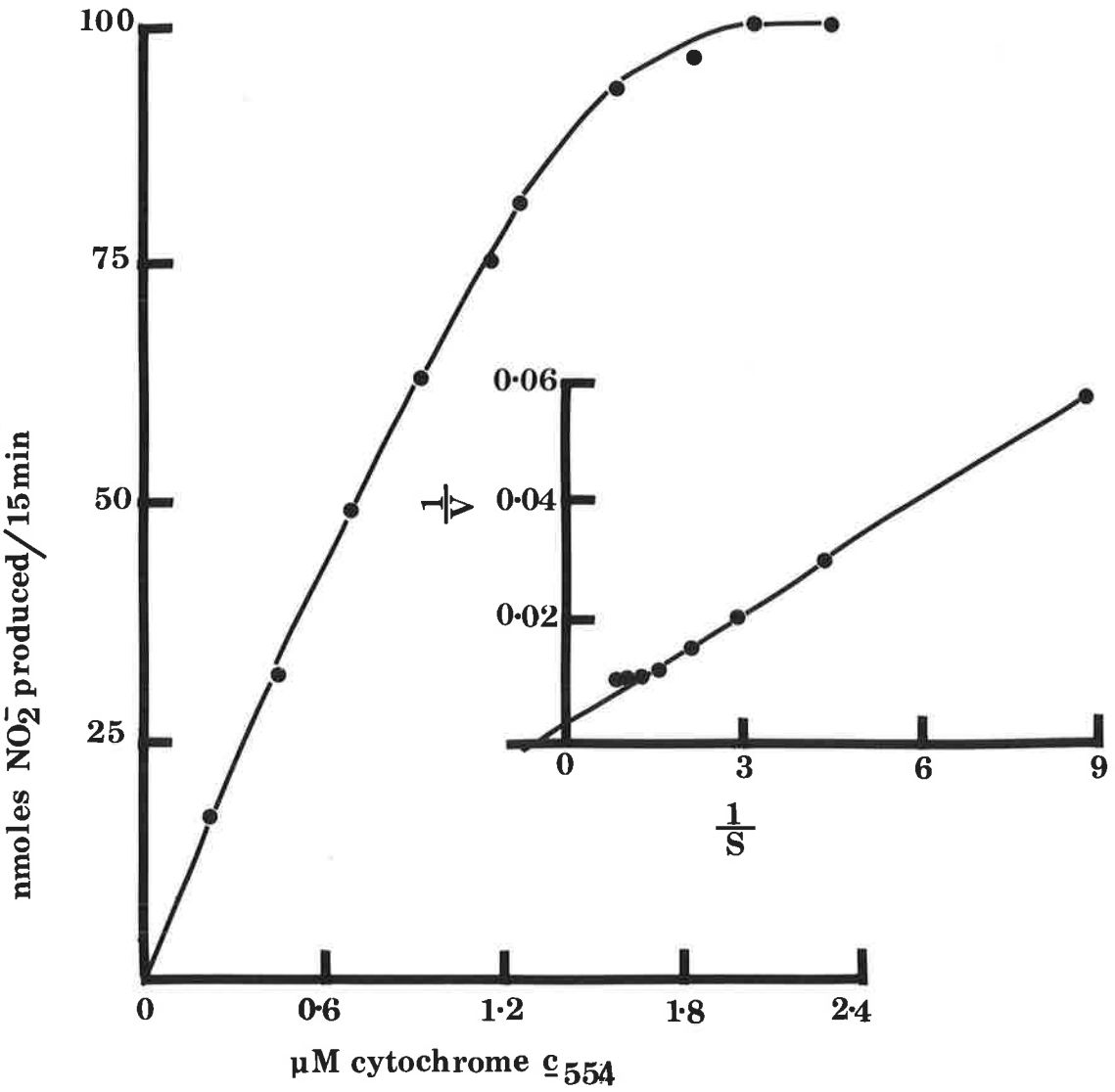

100
75
50
25
nmoles NO_2^- produced/15min
0
0·6
1·2
1·8
2·4
µM cytochrome c_{554}
0·06
0·04
0·02
$\frac{1}{V}$
0
3
6
9
$\frac{1}{S}$

Figure 18. Effect of different concentrations of mammalian cytochrome *c* on nitrate reductase activity.

Experimental details as given in Figure 17 except that reduced mammalian cytochrome *c* was used in place of bacterial cytochrome c_{554}.

Figure 18

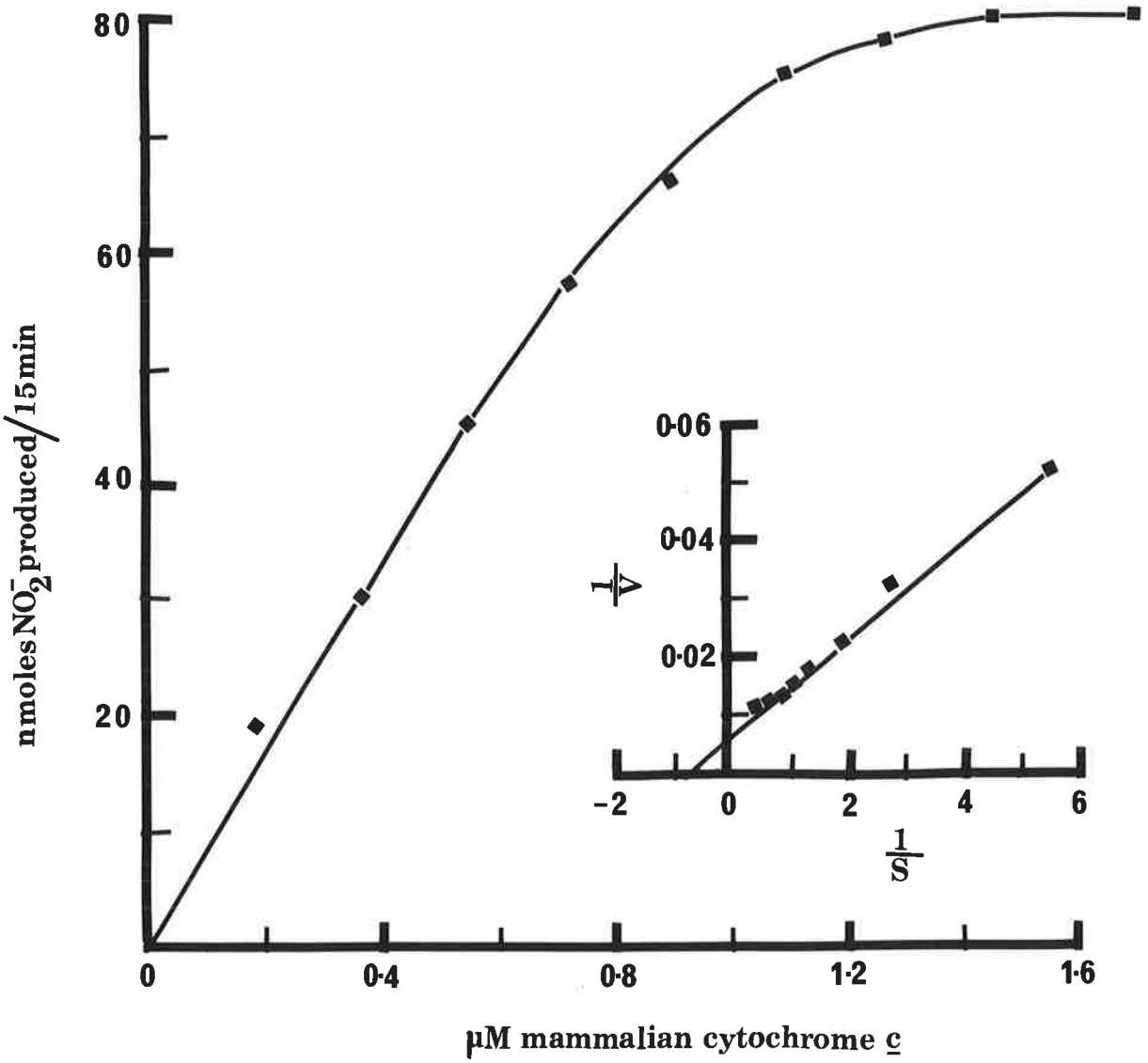

80
60
40
20
0
0·4
0·8
1·2
1·6
nmoles NO_2^- produced/15min
µM mammalian cytochrome c
0·06
0·04
0·02
$\frac{1}{V}$
-2
0
2
4
6
$\frac{1}{S}$

Figure 19. Paper chromatography of flavin isolated from nitrate reductase.

Flavin, from solubilized nitrate reductase (Fraction III, Table 20), was separated by DEAE-column chromatography as described in Section 2.5.7. The isolated flavin along with standards of FAD and FMN was subjected to paper chromatography according to the procedure given in Section 2.6.5.

A : Standard FAD
B : Standard FMN
C : Flavin from nitrate reductase.

Figure 20. High-voltage electrophoresis of the isolated flavin.

Flavin, separated from solubilized nitrate reductase (Section 2.5.7), was incubated at 30° with either snake venom pyrophosphatase for 30 min or with yeast pyrophosphatase for 30 and 60 min. The treated samples of the flavin, as well as an untreated one along with the standards of FMN and FAD, were spotted on to 3 MM Whatman paper. Their relative mobilities, after a high-voltage electrophoresis at 1500 volts for 1 hr, were determined (Section 2.6.6). The flavins were detected under U.V. light.

A : Standard FMN
B : Standard FAD
C : Flavin isolated from nitrate reductase
D : Flavin, from nitrate reductase, incubated with snake venom pyrophosphatase for 30 min
E : Flavin, from nitrate reductase, incubated with yeast pyrophosphatase for 30 min
F : As for E except the incubation time was 60 min.

Figure 19

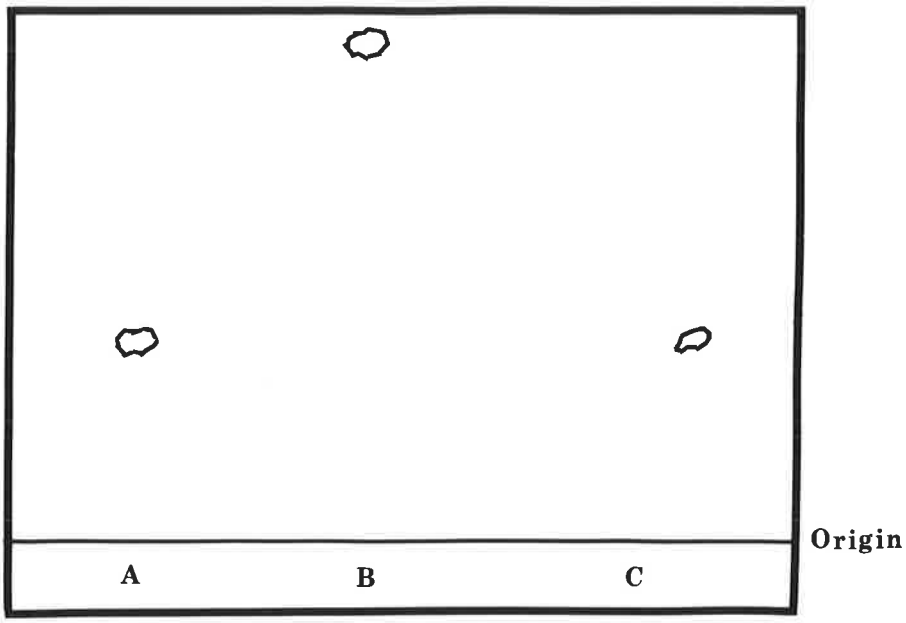
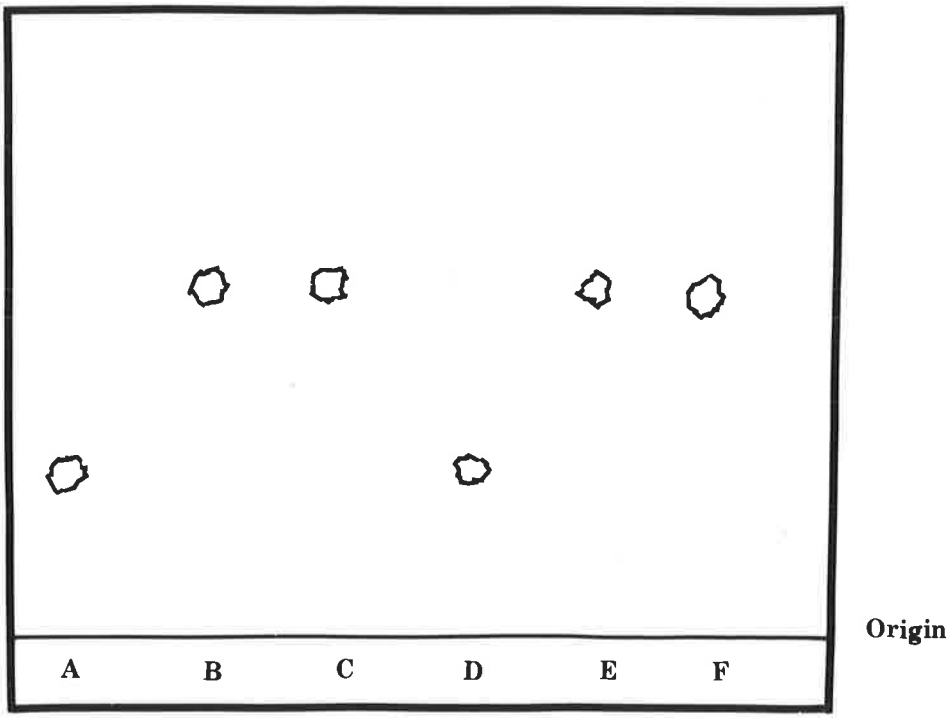


Figure 20



The identity of this flavin as FAD was further confirmed by incubating it with either snake venom pyrophosphatase or yeast pyrophosphatase. These treated samples of the flavin, along with the standards of FAD and FMN, were then subjected to a high voltage electrophoresis as described in Section 2.6.6. The results in Figure 20 show that the isolated flavin, treated with snake venom pyrophosphatase had co-migrated with FMN whereas the untreated sample or that incubated up to 60 min with yeast pyrophosphatase migrated the same distance as the standard FAD. Since snake venom pyrophosphatase hydrolyses FAD to FMN, these results provide additional evidence that the flavin isolated from nitrate reductase was FAD.

3.1.11 Regulation of NADH-linked nitrate reductase

3.1.11.1 Inhibition by NAD^+ and its reversal by NADH and menadione

NADH-linked nitrate reductase activity was progressively inhibited by increasing concentrations of NAD^+ in the reaction mixture as shown in Figure 21. The effect of NAD^+ was influenced by the concentration of NADH in the reaction mixture. Thus a 50% inhibition by 0.3mM NAD^+ in the presence of 0.5mM NADH was reduced to 20% when the concentration of NADH was raised to 1mM.

The inhibitory effect of NAD^+ on NADH-linked nitrate reductase was quite specific since $NADP^+$ did not affect the activity over the range 0.1 - 1.0mM.

The data in Figure 22 indicate that the enzyme activity was dependent on the ratio of NAD^+/NADH rather than on the absolute concentrations of either of the nucleotides. The enzyme activity was not affected when the ratio of NAD^+/NADH was less than 0.1. Above this

Figure 21. Effect of various concentrations of NAD^+ on NADH-linked nitrate reductase activity.

Cell-free extract (S18), prepared as described in Section 2.5.2.1, was dialysed overnight against 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM Na_2-EDTA. The assay procedure for determining the NADH-linked nitrate reductase activity was as described in Section 2.5.4.1. The reaction mixture in a final volume of 2 ml, contained the following: Tris-HCl buffer (pH 7.5), 80 µmoles; KNO_3, 1 µmole; 0.03 ml of dialysed cell-free extract (S18 containing 0.96 mg protein); NADH, either 1 or 2 µmoles as specified and the various concentrations of NAD^+ (0-2.4 µmoles).

The results are expressed as % inhibition with respect to control (without NAD^+) which was 125 nmoles NO_2^- produced/15 min/mg protein.

0.5 mM NADH

1.0 mM NADH

Figure 22. Effect of NAD^+/NADH ratio on NADH-linked nitrate reductase activity.

The data from Figure 21 are plotted as % activity of the control (without NAD^+) against NAD^+/NADH ratio.

0.5 mM NADH

1.0 mM NADH

Figure 21

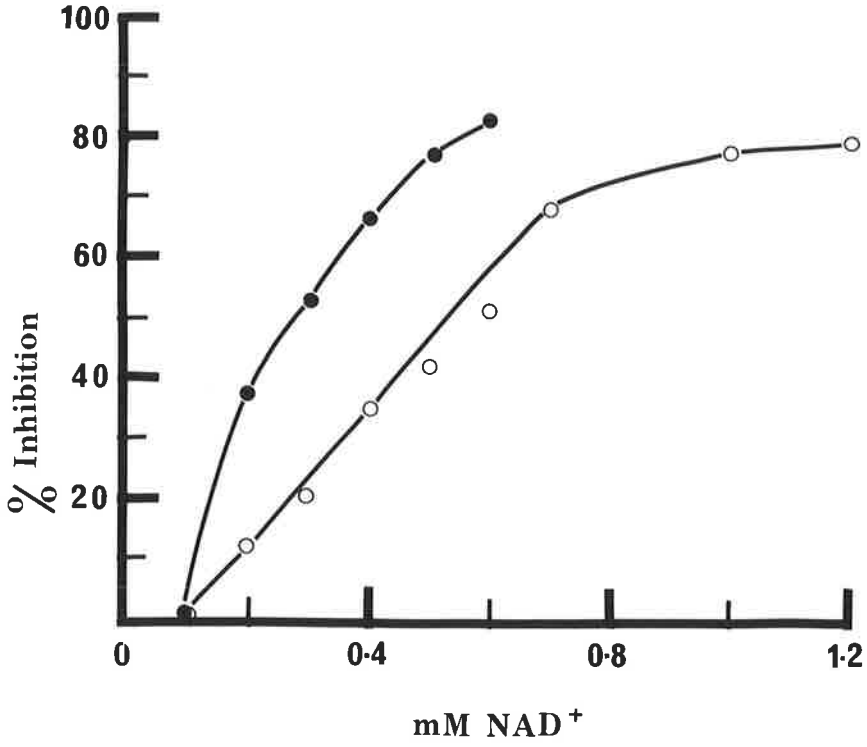
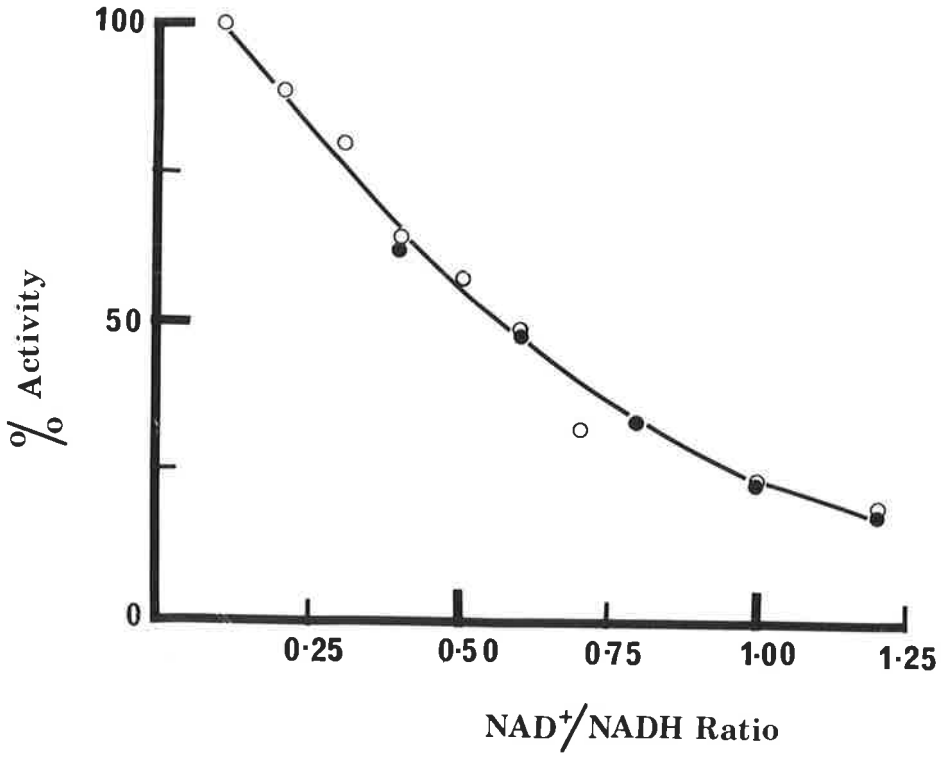


Figure 22



value, the enzyme activity was progressively inhibited and it was virtually inactive when the ratio was 1.2.

As shown in Figure 23, the enzyme activity was not a linear function of the concentrations of NADH. The production of nitrite was markedly stimulated by NADH over the range of 0.10 - 0.75mM. In the presence of NAD^+, this allosteric effect of NADH was, however, significantly suppressed and the maximum velocity also decreased with increasing concentrations of NAD^+.

The data of Figure 23 are presented as a double-reciprocal plot in Figure 24. Non-linear curves which were concave upwards were obtained in the presence of varying concentrations of NADH. However, the curves tended to approach linearity with increasing concentrations of NAD^+ in the reaction mixture. These data also show that the inhibition of the enzyme activity by NAD^+ was reversed by increasing concentrations of NADH. This inhibition by NAD^+ was also reversed by increasing concentrations of menadione (0.05 - 1.0mM) as shown in Figure 25.

The inhibition by NAD^+ was specific for NADH-linked nitrate reductase because even at 1mM, NAD^+ did not affect the sulphite-linked enzyme activity.

3.1.11.2 Effect of various adenine nucleotides

The effects of 5'-AMP, either alone or in the presence of 0.13mM NAD^+, on the NADH-linked nitrate reductase activity are illustrated in Figure 26. The enzyme activity was inhibited by 5'-AMP and at 10mM, it diminished the enzyme activity by more than 60%.

Similar concentrations of 5'-AMP did not affect the sulphite-linked nitrate reductase activity. In addition, neither 5'-ADP nor

Figure 23. Effect of NAD^+ on the NADH-linked nitrate reductase activity at various concentrations of NADH.

Nitrate reductase activity, in the presence of three fixed concentrations of NAD^+, was determined as described in Figure 21 except that the concentration of NADH in the reaction mixture was varied as indicated in the Figure.

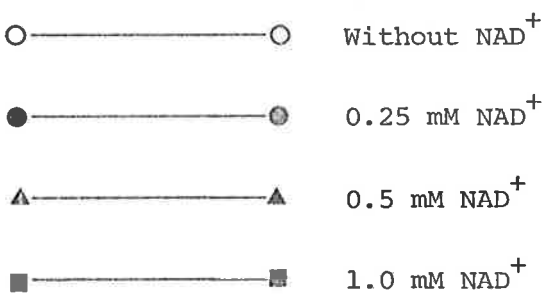


Figure 23

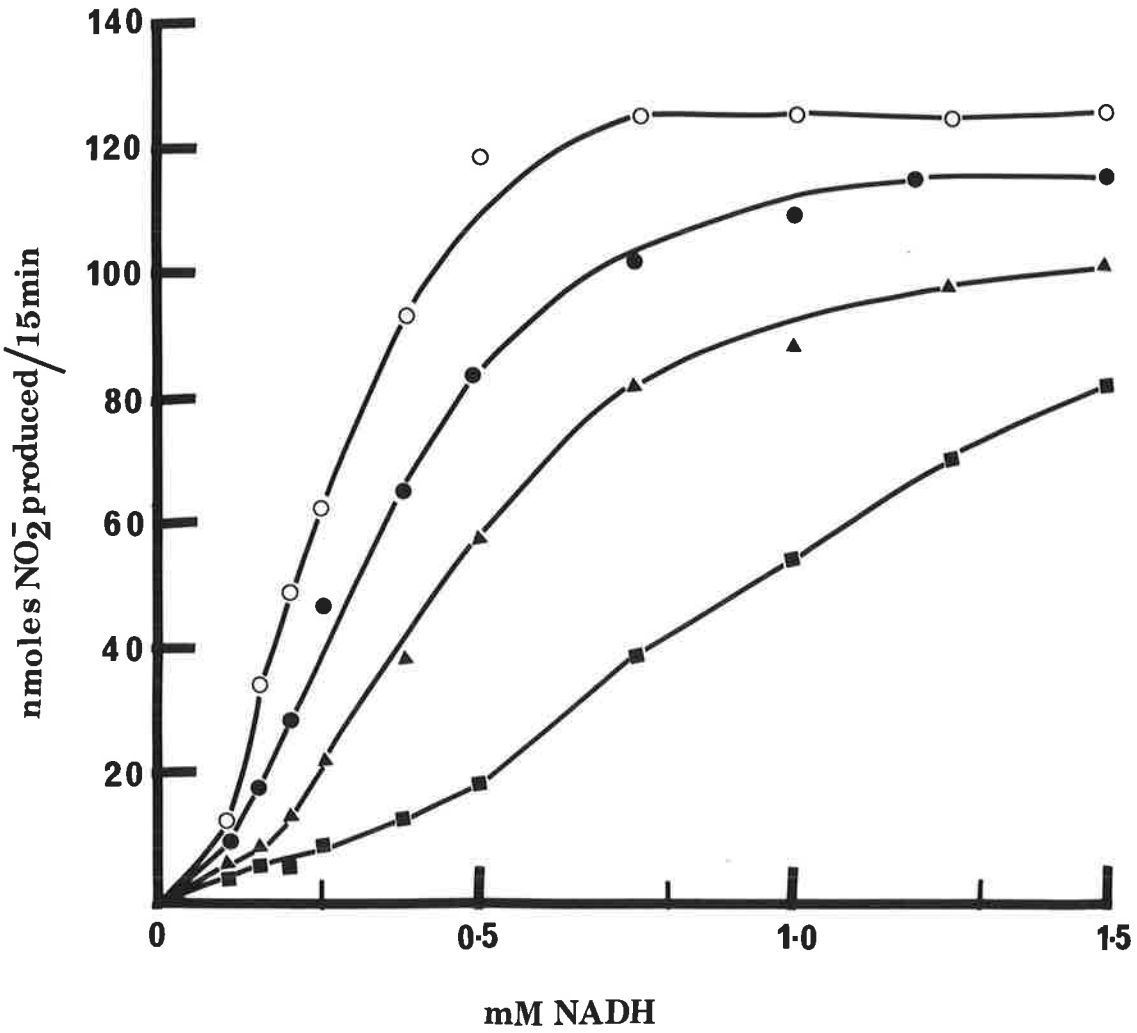

140
120
100
80
60
40
20
0
0·5
1·0
1·5
nmoles NO_2^- produced/15min
mM NADH

Figure 24. Double-reciprocal plot of the effect of NAD^+ at varying concentrations of NADH on nitrate reductase activity.

The data of Figure 23 are presented in the form of a double-reciprocal plot. $\frac{1}{V}$ represents the reciprocal of nmoles NO_2^- produced/15 min.

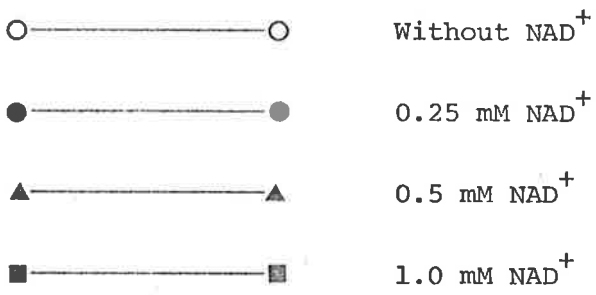


Figure 24

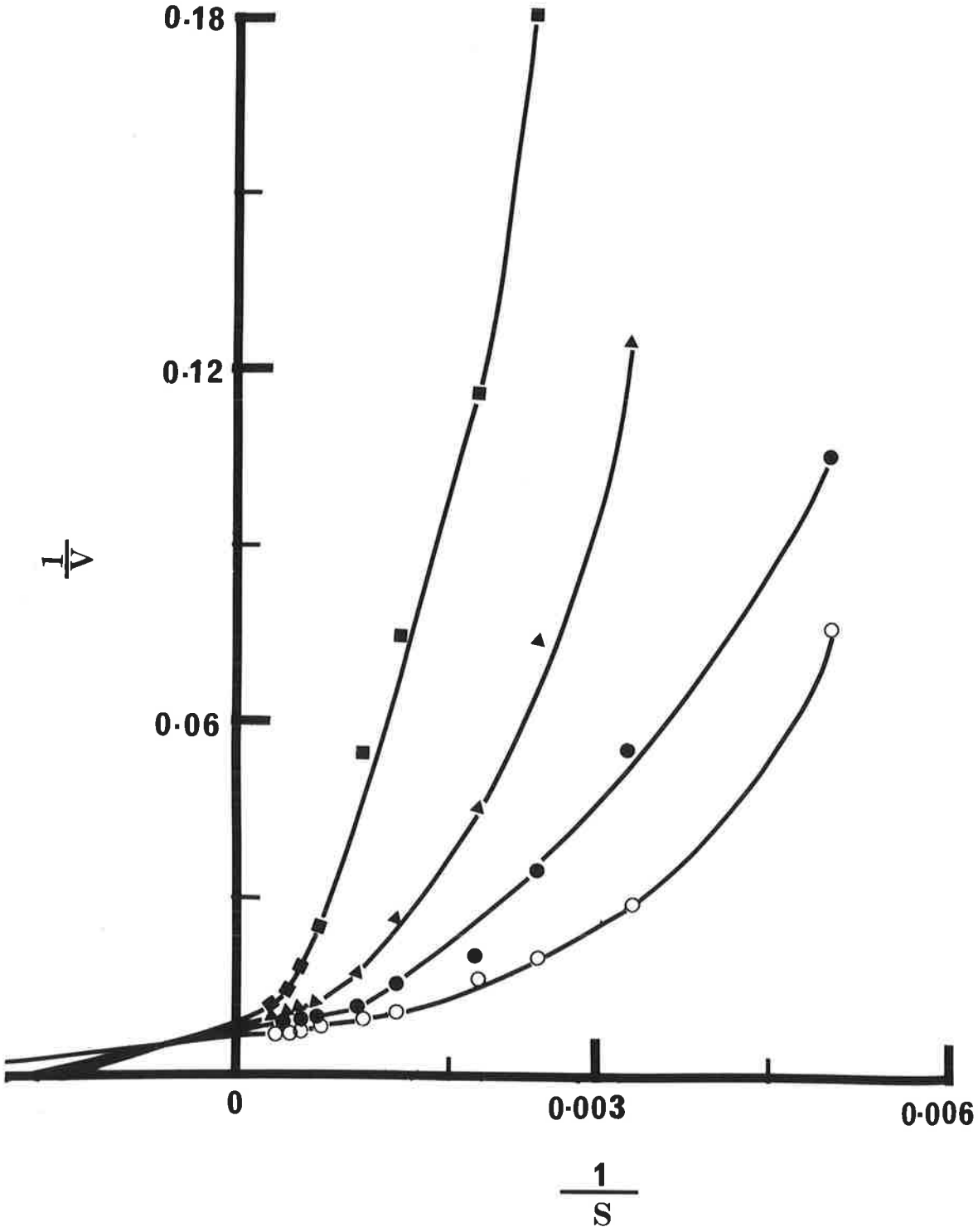


Figure 25. Reversal by menadione of the inhibition of the NADH-linked nitrate reductase by NAD^+.

The enzyme activity in the presence of 0.6 µmole NAD^+ was determined as described in Figure 21 except that specified concentrations of menadione were also included in the assay mixture.

Figure 25

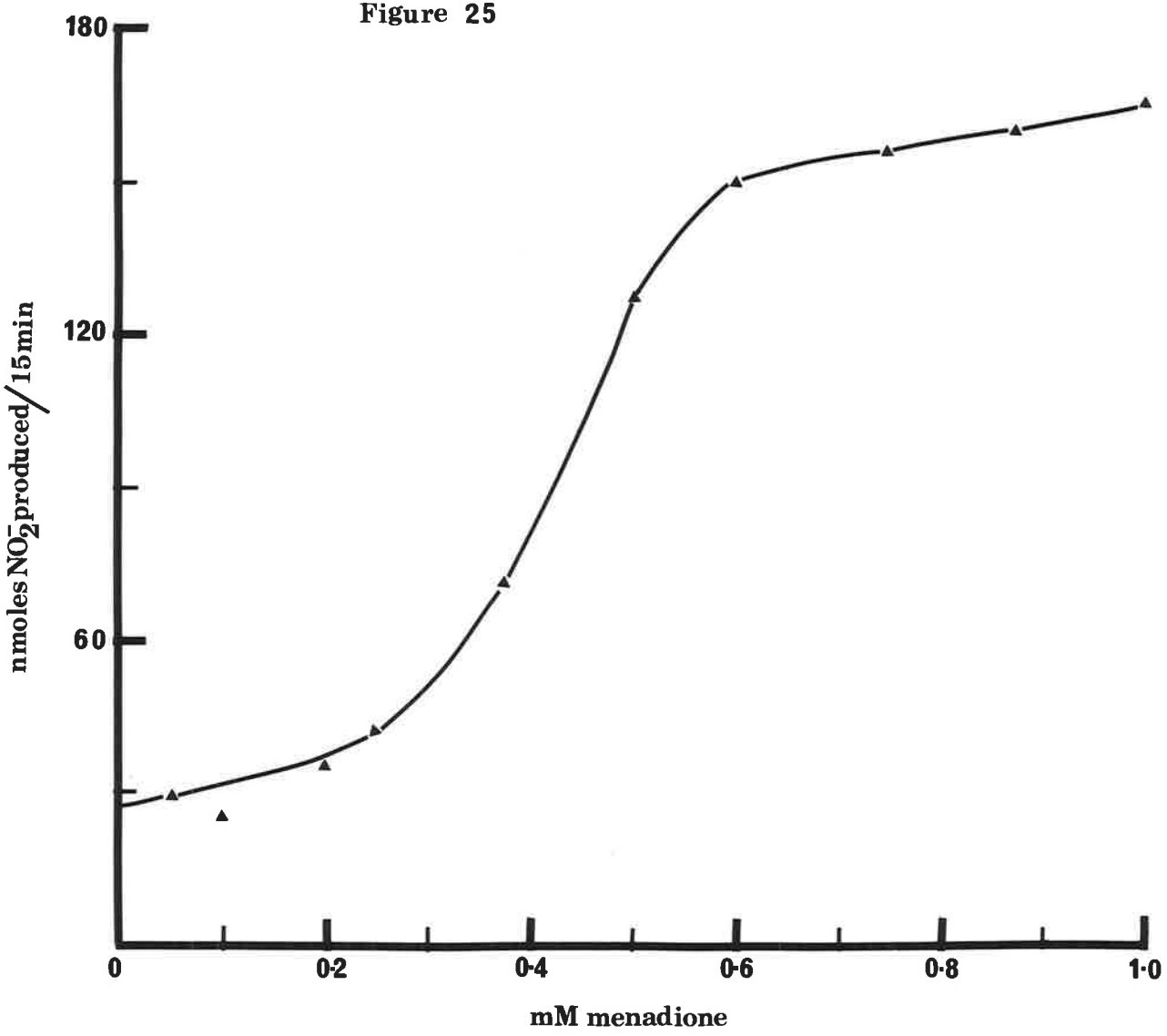

180
120
60
nmoles NO_2^- produced/15min
0
0·2
0·4
0·6
0·8
1·0
mM menadione

Figure 26. Effect of 5'-AMP on nitrate reductase activity.

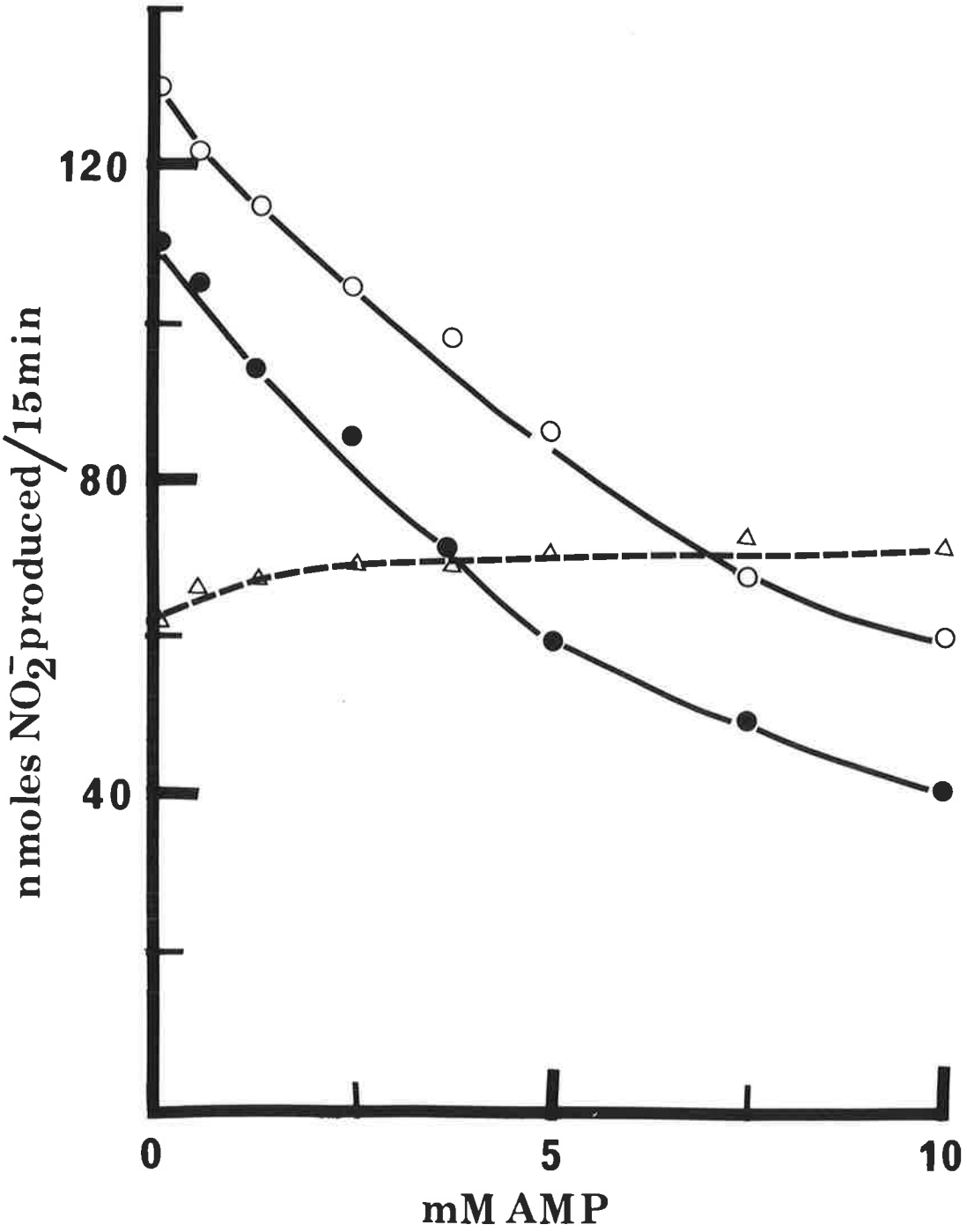
Nitrate reductase activity in the cell-free extract (S18), dialysed against 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM Na_2-EDTA, was determined in presence of specified concentrations of 5'-AMP with either SO_3^{2-} or NADH as the electron donor as described in Section 2.5.4.1. Wherever indicated, 0.6 µmoles NAD^+ was also included in the reaction mixture.

A. Δ – – – – – – – – Δ SO_3^{2-}-linked nitrate reductase

B. O ———————— O NADH-linked nitrate reductase

C. ● ———————— ● Same as B except that 0.3 mM NAD^+ was included in the reaction mixture.

Figure 26



5'-ATP had any effect on either the sulphite- or NADH-linked nitrate reductase activities. Other nucleotide monophosphates viz., 3'-AMP, 5'-UMP, 5'-GMP, 5'-CMP, 5'-TMP and nucleotide triphosphates such as 5'-CTP, 5'-GTP and 5'-UTP, at 5mM each, were also without effect on nitrate reductase activity with either NADH or sulphite as the electron donor. These results thus indicate that NADH-linked nitrate reductase was inhibited specifically by 5'-AMP. The inhibition of NADH-linked nitrate reductase by 5'-AMP was not reversed by either ATP or NADH. However, 0.5mM menadione completely reversed the inhibition by 5'-AMP.

3.1.11.3 Effect of adenylate charge on NADH-linked and sulphite-linked nitrate reductase activities

On varying the adenylate charge from 0.2 - 1.0 in the reaction mixture, the enzyme activity with either NADH or sulphite as an electron donor did not change significantly.

3.1.11.4 Effects of cations on the reversal of inhibition of NADH-linked nitrate reductase by NAD^+ and 5'-AMP

The effects of Mg^{2+} and some other cations on the inhibition of NADH-dependent nitrate reductase by NAD^+ and 5'-AMP are presented in Table 22. In presence of 5mM Mg^{2+}, a 66% inhibition of the enzyme activity by 0.5mM NAD^+ was reduced to 10% and that by 10mM 5'-AMP was lowered from 71 to 57%. Other divalent cations like Ca^{2+} and Ba^{2+} also reversed the inhibitory effect of NAD^+ by about 30%. The inhibition by 5'-AMP was reversed by 50% with Ba^{2+} but not with Ca^{2+}. Monovalent cations like Na^+ and K^+ were not effective in reversing the inhibition of NADH-linked nitrate reductase by either NAD^+ or 5'-AMP.

The inhibition of NADH-linked nitrate reductase by either NAD^+ or 5'-AMP was not influenced by metabolites of the tricarboxylic acid

Table 22. Effects of monovalent and divalent cations on the inhibition by NAD^+ and 5'-AMP of the NADH-linked nitrate reductase activity.

NADH-linked nitrate reductase activity in the dialysed cell-free extract (S18), in the presence of either 0.5 mM NAD^+ or 10 mM 5'-AMP was determined as described in Figure 21. In addition, various cations, as shown in the Table, were included in the reaction mixture. The results are presented as % activity with respect to control (without NAD^+ or 5'-AMP) which was 120 nmoles NO_2^- produced/15 min/mg protein.

Cation	Concentration (mM)	% Activity		
		Without NAD^+ or 5'-AMP	With 0.5 mM NAD^+	With 10 mM 5'-AMP
None	-	100	34	29
$MgCl_2$	5	121	101	53
$MgSO_4$	5	112	93	51
$BaCl_2$	5	110	73	89
$CaCl_2$	5	123	86	36
NaCl	5	103	34	31
	10	109	36	28
KCl	5	109	31	30
	10	110	33	28

cycle, e.g. α-ketoglutarate, oxaloacetate, malate, fumarate and succinate.

3.2 Nitrite reductase

3.2.1 Distribution of sulphide-linked nitrite reductase

The distribution of sulphide-linked nitrite reductase in various cell fractions is shown in Table 23. The supernatant fraction (S100), obtained after centrifuging the crude extract (S10) at 100,000x g for 90 min, had very low enzyme activity. The bulk of the enzyme activity was recovered in the pellet fraction (P100).

3.2.2 Various electron donors

3.2.2.1 Sulphur compounds

Since in P100 fraction nitrite was reduced with sulphide, the effectiveness of some other sulphur compounds for nitrite reduction was also examined. Results in Table 24 show that nitrite was not reduced in the presence of either tetrathionate, thiosulphate or glutathione. Sulphite and thiosulphate plus GSH showed less than 5% of the activity observed with sulphide.

3.2.2.2 Other reductants

The results in Table 25 show that NADH was not an effective electron donor for nitrite reduction in the pellet fraction (P100). However, a significant enzyme activity with NADH was observed when either menadione or PMS was included in the reaction mixture. Similarly ascorbate alone was ineffective but in the presence of either 2,6-dichlorophenol indophenol (DCIP), N,N,N',N'-tetramethylparaphenyl-enediamine (TMPD) or PMS, nitrite reductase activity was markedly increased.

Table 23. Distribution of sulphide-linked nitrite reductase.

The various cell fractions (S10, S100 and P100) were prepared as described in Sections 2.5.2.1 and 2.5.2.3. Nitrite reductase activity, with sulphide as an electron donor, was determined in test-tubes covered with subaseals according to the procedure given in Section 2.5.4.2.

Fraction	Procedure	Specific activity (nmoles NO_2^- utilized/15 min/mg protein)
I	Homogenate obtained after disrupting the washed cells in French pressure cell at 20,000 p.s.i.	155.6
II	Supernatant fraction (S10) obtained by centrifuging the broken cells at 10,000x g for 30 min.	147.6
III	Supernatant fraction (S100) after centrifuging II at 100,000x g for 90 min.	26.0
IV	Pellet fraction (P100) from III, dispensed in 0.05M Tris-HCl buffer (pH 7.5).	428.3

Table 24. Nitrite reductase activity with various sulphur compounds as electron donors.

P100 fraction was used for determining the enzyme activity. The reaction mixture in a final volume of 1.0 ml contained the following, in µmoles: Tris-HCl buffer (pH 7.5), 35; $NaNO_2$, 1; sulphur compounds (as electron donors), 2; and enzyme preparation (P100 fraction containing 3 mg protein). After a 15 min incubation at 30^{o}, the reaction was stopped with 1 ml of 10% (w/v) zinc acetate in 10% (v/v) ethanol except for the incubation mixture containing thiosulphate to which 10% (w/v) mercuric chloride was also added to precipitate thiosulphate.

Sulphur compound	Specific activity (nmoles NO_2^- utilized/15 min/mg protein)
$S_4O_6^{2-}$	0
$S_2O_3^{2-}$	8
GSH	0
$S_2O_3^{2-}$ + GSH	22
SO_3^{2-}	22
S^{2-}	466

Table 25. Nitrite reductase activity with various electron donors.

Details of the reaction mixture and the procedure for determining the enzyme activity in P100 fraction were as described in Section 2.5.4.2. The reaction was started by injecting the electron donor through the subaseal caps fitted on to the test-tubes. MV, BV, FMN, FAD and mammalian cytochrome *c* were reduced with 7.5 µmoles of freshly prepared solution of $Na_2S_2O_4$ in 1.0% (w/v) sodium bicarbonate.

Electron donor	Final concentration (mM)	Specific activity (nmoles NO_2^- utilized/15 min/mg protein)
NADH	1	88
	2	88
NADH + PMS	2 + 2	444
NADH + menadione	2 + 2	1074
NADPH	2	0
$Na_2S_2O_4$	2	0
MVH	0.4	33
BVH	0.4	44
$FMNH_2$	0.2	0
$FADH_2$	0.2	0
Sodium ascorbate	2	94
Ascorbate + 2,6-dichloro-phenol indophenol (DCIP)	2 + 0.15	311
Ascorbate + TMPD	2 + 2	570
Ascorbate + PMS	2 + 2	583
Reduced cytochrome *c*	0.15	155

A relatively small activity was observed with reduced mammalian cytochrome *c*. Other reductants like sodium dithionite, reduced viologen dyes (BVH, MVH) and reduced flavins ($FMNH_2$, $FADH_2$) were ineffective. The maximum enzyme activity was recorded with NADH plus menadione which was two-fold higher than with sulphide. The rate of nitrite reduction with other electron donating systems such as NADH plus PMS or ascorbate plus either DCIP, TMPD or PMS were almost equivalent to that obtained with sulphide.

Since, menadione promoted nitrite reductase activity with NADH as an electron donor, the effect of various concentrations of this carrier on the enzyme activity was examined. As shown in Figure 27, the enzyme activity increased proportionally up to 1mM menadione in the reaction mixture.

3.2.3 Optimum conditions for enzyme activity

3.2.3.1 Effect of incubation time

The time course for nitrite reductase activity in the P100 fraction is illustrated in Figure 28. Utilization of nitrite was linear up to 15 min. In all the subsequent experiments an incubation time of 15 min was used.

3.2.3.2 Effect of enzyme concentration

The effect of varying enzyme concentrations (P100 fraction) on the rate of nitrite reduction at two concentrations of nitrite and sulphide is illustrated in Figure 29. In the presence of sulphide and nitrite, each at 2mM, the enzyme activity was linear up to 3.5 mg protein. However, with 5mM each of sulphide and nitrite, the enzyme activity was linear up to 10 mg protein.

Figure 27. Effect of various concentrations of menadione on nitrite reductase activity with NADH as an electron donor.

The reaction mixture was as described in Section 2.5.4.2 except that in addition it contained menadione at the concentrations given in the Figure. The reaction was started by injecting 1 µmole NADH. After 15 min incubation, the reaction was terminated by adding 1 ml of cold 10% (w/v) zinc acetate in 10% (v/v) ethanol and the residual NO_2^- was determined, in a suitable aliquot of the supernatant, according to the method given in Section 2.7.2.

Figure 27

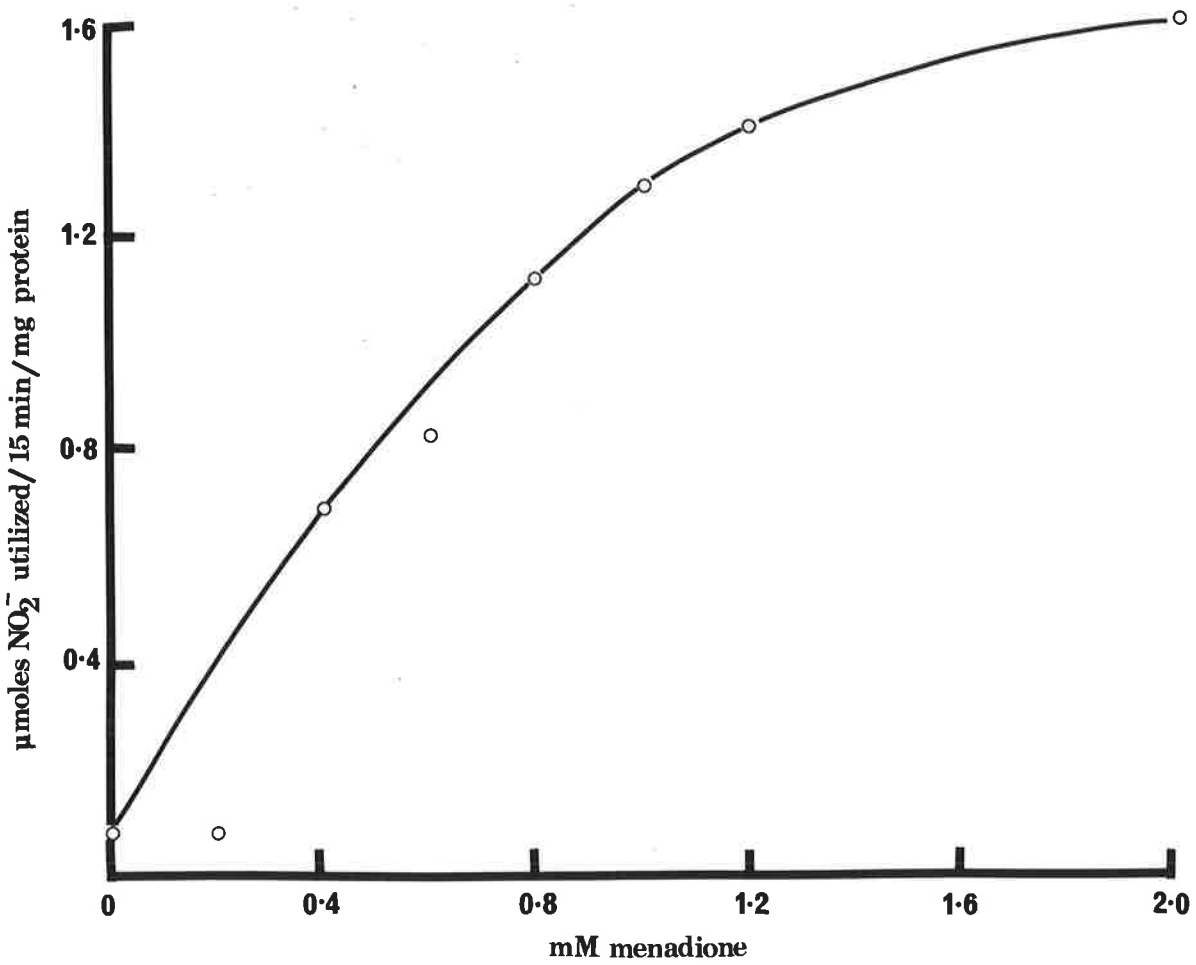

1·6
1·2
0·8
0·4
µmoles NO_2^- utilized/15 min/mg protein
0
0·4
0·8
1·2
1·6
2·0
mM menadione

Figure 28. Effect of incubation time on the sulphide-linked nitrite reductase activity.

The assay was carried out in a Warburg flask fitted with a stopcock as described in Section 2.5.4.2. Reaction mixture in a final volume of 2.5 ml contained the following, in µmoles: Tris-HCl buffer (pH 7.5), 87.5; $NaNO_2$, 2.5 and 0.25 ml enzyme preparation (P100 fraction containing 8.3 mg protein). The flask was evacuated and flushed with N_2 gas. After equilibrating at 30° for 5 min, 5.0 µmoles of freshly prepared solution of sulphide was injected through the subaseal fitted on to the side arm of the Warburg flask. At the times indicated, 100 µl aliquots were withdrawn with an airtight microsyringe and mixed immediately with 100 µl of cold 10% (w/v) zinc acetate solution in 10% (v/v) ethanol. After centrifuging, the residual NO_2^- was determined in 100 µl of the supernatant as described in Section 2.7.2.

Figure 28

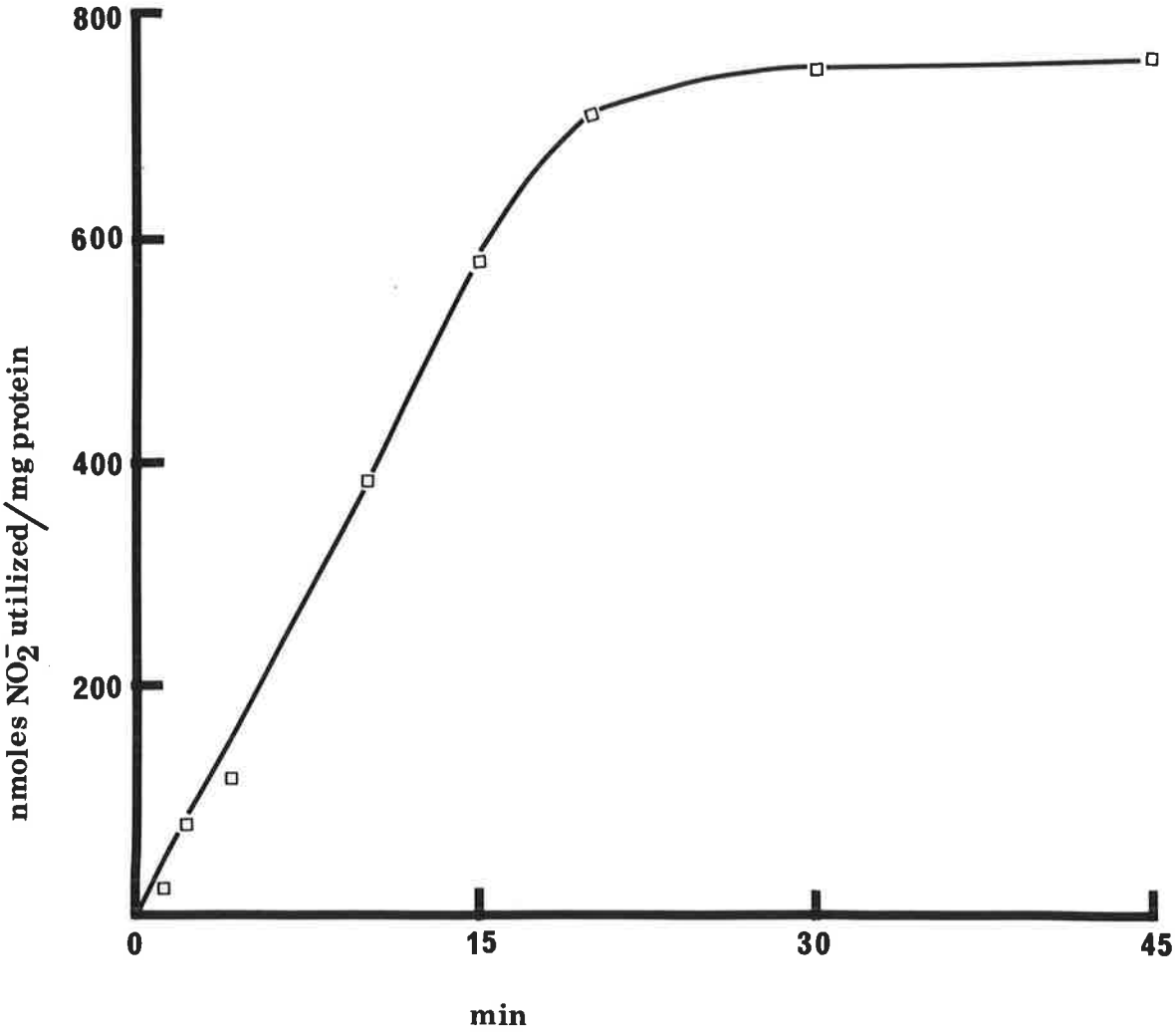


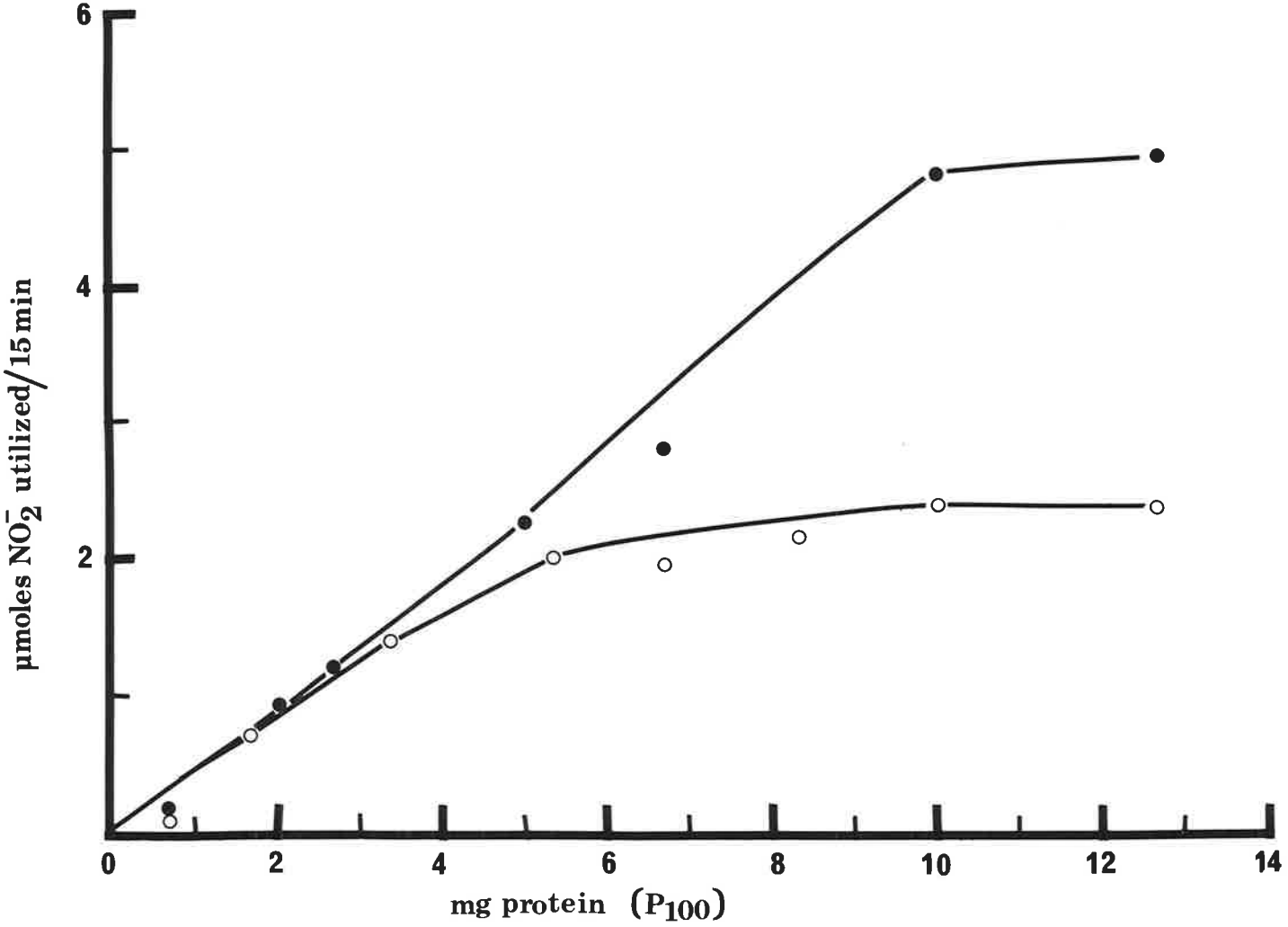
Figure 29. Effect of varying protein concentrations on the sulphide-linked nitrite reductase activity.

The enzyme activity, with S^{2-} as an electron donor, was determined as described in Section 2.5.4.2 except that various amounts of P100 fraction (containing 30 mg protein/ml) were used and the concentrations of NO_2^- and S^{2-} in the reaction mixtures were:

○——————○ 2 µmoles each of S^{2-} and NO_2^-

●——————● 5 µmoles each of S^{2-} and NO_2^-

Figure 29



3.2.3.3 Effect of pH

Nitrite reductase activity showed a broad pH optimum over the range of 6.0 - 7.0 (Figure 30).

3.2.3.4 Heat stability

The effect of heat treatment on the sulphide-linked nitrite reductase is shown in Figure 31. Incubation of the enzyme (P100 fraction) at 70^{o} for 30 min resulted in a 70% loss of activity while at 50^{o} and 40^{o}, the enzyme activity was diminished by 55 and 20% respectively.

3.2.4 K_m values for sulphide and nitrite

At a fixed concentration of 1mM sodium nitrite, the enzyme activity increased proportionally with increasing concentrations of sulphide up to 2mM. However, sulphide at concentrations exceeding 3mM inhibited the enzyme activity (Figure 32). At 1Mm nitrite, the K_m value for sulphide was 1.25mM.

In the presence of 2mM sulphide, nitrite reductase activity was linear up to 1mM nitrite. The apparent K_m value for nitrite, at a fixed concentration of 2mM sulphide, was 1.6mM (Figure 33).

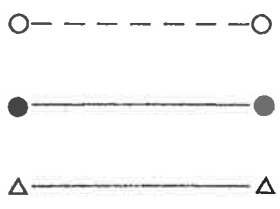
3.2.5 Characterization of the electron-transport chain involved in nitrite reduction

3.2.5.1 Effects of inhibitors

The effects of various inhibitors on nitrite reductase activity in the pellet fraction (P100) were studied to identify the components of the respiratory chain involved in the reduction of nitrite. In addition to sulphide as a donor, the effects of various

Figure 30. Effect of pH on the sulphide-linked nitrite reductase.

The enzyme activity, in P100 fraction, was determined as described in Section 2.5.4.2 except that 0.05 M of either phosphate, Tris-HCl or Tris-maleate buffer of indicated pH was used. The pH values given in the Figure correspond to the final pH of the reaction mixture.



Phosphate

Tris-maleate

Tris-HCl

Figure 30

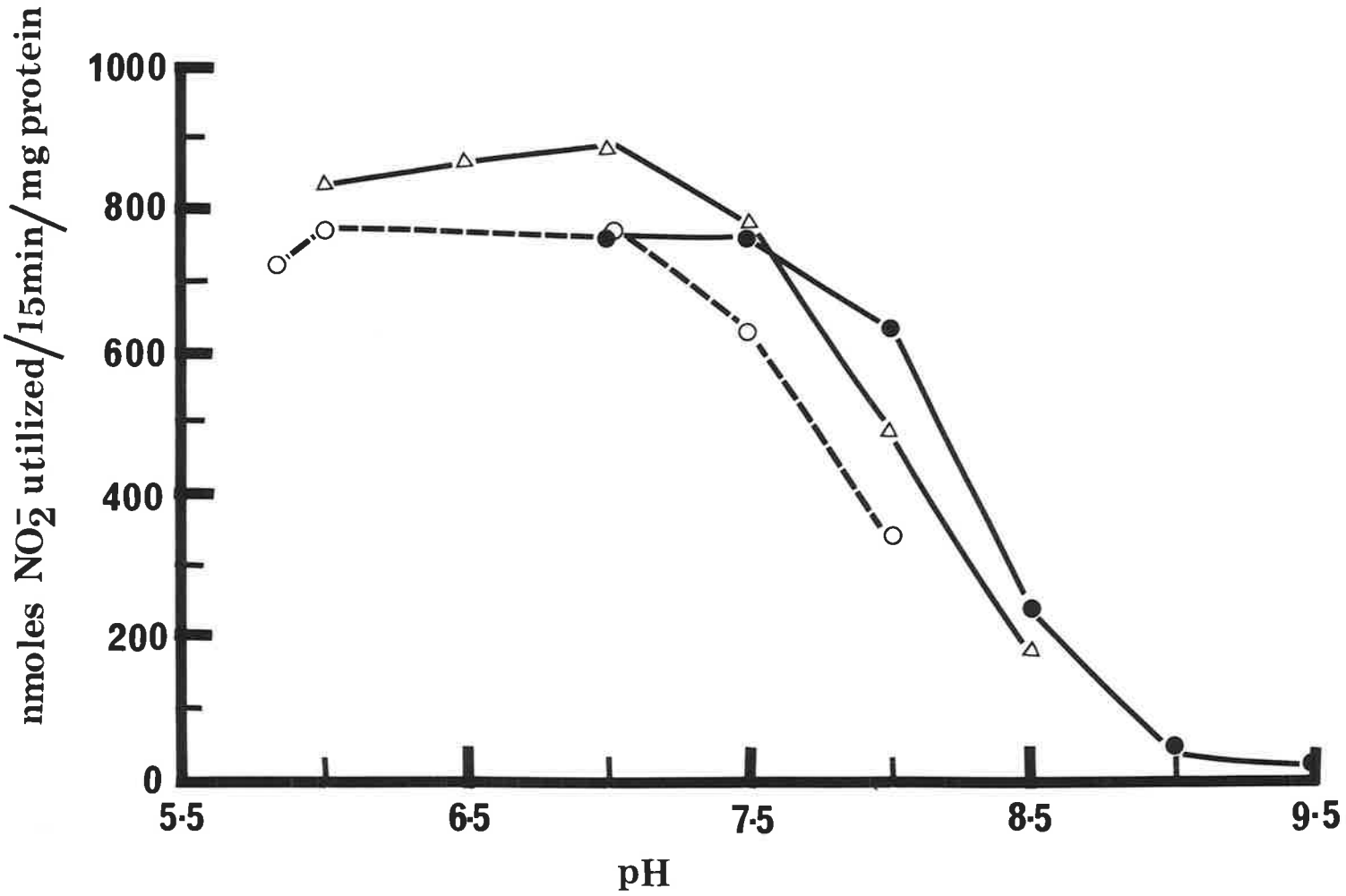
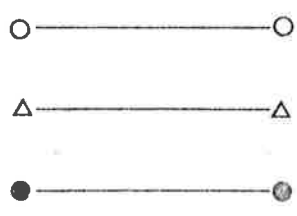

nmoles NO_2^- utilized/15min/mg protein
1000
800
600
400
200
0
5·5
6·5
7·5
8·5
9·5
pH

Figure 31. Thermal stability of the sulphide-linked nitrite reductase.

The enzyme preparation (P100 fraction containing 1.75 mg protein) and 0.7 ml of 0.05 M Tris-HCl buffer (pH 7.5) was pre-incubated at the specified temperatures. At the times indicated, nitrite reductase activity was determined as described in Section 2.5.4.2.

Enzyme activity is expressed as % of the original activity (without heat treatment) which was 503 nmoles NO_2^- utilized/15 min/mg protein.



Pre-incubated at 40°

Pre-incubated at 50°

Pre-incubated at 70°

Figure 31

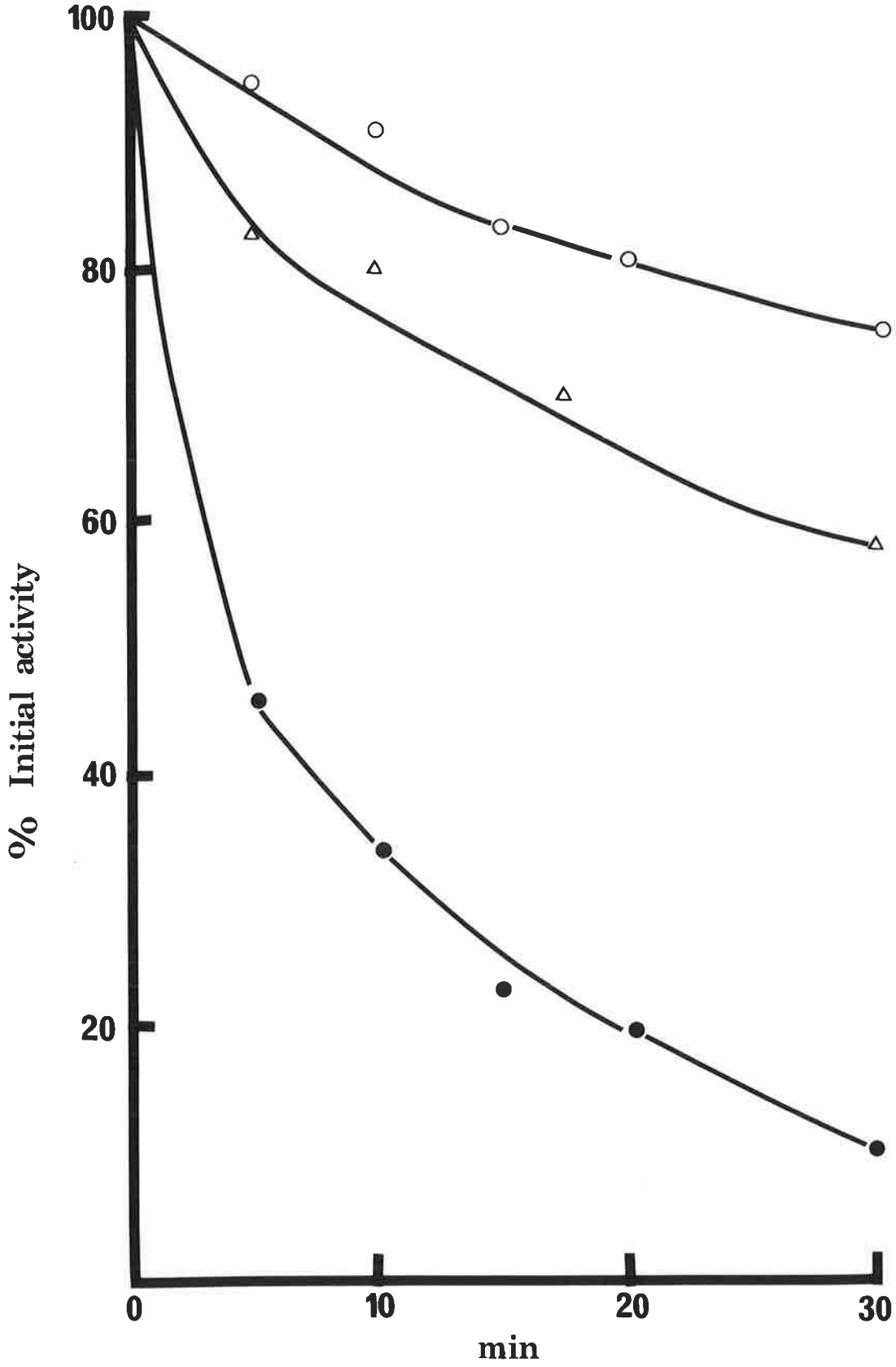


Figure 32. A Lineweaver - Burk's plot of the effect of varying sulphide concentrations on nitrite reductase activity.

Nitrite reductase activity was assayed, in the P100 fraction, as described in Section 2.5.4.2 except that the concentration of sulphide was varied over the range 0.2-4 μmoles. $\frac{1}{V}$ represents reciprocal of nmoles NO_2^- utilized/ 15 min.

Figure 32

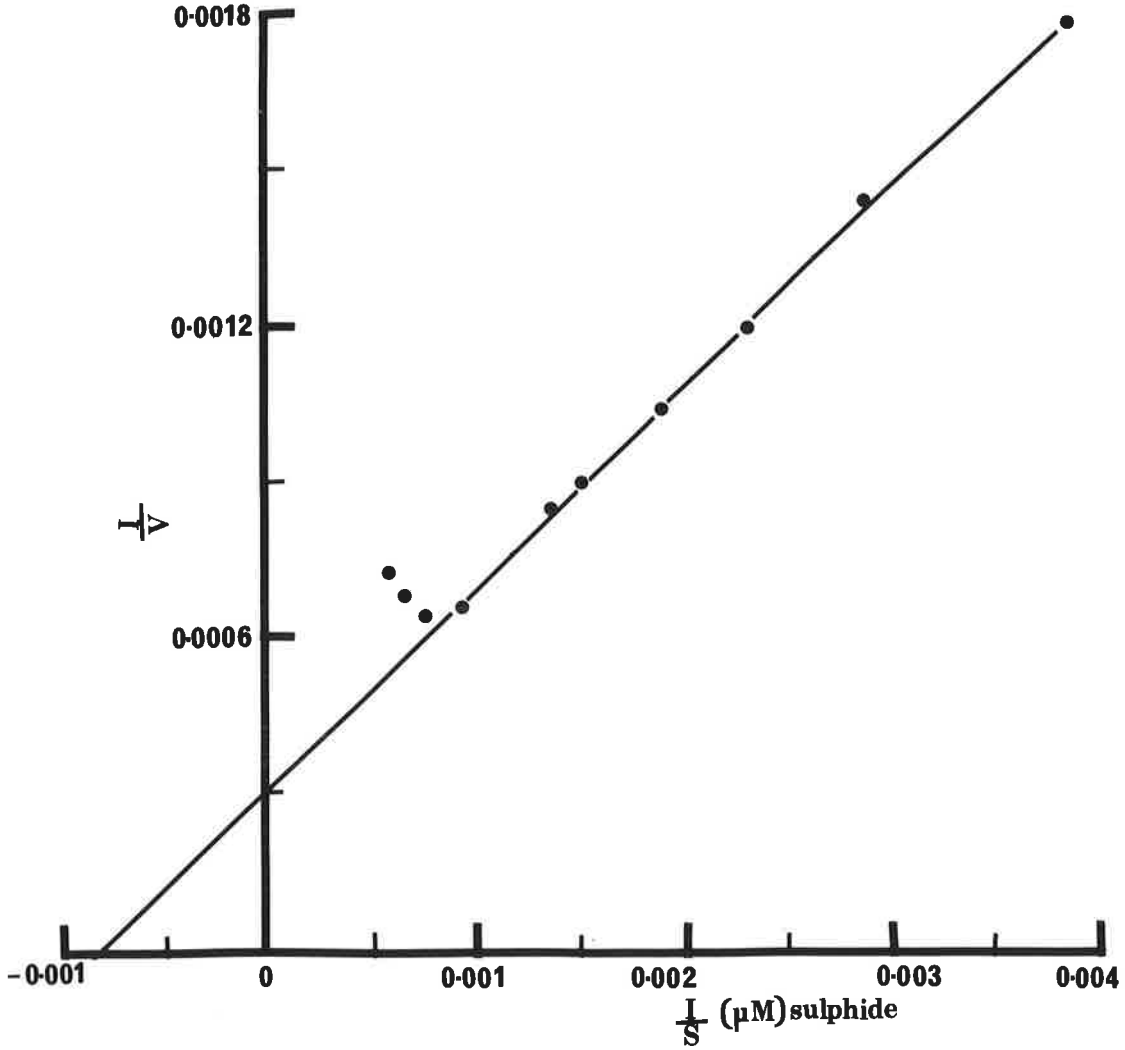
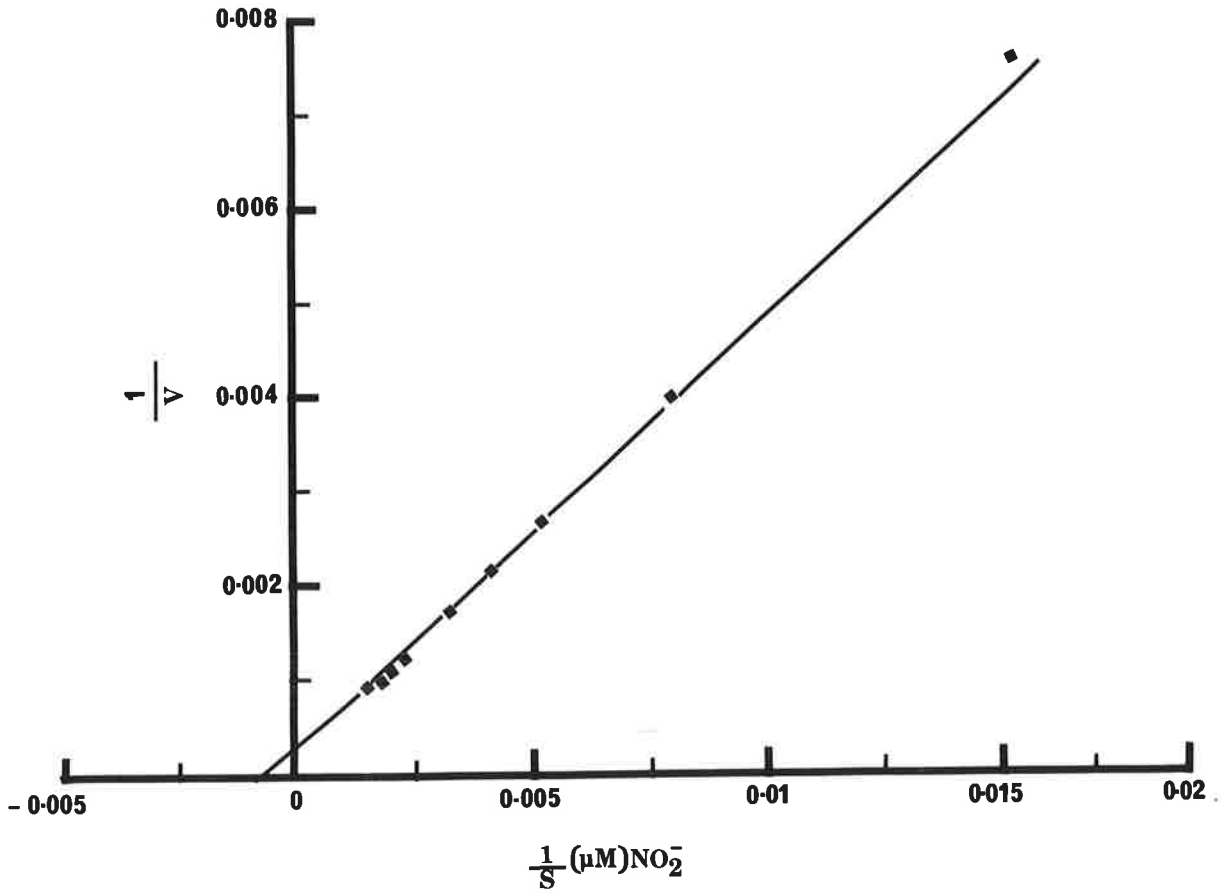

0·0018
0·0012
0·0006
$\frac{1}{v}$
-0·001
0
0·001
0·002
0·003
0·004
$\frac{1}{S}$ (μM) sulphide

Figure 33. Double-reciprocal plot of the effect of varying nitrite concentrations on enzyme activity.

Nitrite reductase activity was determined as described in Section 2.5.4.2. P100 fraction (1.75 mg protein) in 0.05 M Tris-HCl buffer (pH 7.5) was pre-incubated with the indicated concentrations of NO_2^- at 30° for 5 min before starting the reaction by injecting 2 µmoles sulphide solution. $\frac{1}{v}$ represents reciprocal of nmoles NO_2^- utilized/15 min.

Figure 33


0·008
0·006
0·004
0·002
$\frac{1}{V}$
- 0·005
0
0·005
0·01
0·015
0·02
$\frac{1}{S}(\mu M)NO_2^-$

inhibitors on the enzyme activity with NADH plus menadione and ascorbate plus PMS were also examined.

Effect of thiol binding reagents. As shown in Table 26, sulphydryl reagents markedly inhibited the sulphide-linked nitrite reductase activity. The inhibition by N-ethylmaleimide (NEM) was reversed by GSH. The enzyme activity with either NADH plus menadione or ascorbate plus PMS as the reductant, was not affected by either iodoacetamide or NEM, but p-CMB at 1mM inhibited both the activities by 37 and 48% respectively.

Effects of inhibitors of electron transport chain. Rotenone, atabrine and amytal did not effect nitrite reductase activity with either of the three electron donating systems. Antimycin A at 1mM lowered the sulphide-linked nitrite reductase activity by 39%, and HQNO (0.05mM) inhibited by 40% only the enzyme activity with NADH plus menadione (Table 27). The sulphide-linked nitrite reductase activity was markedly inhibited by dicumarol (50% at 0.7mM) and this effect was partially reversed by 0.5mM coenzyme Q_{10} and to a lesser extent by menadione.

Effects of arsenite, azide and cyanide. Arsenite and cyanide, each at 1mM, diminished the sulphide-linked nitrite reductase activity by 100 and 82% respectively (Table 28). Cyanide, at 1mM, also inhibited the NADH plus menadione dependent enzyme activity by 60%. The enzyme activity, with ascorbate plus PMS as the donor system, was not inhibited by any of these inhibitors.

Effects of metal chelators. The effects of some metal-binding reagents on the enzyme activity are given in Table 29. Bathocuproin,

Table 26. Effects of sulphydryl group inhibitors on nitrite reductase activity.

The effects of sulphydryl group inhibitors on nitrite reductase activity in P100 fraction was studied with three electron donating systems (sulphide, NADH plus menadione and ascorbate plus PMS) as described in Section 2.5.4.2. P100 fraction (1.75 mg protein) in 0.025 M Tris-HCl buffer (pH 7.5) was pre-incubated for 10 min at 30° with the inhibitor. After adding NO_2^-, the reaction was started with the appropriate electron donor system.

Results are expressed as % inhibition of the enzyme activity. The specific activities of 444, 488 and 527 nmoles NO_2^- utilized/15 min/mg protein were recorded for the control samples (without inhibitor) with S^{2-}, NADH plus menadione and ascorbate plus PMS as the electron donors respectively.

Addition to the reaction mixture	Final concentration (mM)	% inhibition		
		Electron donor		
		Sulphide	NADH + menadione	Ascorbate + PMS
p-Chloromercuribenzoate	0.1	40	0	6
	0.2	57	17	17
	1.0	75	37	48
p-Chloromercuribenzoate + GSH	0.2 + 0.25	73	20	20
N-Ethylmaleimide (NEM)	1.0	31	0	0
	2.0	55	0	0
	5.0	90	0	0
NEM + GSH	2.0 + 2.0	11	0	0
Iodoacetamide	0.5	75	0	0
	5.0	95	0	0
Iodoacetamide + GSH	0.5 + 2.0	77	0	0

Table 27. Effects of inhibitors of the electron transport chain on nitrite reductase activity.

The experimental details are as given in Table 26. P100 fraction (1.75 mg protein) in 0.025 M Tris-HCl buffer (pH 7.5) was pre-incubated with various inhibitors for 10 min at 30° before adding the other components of the reaction mixture.

The specific activities of the controls (without inhibitor) were 472, 427 and 526 nmoles NO_2^- utilized/15 min/mg protein with S^{2-}, NADH plus menadione and ascorbate plus PMS as the electron donors, respectively.

Addition to the reaction mixture	Final concentration (mM)	% inhibition		
		Electron donor		
		Sulphide	NADH + menadione	Ascorbate + PMS
Rotenone	0.1	10	3	0
	1.0	16	17	0
Atabrine	1.0	21	0	13
	5.0	22	20	30
Amytal	2.0	30	16	0
	5.0	30	36	0
Antimycin A	0.5	22	8	0
	1.0	39	11	0
2-n-Heptyl-4-hydroxy-quinoline-N-oxide (HQNO)	0.005	4	30	0
	0.05	8	39	4
Dicumarol	0.2	35	4	0
	0.5	43	8	0
	0.7	52	20	0
Dicumarol + menadione	0.7 + 1.0	42	-	-
Dicumarol + coenzyme Q_{10}	0.5 + 0.5	19	-	-
Thenoyltrifluoroacetone	2.0	12	0	0

Table 28. Effects of arsenite, azide and cyanide on nitrite reductase activity.

Details of the experimental procedures used are as described in Table 26. The specific activities of the controls (without inhibitor) were the same as in Table 27.

Addition to the reaction mixture	Final concentration (mM)	% inhibition		
		Electron donor		
		Sulphide	NADH + menadione	Ascorbate + PMS
Sodium arsenite	0.1	67	10	0
	0.5	90	15	0
	1.0	100	16	0
Sodium azide	0.5	9	19	0
	1.0	27	28	0
Potassium cyanide	0.1	66	0	0
	0.5	76	28	11
	1.0	82	60	16

Table 29. Effects of various metal chelators on nitrite reductase activity.

The details of the experimental procedures used are given in Table 26. The specific activities of the control samples (without inhibitor) were 583, 566 and 600 nmoles NO_2^- utilized/15 min/mg protein for S^{2-}, NADH plus menadione and ascorbate plus PMS as the electron donors, respectively.

Inhibitor	Final concentration (mM)	% inhibition		
		Electron donor		
		Sulphide	NADH + menadione	Ascorbate + PMS
Bathocuproin	2	34	42	7
	5	45	52	6
O-Phenanthroline	2	44	22	16
	5	50	32	17
2,2'-Bipyridyl	5	43	0	0
8-Hydroxyquinoline	5	36	0	0
Sodium diethyldithio-carbamate	5	0	0	0
Urethane	5	0	0	0

o-phenanthroline, 2,2'-bipyridyl and 8-hydroxyquinoline markedly inhibited the sulphide-linked enzyme while sodium diethyldithiocarbamate (DIECA) and urethane, each at 5mM, had no effect. The enzyme activity with NADH plus menadione was inhibited by bathocuproin and -phen nthroline each at 5mM by 52 and 32% respectively. The enzyme activity with ascorbate plus PMS as an electron donor was not affected by any of these metal chelators.

3.2.5.2 Spectral properties

The sulphide-reduced *versus* oxidized difference spectra of P100 fraction showed absorption bands at 420, 523, 553 and 615 nm and a shoulder around 470-475 nm (Figure 34). The absorption bands at 420, 523 and 553 nm, which are associated with cytochrome *c* and those at 615 and 470-475 nm due to cytochrome *d*, were oxidized on addition of nitrite anaerobically to the sulphide-reduced enzyme. These results thus indicate the involvement of these cytochromes in nitrite reduction. Both cytochromes *c* and *d* of the sulphide-reduced pellet fraction (P100) were also oxidized in air.

3.2.6 Characterization of the electron-transport chain linked to oxygen

3.2.6.1 Comparison of oxygen uptake and nitrite reduction with various electron donors

The data in Table 30 show that either sulphide or NADH plus menadione effectively supported both the O_2 uptake and nitrite reduction in the pellet fraction (P100). Although a significant uptake of O_2 was also observed with either sulphite or NADH alone, these reductants

Figure 34. Sulphide-reduced *versus* oxidized difference spectra of P100 fraction.

The P100 fraction (0.4 ml containing 12 mg protein) and 0.05M Tris-HCl buffer (pH 7.5), in a total volume of 2.5 ml, were dispensed into both sample (Thunberg) and reference (ordinary) cuvettes. The side arm of the sample cuvette contained 4 µmoles of sulphide. This cuvette was thoroughly evacuated and then flushed with a high purity N_2 gas as described in Section 2.6.3. Sulphide solution was tipped from the side arm into the cuvette and mixed thoroughly. The spectra of the sample was recorded against the reference cuvette. The sample cuvette was then opened, shaken in air for 1 min and its spectra was again recorded. In another experiment, instead of shaking in air, 40 µmoles of $NaNO_2$ was added anaerobically.

A. ———————— Reduced *versus* oxidized difference spectra

B. — — — — — — — After either readmitting air or adding 40 µmoles of NO_2^- anaerobically to A.

Figure 34

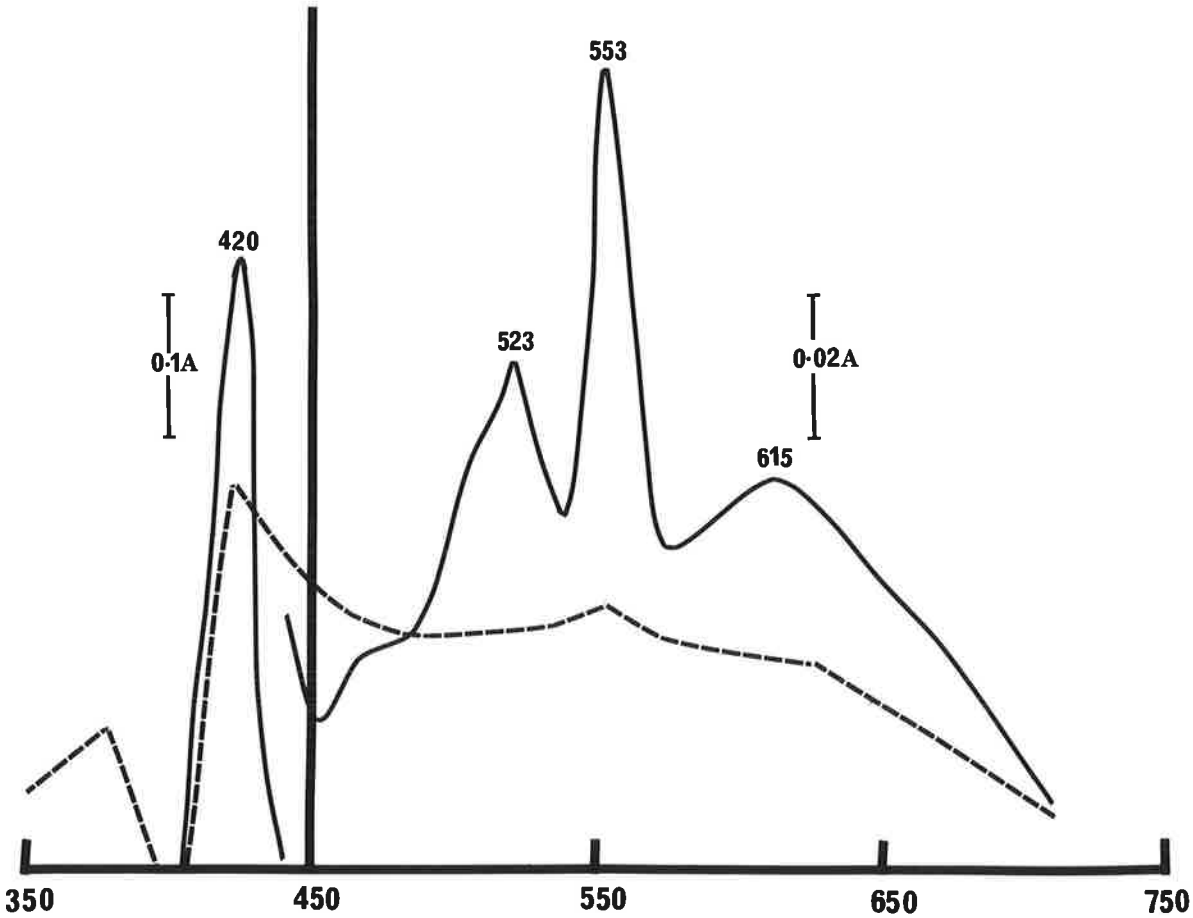


Table 30. Comparison of oxygen uptake and nitrite reduction with various electron donors.

Oxygen uptake was monitored using an oxygen electrode as described in Section 2.6.1. Nitrite reductase activity was assayed as described in Section 2.5.4.2. For each assay, 0.1 ml P100 fraction containing 2.15 mg protein was used.

Electron donor	Final concentration (mM)	O_2 uptake (nmoles O_2 utilized/min/mg protein)	Nitrite reductase (nmoles NO_2^- utilized/min/mg protein)
Sulphide	2	22	31
Sulphite	2	31	1
NADH	1	12	6
	2	17	6
NADH + menadione	2 + 1	17	57
	2 + 2	49	85
Sodium ascorbate	2	6	6
Ascorbate + PMS	2 + 2	-	39

were not effective in reducing nitrite. Ascorbate alone did not promote either O_2 uptake or nitrite reduction, but in the presence of PMS, it stimulated the latter reaction by about six-fold. The effect of ascorbate plus PMS on oxygen uptake could not, however, be determined because PMS is autooxidizable, resulting in a substantial non-enzymic uptake of oxygen.

3.2.6.2 Effects of various inhibitors on oxygen uptake with sulphide as reductant

Sulphydryl group inhibitors. The effects of various thiol-binding agents on O_2 uptake with sulphide as an electron donor are shown in Table 31. NEM at 5mM inhibited O_2 uptake by 90% while 1mM p-CMB and 5mM iodoacetamide reduced the activity by 40 and 55% respectively. The inhibition of O_2 uptake by either NEM or p-CMB was partially relieved by GSH.

Inhibitors of electron transport chain. Amytal, atabrine, rotenone, antimycin A and HQNO did not inhibit O_2 uptake. However, dicumarol, at 1mM reduced the activity by about 60% (Table 32).

Metal chelators and other inhibitors. The effects of various metal chelating agents on the sulphide-dependent O_2 uptake are presented in Table 33. 2,2'-bipyridyl and 8-hydroxyquinoline, each at 5mM, inhibited the uptake of O_2 by about 50%. Cyanide was strongly inhibitory and at 0.1mM, it reduced the activity by 80%. Diethyldithiocarbamate (DIECA), bathocuproin and urethane did not have any effect whereas azide at 1mM was only slightly inhibitory.

Table 31. Effects of inhibitors of -SH groups on oxygen uptake with sulphide as an electron donor.

The details of the assay procedures are the same as given in Section 2.6.1 except that P100 fraction (2 mg protein) and 42.5 µmoles of Tris-HCl buffer (pH 7.5) were pre-incubated with each inhibitor for 10 min prior to adding the electron donor. The results are expressed as % inhibition as compared to the control (without inhibitor) which was 20 nmoles O_2 utilized/min/mg protein.

Addition to the reaction mixture	Final concentration (mM)	% inhibition
p-Chloromercuribenzoate	0.1	32
	1.0	39
p-Chloromercuribenzoate + GSH	1.0 + 1.0	21
N-Ethylmaleimide (NEM)	1.0	37
	2.0	45
	5.0	90
NEM + GSH	2.0 + 2.0	34
Iodoacetamide	1.0	30
	2.0	43
	5.0	55
Iodoacetamide + GSH	5.0 + 5.0	52

Table 32. Effects of inhibitors of electron transport chain on sulphide-linked oxygen uptake.

Aliquots of P100 fraction were pre-incubated with the inhibitors for 10 min as described in Table 31. The specific activity of the control (without inhibitor) was 19 nmoles O_2 utilized/min/mg protein.

Inhibitor	Final concentration (mM)	% inhibition
Amytal	2.0	23
	5.0	27
Atabrine	2.0	11
	5.0	23
Rotenone	0.5	27
	1.0	34
Antimycin A	0.1	16
	0.5	35
HQNO	0.01	25
	0.05	25
Dicumarol	0.5	45
	1.0	58

Table 33. Effects of metal chelators and other inhibitors on sulphide-linked oxygen uptake.

The details of the experimental procedure are described in Table 31. The specific activity of the control sample, without inhibitor, was 20 nmoles O_2 utilized/min/mg protein.

Inhibitor	Final concentration (mM)	% inhibition
2,2'-Bipyridyl	5.0	48
8-Hydroxyquinoline	5.0	44
Sodium diethyldithiocarbamate	5.0	0
Urethane	5.0	4
Bathocuproin	5.0	0
o-Phenanthroline	5.0	23
Sodium azide	1.0	30
Potassium cyanide	0.05	59
	0.1	80
Sodium arsenite	1.0	0

3.2.7 Purification of sulphide-linked nitrite reductase

3.2.7.1 Solubilization

As mentioned earlier (Table 23), nitrite reductase is associated with membraneous fraction (P100). Attempts to solubilize the enzyme from P100 fraction by treatment with detergents namely, sodium deoxycholate, SDS, Triton X-100 or Tween 80 or with lipases and proteolytic enzymes were ineffective. However, the enzyme from acetone-dried cells was recovered in the supernatant fraction. Thus an acetone treatment of the cells was effective in dissociating the sulphide-linked nitrite reductase from the membranes. As shown in Figure 35, addition of sulphide to the high speed supernatant fraction (S100) of the acetone-dried cells results in the appearance of absorption bands, corresponding to reduced cytochrome *c* (420, 523 and 553 nm) and cytochrome *d* (615 and a shoulder at 470-475 nm). In contrast, sulphite did not cause any spectral changes thus indicating that the acetone treatment of the cells did not dissociate the sulphite-linked nitrate reductase from the membranes. However, the addition of sulphite to the S100 fraction, prepared after treating the crude extract of the acetone-dried cells with sodium deoxycholate (0.06 mg/mg protein) for 30 min at 30^{o}, showed absorption bands at 424, 524 and 554 nm. This absorption spectra is identical to that observed in Figure 9 for sulphite-linked nitrate reductase.

3.2.7.2 Purification

Enzyme activity at the various stages of purification was assayed by determining the rate of nitrite reduction with sulphide as the reductant. The buffer used during purification was 0.025M Tris-HCl (pH 7.5) and unless stated otherwise, all the procedures were carried out at 4^{o}.

Figure 35. Effect of sodium deoxycholate treatment on reduced *versus* oxidized spectra of S100 fraction prepared from acetone-dried cells.

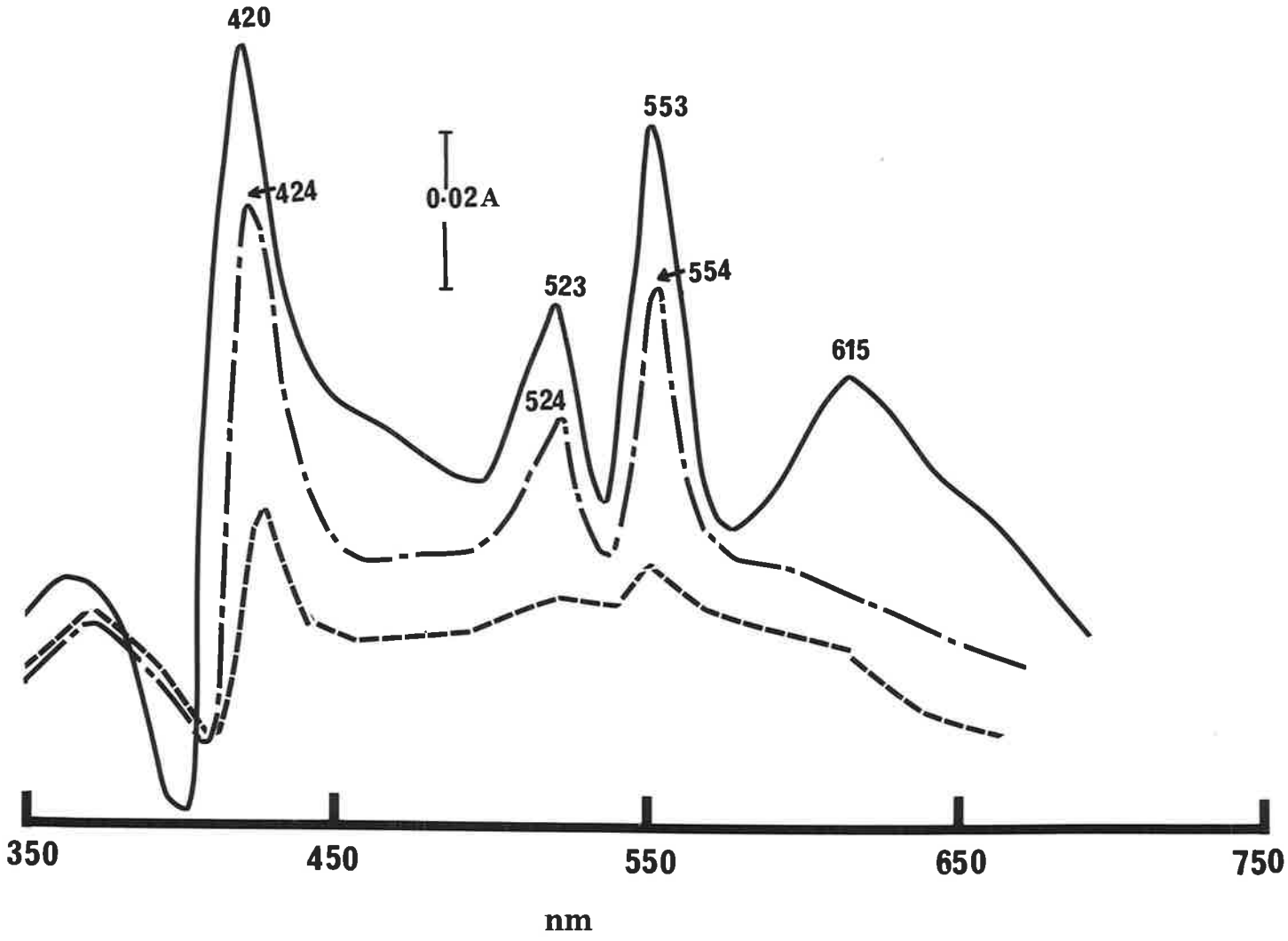
The acetone-dried cells, disrupted as described in Section 2.5.1.1, were centrifuged at 18,000x g for 45 min. The supernatant fraction thus obtained was further centrifuged at 100,000x g for 90 min either without any treatment or after incubating it with sodium deoxycholate (0.1%, w/w) at 30° for 30 min. Reduced *versus* oxidized difference spectra of the supernatant fractions (S100) were recorded as described in Section 2.6.3.

- - - - - - - - SO_3^{2-}-reduced *versus* oxidized difference spectra of S100 fraction

— - — - — - SO_3^{2-}-reduced *versus* oxidized difference spectra of S100 fraction of the deoxycholate-treated sample

——————— S^{2-}-reduced *versus* oxidized difference spectra of S100 fraction.

Figure 35



The cell-free extract (S18), prepared from acetone-dried cells as described in Section 2.5.2.2, was further centrifuged at 100,000x g for 90 min. To the supernatant fraction (S100) thus obtained, solid ammonium sulphate was added gradually with continuous stirring to bring it to 40% saturation. The suspension was centrifuged at 20,000x g for 20 min. The supernatant fraction was then brought to 95% saturation by further addition of solid ammonium sulphate. The pellet, obtained after centrifuging at 20,000x g, was dispensed in a minimum volume of 0.025M Tris-HCl buffer (pH 7.5) and dialyzed for 16 hr against three changes of double-distilled water.

A DEAE-32 cellulose column (2.5 x 26 cm), prepared as described in Section 2.6.8.1, was equilibrated with 0.025M Tris-HCl buffer (pH 7.5) until the pH of the effluent was the same as that of the equilibrating buffer. The enzyme preparation, from the preceding step (Fraction III, Table 34), was then loaded onto the column and eluted at a flow rate of 60 ml/hr with 0.025M Tris-HCl buffer (pH 7.5). Nitrite reductase, which was not adsorbed on to the column, was recovered after one column volume in the greenish brown fractions (Figure 36). A reddish brown band retained at the top of the column was eluted with 0.025M Tris-HCl buffer (pH 7.5) containing 0.5M KCl. The spectral characteristics of this fraction are presented in Figure 37. In its oxidized state, it had absorption maxima at 408 and 525 nm and on reduction with sodium dithionite at 416, 524 and 554 nm. These absorption bands indicated that this reddish brown fraction contained a cytochrome of the *c* type.

The fractions (15-26) from DEAE-cellulose column, which contained most of the nitrite reductase activity, were pooled and concentrated in an Aminco ultrafiltration unit using PM-10 membrane filter under N_2 gas as described in Section 2.6.9.

Figure 36. Elution profile of nitrite reductase from a DEAE-cellulose column.

A DEAE-cellulose column (DE32; 2.5 x 26 cm) was equilibrated with 0.025 M Tris-HCl buffer (pH 7.5) at 4°. Fraction III (Table 34) was loaded on to the column and eluted with the same buffer at a flow rate of 60 ml/hr. Fractions (3 ml) were collected in an automatic fraction collector and nitrite reductase activity was determined in 0.2 ml aliquots of the each fraction as described in Section 2.5.4.2. E_{280} of a suitably diluted fraction, in a final volume of 2.5 ml, was recorded. The values given for E_{280} in the Figure have been calculated for the absorbance/ml of each fraction.

▲——————▲ E_{280}

△– – – – – – –△ Nitrite reductase activity

Figure 36

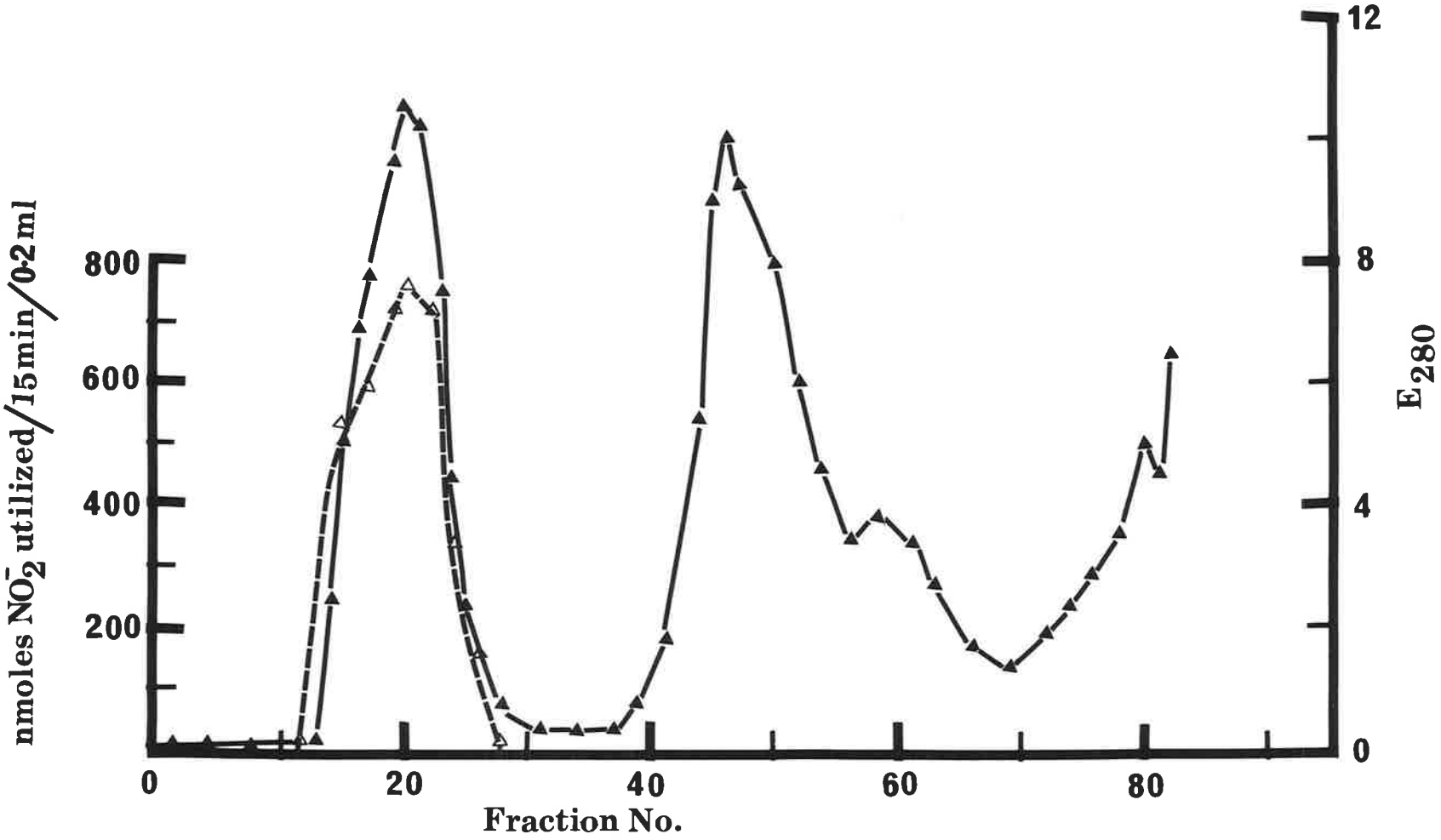

nmoles NO_2^- utilized/15min/0·2ml
800
600
400
200
0
20
40
60
80
Fraction No.
12
8
4
0
E_{280}

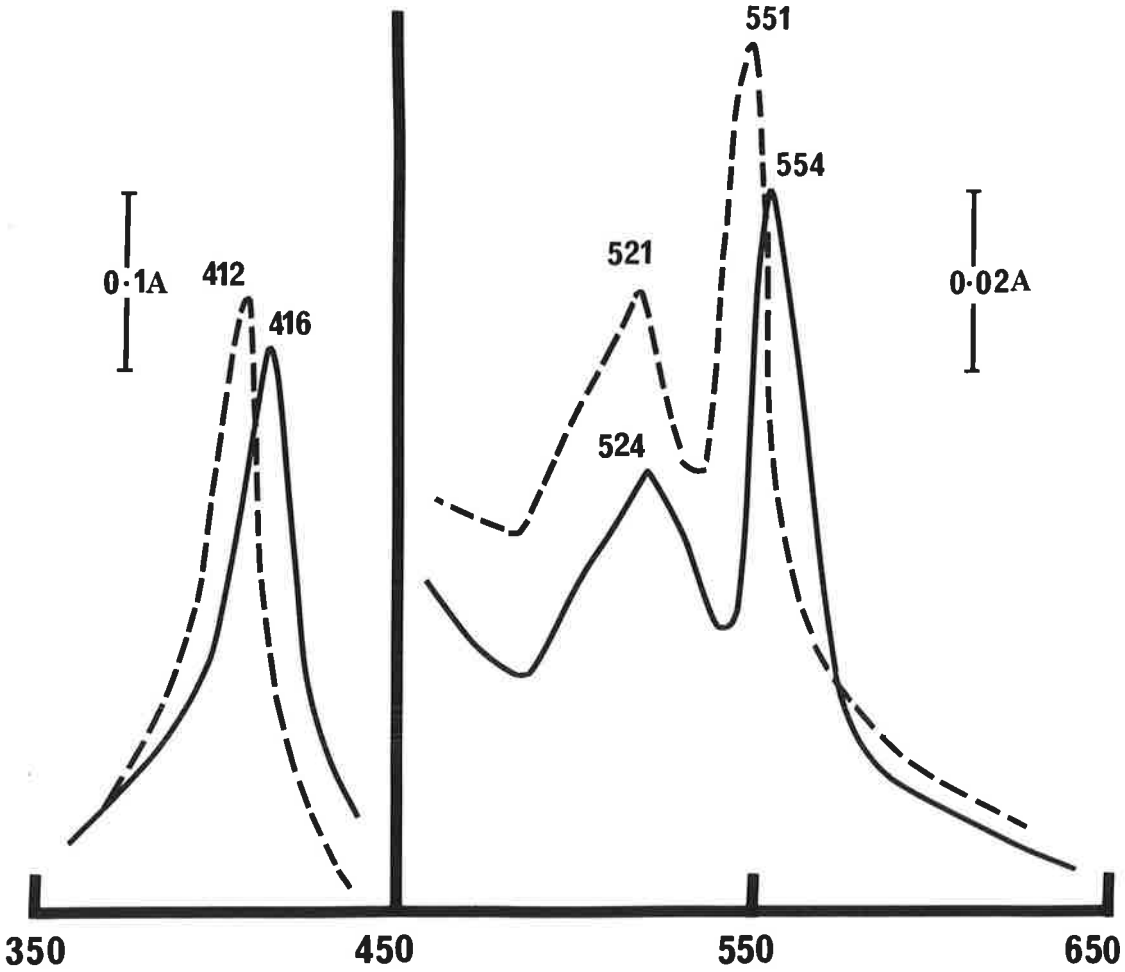
Figure 37. Dithionite-reduced *versus* oxidized difference spectra of cytochromes c_{551} and c_{554}.

Cytochromes c_{551} and c_{554} were isolated from *T. denitrificans* during purification of nitrite reductase as described in Section 4.2.7.2. 200 µl of either cytochrome c_{551} or c_{554} and 0.025 M Tris-HCl (pH 7.5), in a final volume of 2.5 ml, were dispensed in to both sample and reference cuvettes. After adding a small amount of $Na_2S_2O_4$ to the sample cuvette and a crystal of $K_3Fe(CN)_6$ to the reference cuvette, the difference spectra were recorded.

– – – – – – – $Na_2S_2O_4$-reduced *versus* oxidized spectra of cytochrome c_{551}

——————— $Na_2S_2O_4$-reduced *versus* oxidized spectra of cytochrome c_{554}.

Figure 37



A Sephadex G-100 column (2.5 x 45 cm), prepared as described in Section 2.6.8.2, was equilibrated with 0.025M Tris-HCl buffer (pH 7.5). The concentrated enzyme from DEAE-cellulose (Fraction IV, Table 34) was applied carefully onto the column and the sample was eluted at a flow rate of 15 ml/hr with the equilibrating buffer and 3 ml fractions were collected using L.K.B. fraction collector. The elution profile of the sample from this column indicated that Fraction IV was resolved into two components (Figure 38). Nitrite reductase was recovered in the fractions immediately after the void volume. The second fraction contained a cytochrome of *c* type with absorption maxima at 408 and 525 nm in the oxidized form and at 412, 521 and 551 nm after its reduction with sodium dithionite (Figure 37).

This purification procedure, summarized in Table 34, resulted in a twenty-fold increase in specific activity of nitrite reductase compared to the crude extract (S18) and 36% of the original enzyme activity was recovered. The purified enzyme had an A_{280}/A_{405} nm ratio of 0.75.

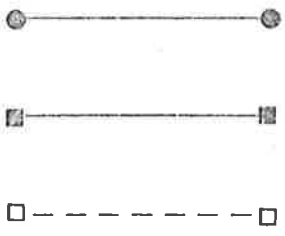
3.2.8 Properties of the purified enzyme

3.2.8.1 Molecular weight

Molecular weight of the purified enzyme (Fraction V, Table 34) was determined by gel filtration through a Sephadex G-150 column and using the equation of Squire (1964) as described in Section 2.5.6.1. Assuming that the enzyme was a globular protein, its estimated molecular weight was 120,000 daltons with a standard error of $\pm$5,000.

Figure 38. Elution profile of nitrite reductase from Sephadex G-100 column.

Fraction IV (Table 34) was loaded onto a Sephadex G-100 column (2.5 x 45 cm), pre-equilibrated with 0.025 M Tris-HCl buffer (pH 7.5). The sample was eluted with the same buffer at the flow rate of 15 ml/hr and 3 ml fractions were collected. Nitrite reductase activity was determined in 0.2 ml aliquots of each fraction according to the procedure given in Section 2.5.4.2. Haem *c* content of each fraction was measured at 405 nm.



E_{280}

E_{405}

Nitrite reductase activity

Figure 38

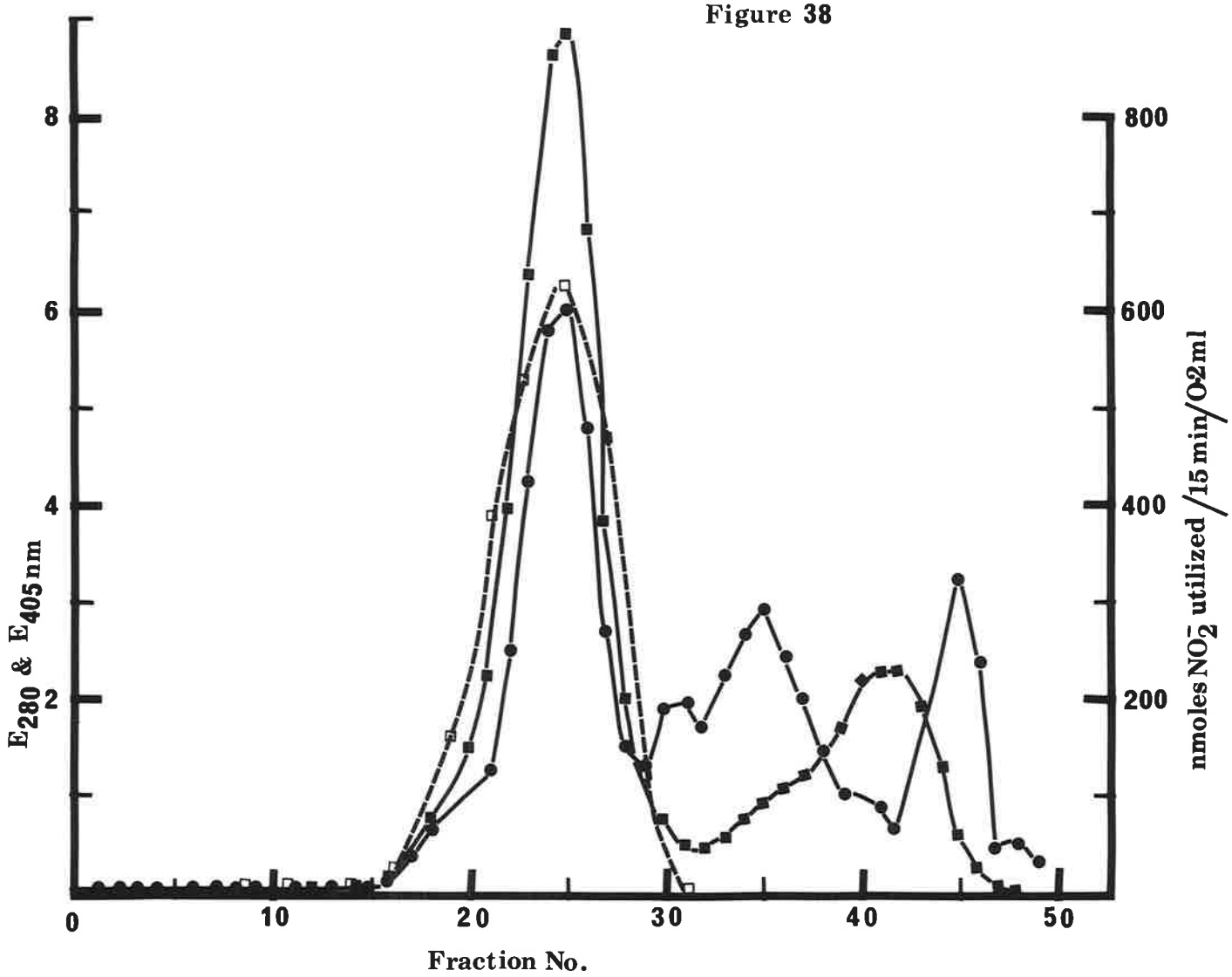

8
6
4
2
0
10
20
30
40
50
800
600
400
200
E_{280} & $E_{405\,nm}$
nmoles NO_2^- utilized /15 min/0·2 ml
Fraction No.

Table 34. Purification of nitrite reductase.

Acetone-dried powder of the washed cells was prepared as described in Section 2.5.1. The acetone-dried cells, suspended in 0.025 M Tris-HCl buffer (pH 7.5), were stirred at 4^{o} for 3 hr. The suspension was passed through a French pressure cell (Section 2.5.2.2). The broken cells were centrifuged at 18,000x g for 45 min and the supernatant (S18) fraction was used for the further purification of the enzyme.

Nitrite reductase activity, in various fractions obtained, was determined as described in Section 2.5.4.2.

Total activity : µmoles NO_2^- utilized/15 min

Specific activity : µmoles NO_2^- utilized/15 min/mg protein

Table 34. Purification of nitrite reductase.

Fraction	Procedure	Total activity	Total protein (mg)	Specific activity	Fold Purification	Recovery (%)
I	S18, supernatant fraction left after centrifuging broken cells at 18,000x g for 45 min	507.5	9207	0.05	1	100
II	S100, supernatant fraction obtained after centrifuging the S18 fraction at 100,000x g for 90 min	384	4800	0.08	1.5	75
III	Fraction II precipitated with ammonium sulphate between 40-95% saturation and dialyzed against double distilled water overnight	184.8	1680	0.11	1.9	36
IV	Fraction III eluted with 0.025 M Tris-HCl buffer (pH 7.5) from a DEAE-cellulose column and concentrated by ultra-filtration	173.8	245	0.71	12.8	34
V	Fraction IV eluted with 0.025 M Tris-HCl buffer (pH 7.5) from Sephadex G-100 column	132.3	118	1.12	20.4	26

3.2.8.2 Copper and iron contents

Copper was determined by atomic absorption spectrometry as described in Section 2.7.3. With this technique up to 0.1 µg of copper can be determined. However, copper was not detected in the 12.5 mg protein used for the assay. Results in Table 35 show that the purified enzyme contained 29 µmoles of iron per mg protein.

3.2.8.3 Relative concentrations of haems *c* and *d* in purified nitrite reductase

It was shown earlier that the reducing equivalents from sulphide were transferred to nitrite *via* cytochromes *c* and *d* of purified nitrite reductase. The relative concentrations of haems *c* and *d* in the purified enzyme was, therefore, determined from the respective molecular extinction coefficients of their pyridine haemochromogen derivatives. The results of three determinations carried out with different enzyme preparations indicate that these two haems were present in an equimolar amount (Table 36).

3.2.8.4 Sequence of reduction and oxidation of cytochromes *c* and *d*

As mentioned in the preceding Section (3.2.8.3), both haems *c* and *d* were present in the purified nitrite reductase in the ratio of 1 : 1. An attempt was, therefore, made to establish the sequence of electron transfer from sulphide to nitrite *via* nitrite reductase. Although both the cytochromes were reduced after adding sulphide, the rate of reduction of cytochrome *c* during the first few minutes was much faster than that of cytochrome *d*. Thus 1 min after adding sulphide to the enzyme, 50% of cytochrome *c* was reduced as compared

Table 35. Iron content of purified nitrite reductase (cytochrome *cd* complex), dissociated cytochrome *c* and haem *d*.

Purified nitrite reductase (1.0 ml containing 12.5 mg protein of Fraction V, Table 34), dissociated cytochrome *c* (1.5 ml containing 9.2 mg protein) and haem *d* (1.5 ml containing 3.15 mg protein) were dried separately in acid-washed pyrex test-tubes. The dried samples were digested in an acid mixture of HNO_3, $HClO_4$ and H_2SO_4 (in ratio of 40 : 4 : 1) and their iron contents were estimated by Varian atomic absorption spectrophotometer as described in Section 2.7.3.

Sample	Concentration (μmoles/gm protein)
Purified nitrite reductase	29.0
Dissociated cytochrome *c*	16.8
Haem *d*	16.8

Table 36. Relative concentrations of haem *c* and haem *d* in the purified nitrite reductase.

The concentrations of haem *c* and haem *d* of the purified nitrite reductase (Fraction V, Table 34) were determined spectrophotometrically in an alkaline pyridine solution as described in Section 2.7.4. The extinction coefficient values of 30.2 and 19.6 mM^{-1} cm^{-1} were used for calculating the amounts of haem *c* and haem *d* respectively.

Preparation No.	Haem *c* (μM)	Haem *d* (μM)	$\frac{\text{Haem } d}{\text{Haem } c}$ ratio
1	104	110	1.05
2	92	96	1.04
3	100	105	1.05

to 25% of cytochrome *d* (Figure 39). After 13 min both the cytochromes were fully reduced. The relatively faster rate of cytochrome *c* reduction during early stages indicates that the reducing equivalents were first transferred to cytochrome *c* and then to cytochrome *d*.

The rates of oxidation of the sulphide-reduced cytochromes *c* and *d* with nitrite were also examined. It is clear from Figure 40 that on adding nitrite, cytochrome *d* was oxidized at a much faster rate than cytochrome *c*. Thus, after 15 min more than 75% of cytochrome *d* was oxidized compared with 34% of cytochrome *c*. The sequence of the oxidation of cytochromes *c* and *d* indicates that cytochrome *d* functions as the immediate electron donor for nitrite reduction.

3.2.8.5 Difference in redox-potential of cytochromes *c* and *d*

The experimental values for the relative concentrations of reduced and oxidized cytochromes *c* and *d* given in Table 37, for calculating the values of equilibrium constant, were derived from the data presented in Figure 39. On reduction of the enzyme with sulphide, an equilibrium was established between the haem *c* and *d* moieties. The difference in the redox-potential of the two cytochromes was calculated by substituting the values of the equilibrium constant in the equation given in Section 2.6.4. The redox-potential of cytochrome *d* was 22 mV more positive than that of cytochrome *c*.

3.2.8.6 Electron donors

Nitrite reductase activity of the purified enzyme was examined in the presence of a variety of electron donor systems. The results in Table 38 indicate that as in P100 fraction (Table 25), sulphide as well as ascorbate plus PMS were effective electron donors.

Figure 39. Rate of reduction of cytochromes *c* and *d* of *cd* complex of purified nitrite reductase.

Purified nitrite reductase (200 µl of Fraction V, Table 34) and 0.025 M Tris-HCl buffer (pH 7.5), in a total volume of 2.5 ml, were taken in a Thunberg cuvette which contained 5 µmoles of sulphide solution in the side arm. The cuvette was vigorously evacuated and then filled with pure N_2 gas. This procedure was repeated twice (Section 2.6.3). Sulphide solution, from the side arm, was then mixed with the contents of the cuvette and change in absorbance at 554 nm (for cytochrome *c*) and at 610 nm (for cytochrome *d*) was recorded at various time intervals. The % reduction of cytochromes *c* and *d* respectively, was calculated on the basis of maximum reduction of the cytochromes obtained after prolonged incubation period.

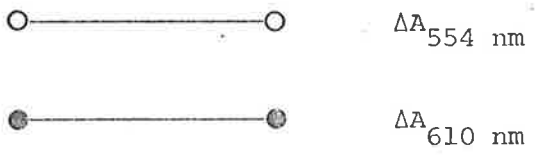


Figure 40. Rate of oxidation of sulphide-reduced cytochromes *c* and *d* of *cd* complex of purified nitrite reductase with nitrite.

Experimental procedure was as described in Figure 39 except that 5 µmoles of sulphide solution was added to the Thunberg cuvette and the side arm contained 10 µmoles of nitrite solution. The Thunberg cuvette was made anaerobic as described in Section 2.6.3. Optical density at 554 and 610 nm for cytochromes *c* and *d* respectively, of the reduced enzyme was recorded until maximum absorbance was obtained. Nitrite was then tipped from the side arm and mixed thoroughly with the contents of the cuvette. Changes in absorbance at 554 and 610 nm were recorded at various time intervals. % oxidation of each cytochrome was calculated on the basis of the respective amounts of the fully reduced cytochromes *c* and *d* present before adding nitrite.

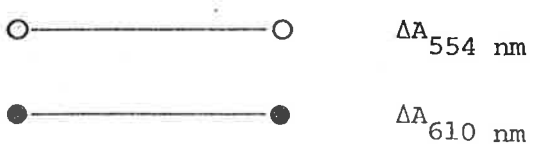


Figure 39

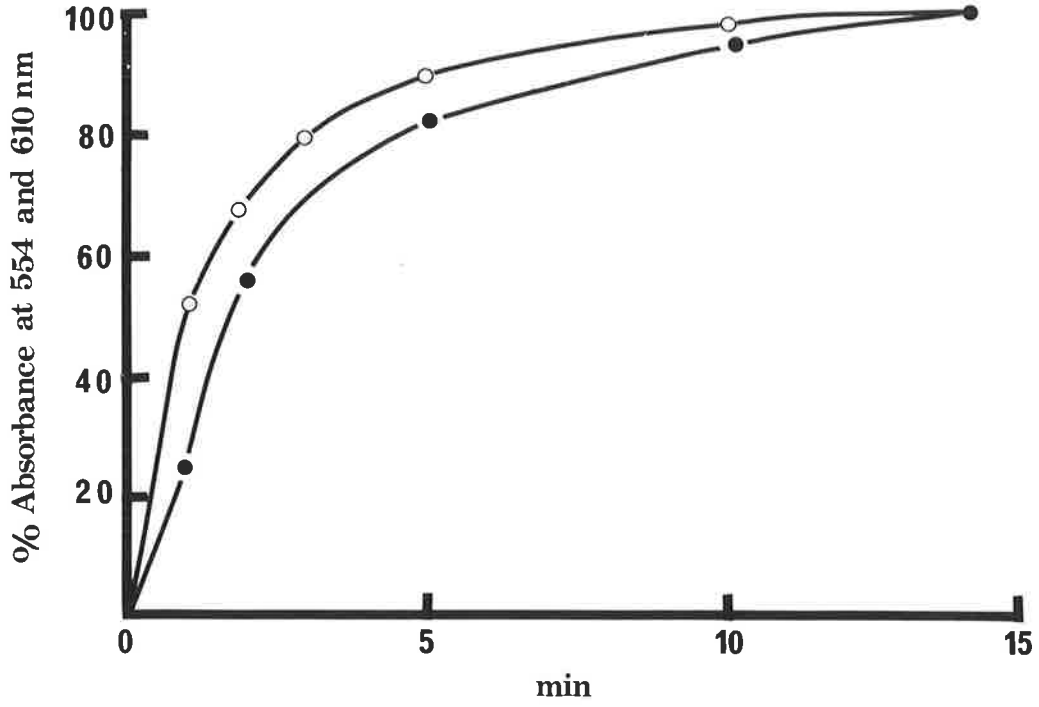


Figure 40

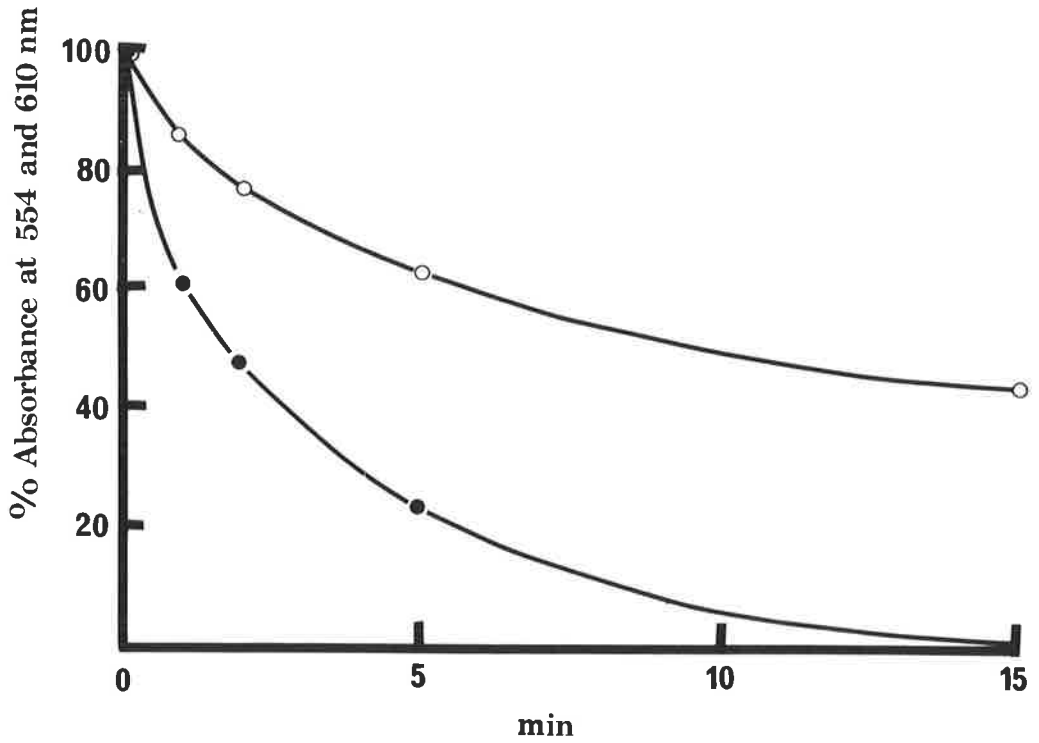


Table 37. Difference in redox-potentials of cytochromes *c* and *d* of the *cd* complex of nitrite reductase.

The difference in the redox-potentials of cytochromes *c* and *d* of purified nitrite reductase (Fraction, Table 34) was calculated, as described in Section 2.6.4, from the data given in Figure 39.

Time (min)	Cytochrome *c*		Cytochrome *d*		Equilibrium constant (K) $= \frac{(c^{3+})(d^{2+})}{(c^{2+})(d^{3+})}$	Em(cyt.*d*) - Em(cyt.*c*) = 0.059 log x K (mV)
	% Oxidized (c^{3+})	% Reduced (c^{2+})	% Oxidized (d^{3+})	% Reduced (d^{2+})		
1.0	48.06	51.94	75.00	25.00	3.24	+30
2.0	32.47	67.53	45.47	54.52	1.72	+14
3.0	20.80	79.20	29.55	70.45	1.59	+12
5.0	9.10	90.90	17.30	82.70	2.08	+19
9.0	1.30	98.70	4.50	94.45	3.56	+32
15.0	0.00	100.00	0.00	100.00	-	
					Mean =	+22

Table 38. Effect of various electron donors on nitrite reductase activity in the purified enzyme preparation.

Nitrite reductase activity in the purified preparation (Fraction V, Table 34), with various electron-donating systems, was assayed in Warburg flasks fitted with stopcocks as described in Section 2.5.4.2.

Electron donor	Final concentration (mM)	Specific activity (nmoles NO_2^- utilized/15 min/mg protein)
Sulphide	2	660
NADH + menadione	2 + 2	0
Ascorbate + PMS	2 + 2	1040

Nitrite, however, was not reduced with NADH plus menadione as the reductant.

The effectiveness of various redox-dyes, with potentials ranging from -0.08 to +0.217 V, as electron carriers for nitrite reduction in the presence of ascorbate was examined (Table 39). Maximum enzyme activity was obtained with PMS and TMPD, while methylene blue, thionine, or 2,6-dichlorophenol indophenol (DCIP) gave 75% of the activity obtained with TMPD. The inclusion of cytochrome *c*, isolated from *T. denitrificans*, along with DCIP did not increase the enzyme activity markedly. Thus, nitrite reductase activity was maximal with dyes with redox-potentials within the range of +0.08 to +0.197 V.

3.2.8.7 Effects of mammalian and bacterial cytochromes *c* on nitrite reductase activity

Results in Table 40 indicate that the inclusion of 2.2 μM cytochrome c_{551} (see Section 3.2.7.2) in the reaction mixture enhanced the sulphide-linked nitrite reductase activity by about two-fold. The presence of either cytochrome c_{554}, also isolated from *T. denitrificans* (Section 3.2.7.2, Figure 37), or mammalian cytochrome *c* had no effect on the enzyme activity. Thus, sulphide-linked nitrite reductase activity was stimulated only by the bacterial cytochrome c_{551}.

The effects of various concentrations of cytochrome c_{551} on the enzyme activity were examined. The maximum stimulation was observed with 2 μM cytochrome c_{551} (Figure 41) and higher amounts, up to 10 μM, did not enhance the enzyme activity further.

Reduced cytochrome c_{551} served as an effective electron donor for nitrite reduction by the purified enzyme (Figure 42). The

Table 39. Effects of various electron mediators (dyes) on the activity of nitrite reductase in the purified enzyme (Fraction V, Table 34).

The details of assay procedure are as described in Section 2.5.4.2 except that instead of sulphide, 1 mM of the specified electron mediators were used. Methylene blue was, however, used at a final concentration of 0.5 mM. These mediators were reduced by injecting 2 μmoles of neutralized ascorbic acid into the Warburg flasks. After a 15 min incubation at 30°, residual nitrite was determined colorimetrically as described in Section 2.7.2.

Electron mediator	E'_o of mediator (V)	Specific activity (nmoles NO_2^- utilized/15 min/mg protein)
Triphenyltetrazolium	-0.08	130
Methylene blue	+0.011	640
Thionine	+0.065	640
Phenazine methosulphate (PMS)	+0.08	820
N,N,N',N'-tetramethylpara-phenylenediamine (TMPD)	+0.197	840
TMPD + cytochrome *c**		860
2,6-dichlorophenol indophenol (DCIP)	+0.217	696
DCIP + cytochrome c_{551}*		756

* cytochrome c_{551} was separated from *T. denitrificans* (Section 4.2.7.2, Figure 37).

Table 40. Effect of cytochrome *c* from various sources on nitrite reductase activity in purified enzyme preparation (Fraction V, Table 34).

The composition of the reaction mixture was as given in Section 2.5.4.2 except that cytochromes c_{551} and c_{554}, both isolated from *T. denitrificans* (Section 4.2.7.2, Figure 37), and mammalian cytochrome *c*, at indicated concentrations, were included in the reaction mixtures. The reaction was started by injecting sulphide solution through the subaseals-fitted side arms of the Warburg flasks.

Addition to the reaction mixture	Final concentration (mM)	Specific activity (nmoles NO_2^- utilized/15 min/mg proteins)
None	-	569
Cytochrome c_{551}	1.1	1015
	2.2	1123
Cytochrome c_{554}	1.0	569
	2.0	560
Mammalian cytochrome *c*	1.0	550
	2.0	573

Figure 41. Effect of various concentrations of cytochrome c_{551} on the activity of purified nitrite reductase (Fraction V, Table 34).

The enzyme activity was assayed as described in Section 2.5.4.2 except that cytochrome c_{551}, at the concentrations specified in the Figure, was included in the reaction mixture.

Figure 42. Reduced cytochrome c_{551} and mammalian cytochrome *c* as electron donors for the activity of purified reductase.

Concentrated solutions of cytochrome c_{551} and mammalian cytochrome *c* respectively were reduced with an excess of $Na_2S_2O_4$. Each of the reduced cytochromes was then dialysed overnight against 0.025 M Tris-HCl buffer (pH 7.5). The enzyme activity was assayed in Warburg flasks fitted with stopcocks. The reaction mixture in a final volume of 1 ml contained the following, in µmoles: Tris-HCl buffer (pH 7.5), 35; $NaNO_2$, 0.2 and 50 µl of purified nitrite reductase (Fraction V, Table 34) containing 0.625 mg protein. The flasks were repeatedly evacuated and filled with N_2 gas. The reaction was started by injecting different amounts of either reduced cytochrome c_{551} or reduced mammalian cytochrome *c*, through subaseals fitted on to the side arms of the Warburg flasks.

After 15 min incubation, 1 ml of 10% (w/v) zinc acetate in 10% (v/v) ethanol was added to stop the reaction. 0.5 ml aliquots were used for estimating residual nitrite according to the procedure given in Section 2.7.2.

Reduced cytochrome c_{551} as the reductant

Reduced mammalian cytochrome *c* as the reductant.

Figure 41

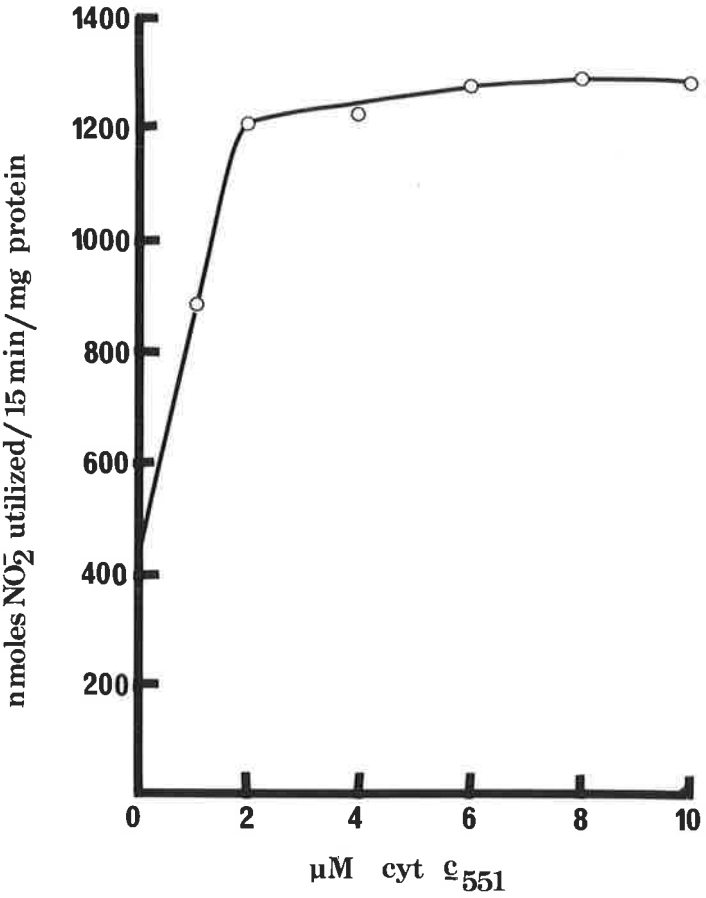
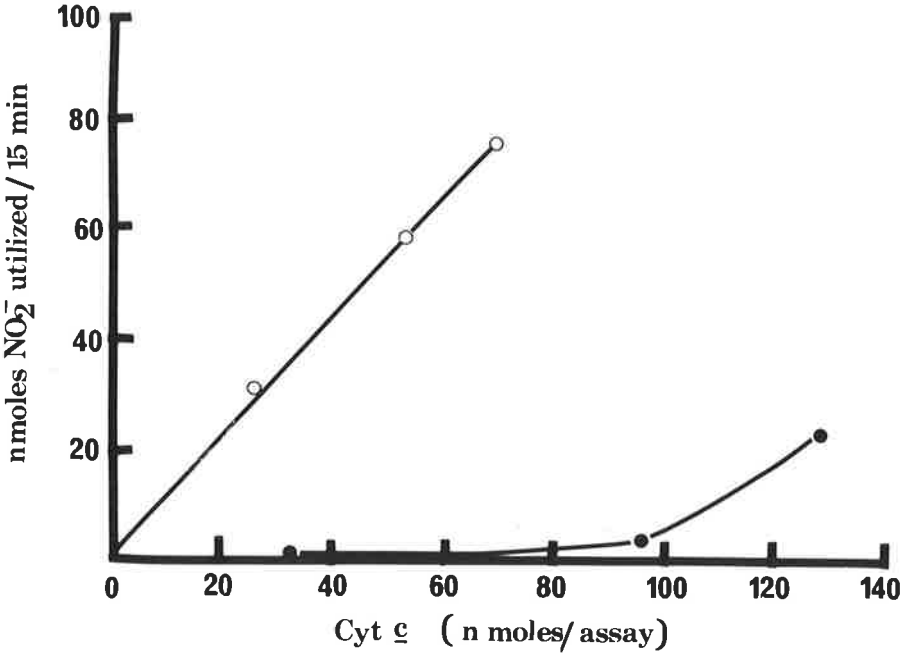


Figure 42



stoichiometry of approximately 1 : 1 was obtained for the amount of nitrite reduced to the amount of cytochrome c_{551} added to the reaction mixture. In contrast, reduced mammalian cytochrome *c* was an ineffective electron donor. Only 25 nmoles of nitrite were reduced in the presence of 130 nmoles of reduced mammalian cytochrome *c*.

3.2.9 Cytochrome oxidase activity of the purified nitrite reductase

Cytochrome oxidase activity was determined in the various fractions obtained during the purification of nitrite reductase (Table 34). Cytochrome oxidase activity was measured by following the rate of O_2 uptake with sulphide as an electron donor as described in Section 2.5.4.3. Results in Table 41 show that the specific activity of cytochrome oxidase increased progressively during the successive stages of purification of nitrite reductase.

3.2.9.1 Comparison of nitrite reductase and cytochrome oxidase activities

Some of the properties of nitrite reductase and cytochrome oxidase activities in the purified enzyme were compared. Both the activities, with sulphide as an electron donor, were stimulated two- to three-fold by adding 2 μM cytochrome c_{551} to the reaction mixture. Reduced cytochrome c_{551} was also an effective electron donor for O_2 uptake and nitrite reduction. For each mole of reduced cytochrome c_{551} added, one mole of either nitrite or O_2 was reduced. Glutathione, at 2mM, inhibited the cytochrome oxidase activity by about 40% while nitrite reductase was not affected (Table 42).

Table 41. Cytochrome oxidase activity in the various fractions obtained during the purification of nitrite reductase.

The fractions were prepared as described in Table 34 (Section 4.2.7.2) and the cytochrome oxidase activity was monitored in an O_2 electrode cell using sulphide as the electron donor (Section 2.5.4.3).

Fraction	Procedure	Total activity (μmoles O_2 utilized/min)	Specific activity (μmoles O_2 utilized/min/mg protein)
I	Crude extract (S18) left after centrifuging the disrupted acetone-dried cells at 18,000x g for 45 min.	74.1	8.0
II	Supernatant fraction (S100) obtained after centrifuging S18 at 100,000x g for 90 min.	37.6	7.89
III	Fraction II precipitated with ammonium sulphate (40-95% saturation) and dialyzed against 0.025 M Tris-HCl buffer (pH 7.5) overnight.	19.2	11.4
IV	Fraction III loaded on to a DEAE-cellulose column and eluted with 0.025 M Tris-HCl buffer (pH 7.5). The active fractions concentrated by ultrafiltration.	6.5	26.4

Table 42. Comparison of nitrite reductase and cytochrome oxidase activities in the purified nitrite reductase.

A. Nitrite reductase and cytochrome oxidase activities in the purified nitrite reductase enzyme (Fraction V, Table 34) were determined, with sulphide as a donor, as described in Section 2.5.4.2 and 2.5.4.3 respectively. Wherever indicated either cytochrome c_{551} (Section 4.2.7.2, Figure 37) or GSH were included in the reaction mixture.

B. Reduced cytochrome c_{551}, in place of sulphide, was used as the electron donor.

	Electron donor	Addition to the basal reaction mixture	Nitrite reductase (nmoles NO_2^- utilized/min/mg protein)	Cytochrome oxidase (nmoles O_2 utilized/min/mg protein)
A.	Sulphide (2 µmoles)	-	35	18
		Cytochrome c_{551} (2 µM)	107	48
		GSH (2 mM)	35	11
B.	Reduced cytochrome c_{551}			
	20 nmoles	-	21	20
	53 nmoles	-	59	53

3.2.10 Spectral properties of purified nitrite reductase

3.2.10.1 Absorption spectra

The absorption spectra of purified nitrite reductase (Fraction V, Table 34) showed absorption bands at 405, 525 and 645 nm and on reduction with sodium dithionite at 418, 468, 523, 549-554, 615 nm and a broad plateau around 665-700 nm (Figure 43). The absorption bands at 418, 523, 549-554 nm are associated with cytochrome *c* while those at 468, 615 and a plateau between 665-700 nm were due to cytochrome *d*. The ratio of α/β peaks of reduced cytochrome *c* was 1.0. The ratio of the absorbance bands at 468 and 615 nm of reduced cytochrome *d* was 0.7. On reduction with sodium dithionite, the absorption of the purified enzyme at 645 nm disappeared.

Addition of sulphide to the purified nitrite reductase, resulted in the following changes in the absorption spectra: absorption bands of reduced cytochrome *c* appeared within a minute of adding the reductant and their peak heights continued to increase with time. The reduction of cytochrome *d* was relatively slower than that of cytochrome *c*. Both the cytochromes were fully reduced after 15 min. The sulphide-reduced enzyme showed absorption maxima at 418, 462, 523, 549-554, 610 and 664-667 nm (Figure 44). The plateau at 665-700 nm, observed in the sodium dithionite-reduced enzyme, was replaced by a broad band between 664-667 nm. Moreover, instead of a peak at 468 nm obtained with sodium dithionite, there was a sharp peak at 462 nm in the sulphide-reduced enzyme. The absorption ratio of 462 nm and 610 nm was 2.1 which was three times greater than in the dithionite-reduced enzyme. The absorption characteristics of cytochrome *c* with either of these reductants were, however, the same. On reduction of the enzyme with

Figure 43. Absolute and difference spectra of purified nitrite reductase.

Absorption spectra of purified nitrite reductase (Fraction V, Table 34) were recorded as described in Section 2.6.3.

– – – – – – – – Absolute spectra

———————— Difference spectra, $Na_2S_2O_4$ reduced *versus* oxidized.

Figure 43

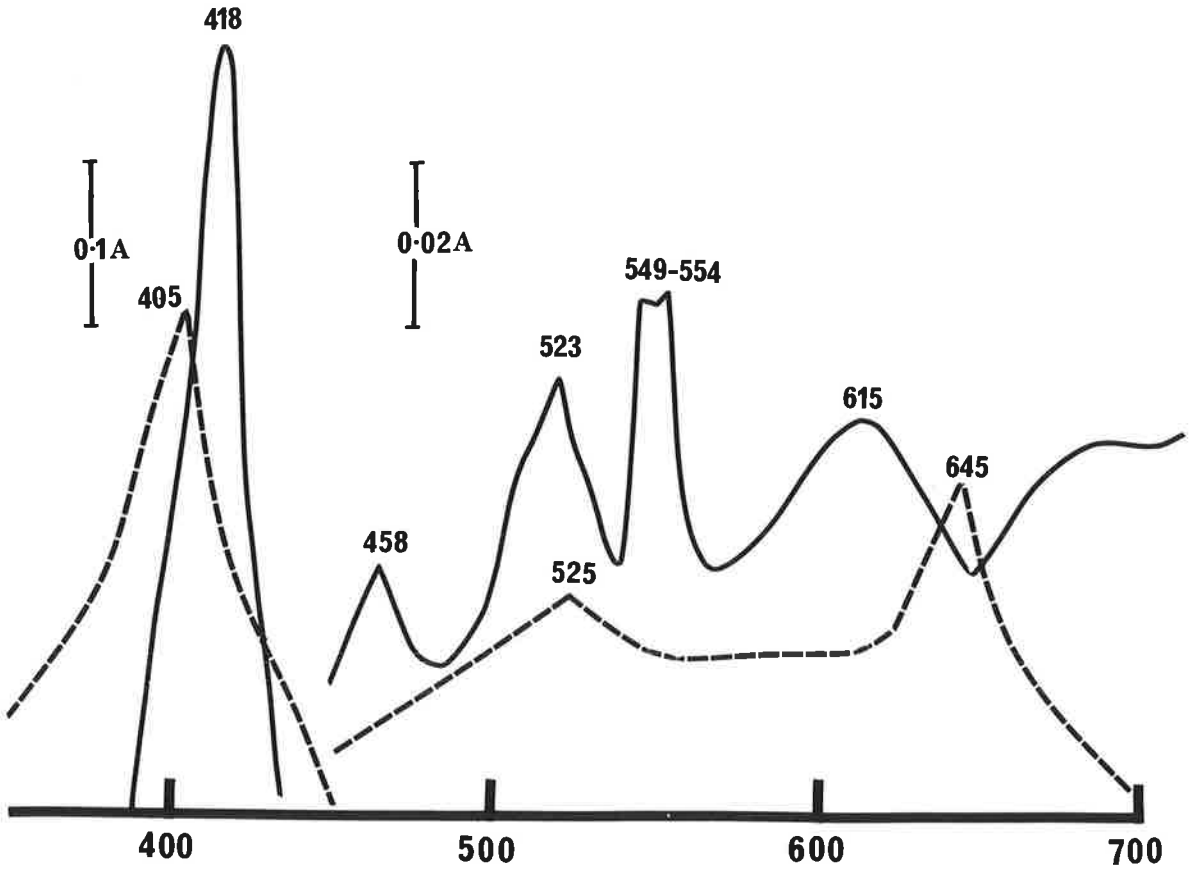


Figure 44. Time course of reduction of the cytochrome *cd* complex of purified nitrite reductase with sulphide.

The experiment was done in Thunberg cuvettes. 150 µl of purified enzyme (Fraction V, Table 34) and 0.025 M Tris-HCl buffer (pH 7.5), in a final volume of 2.5 ml, were dispensed into each of the reference and sample cuvettes. Freshly prepared solution of sulphide (15 µmoles) was placed in the side arm of the sample cuvette which was vigorously evacuated and then filled with N_2 gas as described in Section 2.6.3. The reaction was started by adding sulphide solution to the cuvette from the side arm and the spectra was recorded against the reference cuvette after following time intervals:

— — — — — — — — 1 min

— — — — — — — — — 5 min

——————— 15 min

Figure 45. Time course of reduction of cytochrome *cd* complex of purified nitrite reductase with ascorbate *plus* PMS.

Experimental details as described in Figure 44 except that instead of sulphide, 5 µmoles of neutralized solution of ascorbate and 0.5 µmoles of PMS were used. The same amount of PMS was also included in the reference cuvette. The spectra were recorded after the following time intervals:

——————— 1 min

— — — — — — — — 5 min

— — — — — — — — — 15 min

Figure 44

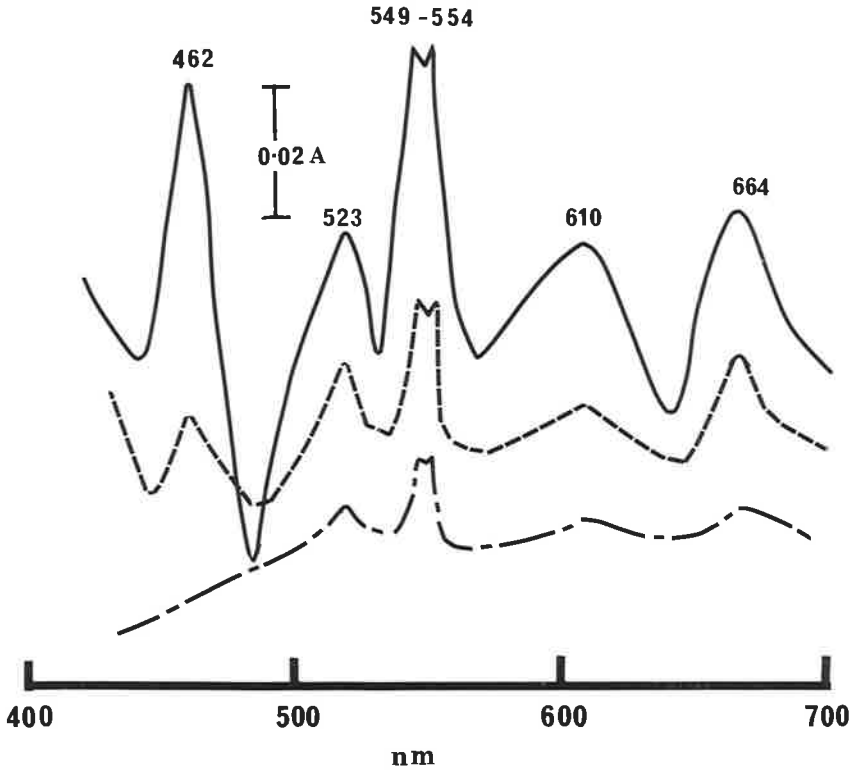
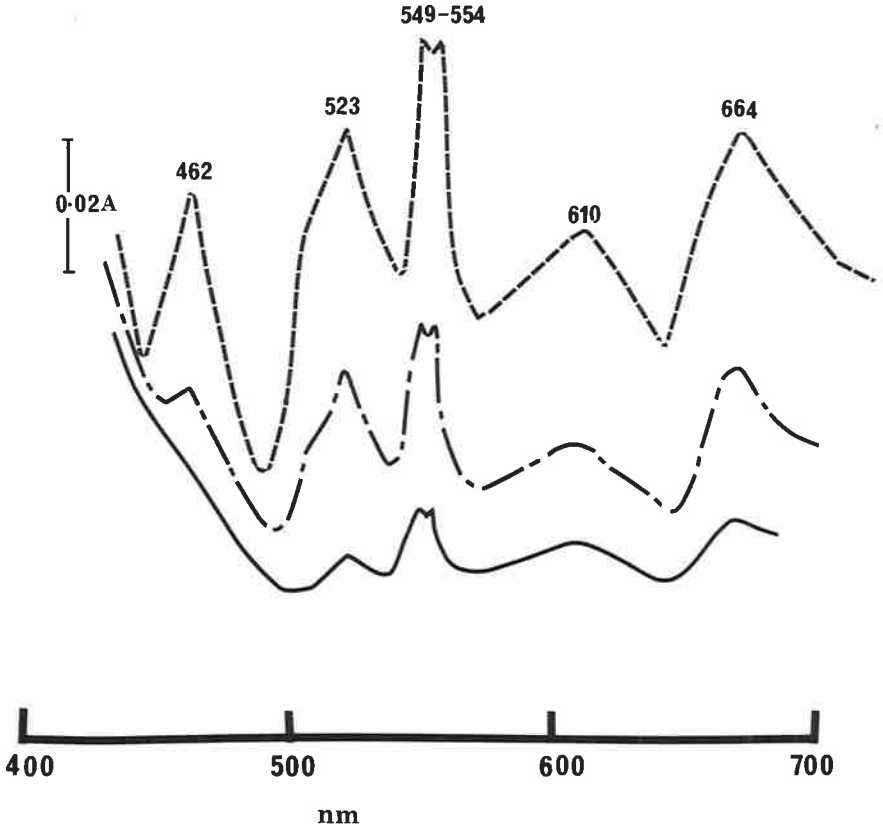


Figure 45



sulphide as well as with dithionite, the enzyme turned dark green from its original reddish brown colour. Spectral changes similar to those with sulphide were recorded when ascorbate plus PMS was used as the reductant (Figure 45).

The results in Figure 46 show that on adding nitrite anaerobically to the sulphide-reduced enzyme, both cytochromes *c* and *d* were oxidized; cytochrome *c*, however, remained in a partially reduced state even after 15 min of adding nitrite. The absorption bands at 462 and 664-667 nm disappeared immediately on adding nitrite, followed by the one at 610 nm. The peaks associated with cytochrome *c* were, however, oxidized at a relatively slower rate. The oxidation of cytochrome components of the enzyme was accompanied by an appearance of a peak at 573 nm. Oxidation of either the sulphide- or the dithionite-reduced enzyme with nitrite, resulted in restoration of the original reddish brown colour of the purified enzyme. The cytochromes *c* and *d* were not oxidized when nitrate, instead of nitrite, was added to the sulphide-reduced enzyme.

Absorption spectrum of cytochrome *d* of the sulphide-reduced enzyme was markedly influenced by the pH of the reaction mixture whereas that of cytochrome *c* remained unaffected. The absorption peak of cytochrome *d* at 645 nm of the purified nitrite reductase was also not influenced over the pH range of 5.5 - 8.0. As shown in Table 43, the ratio of the absorbance at 610/645 nm increased from 0.93 to 1.8 on lowering the pH of the reaction mixture from 8.0 to 5.5. The γ-band of cytochrome *d* at 462 nm was less prominent at the acid pH. Thus, the ratio of 462/610 nm declined from 1.7 at pH 8.0 to 0.42 at pH 5.5.

Figure 46. Oxidation of the sulphide-reduced cytochrome *cd* complex of purified nitrite reductase with nitrite.

Purified nitrite reductase (150 µl of Fraction V, Table 34), 0.025 M Tris-HCl (pH 7.5) and 10 µmoles of sulphide, in final volume of 2.5 ml, were dispensed into a sample cuvette (Thunberg) and 20 µmoles of $NaNO_2$ was placed in the side arm of the cuvette. The cuvette was vigorously evacuated and filled with pure N_2 gas as described in Section 2.6.3. After recording the spectra of the fully reduced enzyme, nitrite was tipped from the side arm and mixed thoroughly with the contents of the cuvette. The spectra was recorded at the times indicated below:

———————— Reduced with S^{2-}

– – – – – – – – – 1 min after adding NO_2^-

— –Δ– — –Δ– — 5 min after adding NO_2^-

— – — – — – — 15 min after adding NO_2^-

Figure 46

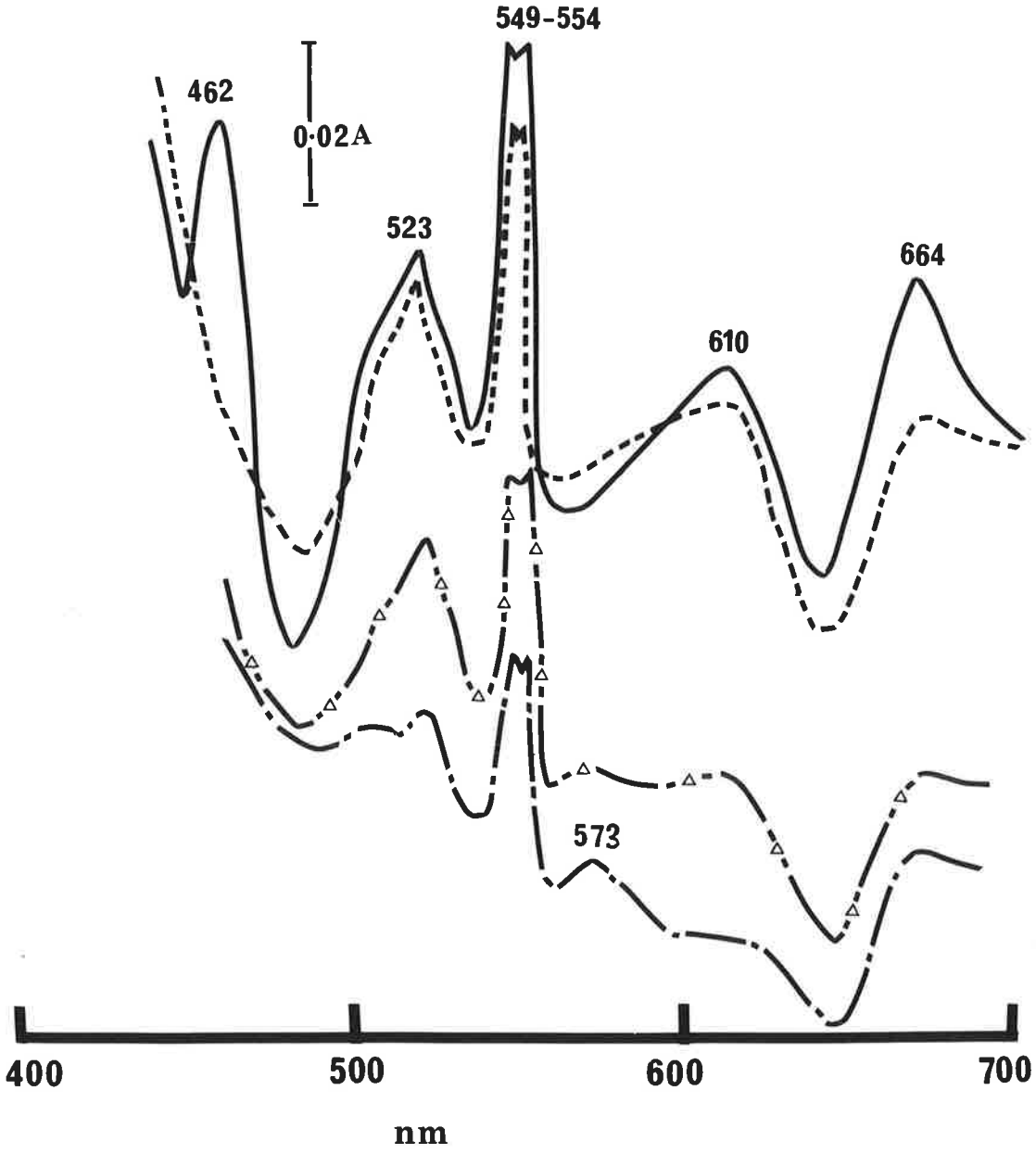


Table 43. Effect of pH on the absorbance characteristics of cytochrome *d* component of sulphide reduced cytochrome *cd* complex of the purified nitrite reductase (Fraction V, Table 34).

Nitrite reductase enzyme (200 µl containing 2.5 mg protein) and 2.5 ml of 0.05M phosphate buffer of various pH values were dispensed in Thunberg cuvettes. The enzyme was then reduced with 10 µmoles of sulphide solution. The absorbance of 645 nm before adding sulphide and at 462 nm (γ - peak) and at 610 nm (α - peak) of the sulphide-reduced cytochrome *d* component of the purified nitrite reductase were noted.

pH	Absorbance ratios	
	Reduced γ (462 nm) / Reduced α (610 nm)	Reduced α (610 nm) / Oxidized (645 nm)
8.0	1.7	0.93
7.6	1.1	1.15
7.0	1.0	1.20
5.5	0.42	1.80

3.2.10.2 Effects of cyanide and carbon monoxide on the absorption spectra of purified nitrite reductase

The results in Figure 47 show that cyanide had a profound effect on spectra of the sulphide-reduced enzyme. The γ-band of cytochrome *d* at 462 nm was abolished when the enzyme was reduced with sulphide in the presence of 1mM KCN and instead, two bands at 443 and 482 nm appeared. In addition, the α-peak of cytochrome *d* shifted to 627 nm and the absorbance peak at 523 nm diminished significantly. The 554 nm component of the bifurcated α-band of the reduced cytochrome *c* became more prominent as compared to the one at 549 nm. Cyanide had no effect on the absorption band at 418 nm. The spectra of the oxidized enzyme was not altered in the presence of cyanide.

On reducing the enzyme with sulphide, in the presence of CO, the γ-peak of cytochrome *d* was suppressed and the absorption band at 610 nm was replaced by a broad plateau around 650 nm (Figure 48). The bands of reduced cytochrome *c* remained unaffected by CO.

3.2.10.3 Pyridine haemochromogen

Cytochrome *d* was reduced by akaline pyridine even in the absence of a reductant; a sharp absorption band appeared at 620 nm in place of a broad band at 610 nm which was present in the sulphide-reduced enzyme. The 664-667 nm band was not detected in the alkaline pyridine preparation (Figure 49). In contrast, the cytochrome *c* component of the enzyme was not reduced by alkaline pyridine unless sodium dithionite was added. Under these conditions, the bifurcated α-peak of cytochrome *c* at 549-554 nm was replaced by a sharp peak at 549 nm. In addition, the ratio of α/β bands of reduced cytochrome *c* increased to 2.4 as compared to 1.0, recorded in the absence of

Figure 47. Effect of cyanide on the spectra of purified nitrite reductase (Fraction V, Table 34).

Details of the procedure are the same as in Figure 44 except that 2.5 µmoles of KCN was included in the sample cuvette (Thunberg). After evacuating and flushing the cuvette with pure N_2 gas, 10 µmoles of sulphide was added from the side arm of the Thunberg cuvette and difference spectra recorded after 15 min.

Figure 48. Effect of CO on the spectra of purified nitrite reductase (Fraction V, Table 34).

Experimental details as described in Figure 44 except that CO was bubbled through the sample cuvette for 5 min and the following spectra were recorded:

- – – – – – – – – Absolute spectrum of CO-treated enzyme

———————— CO-sulphide-reduced *versus* oxidized, difference spectra

— – — – — – — CO-sulphide-reduced *versus* sulphide reduced, difference spectra.

Figure 47

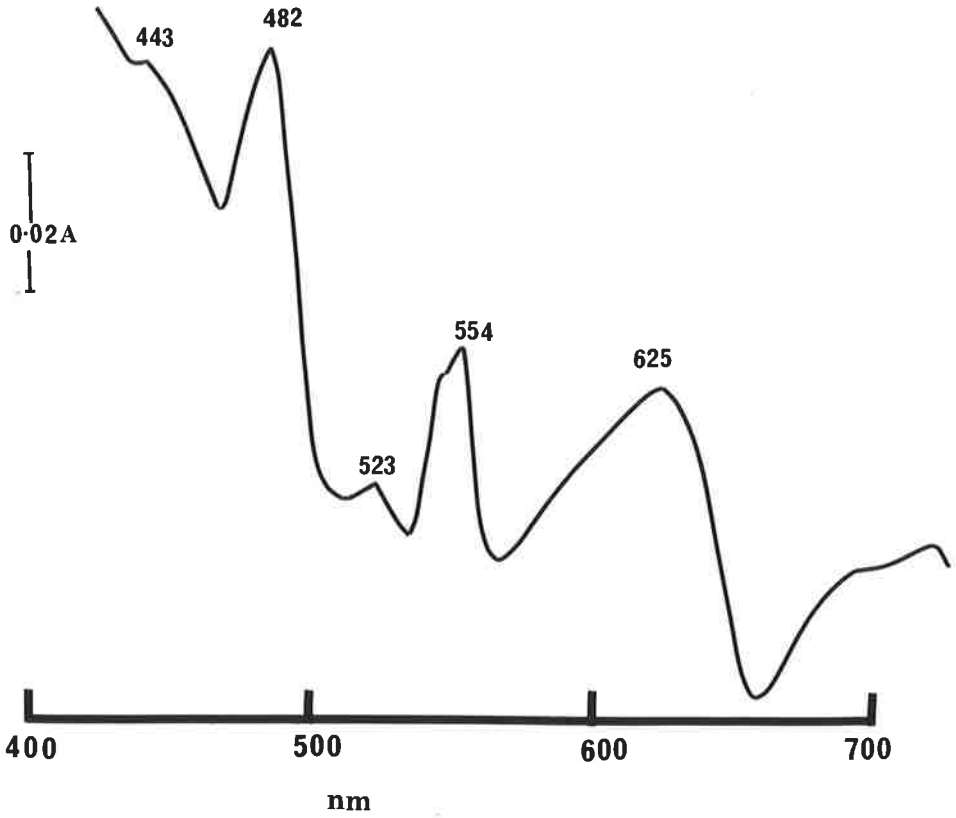


Figure 48

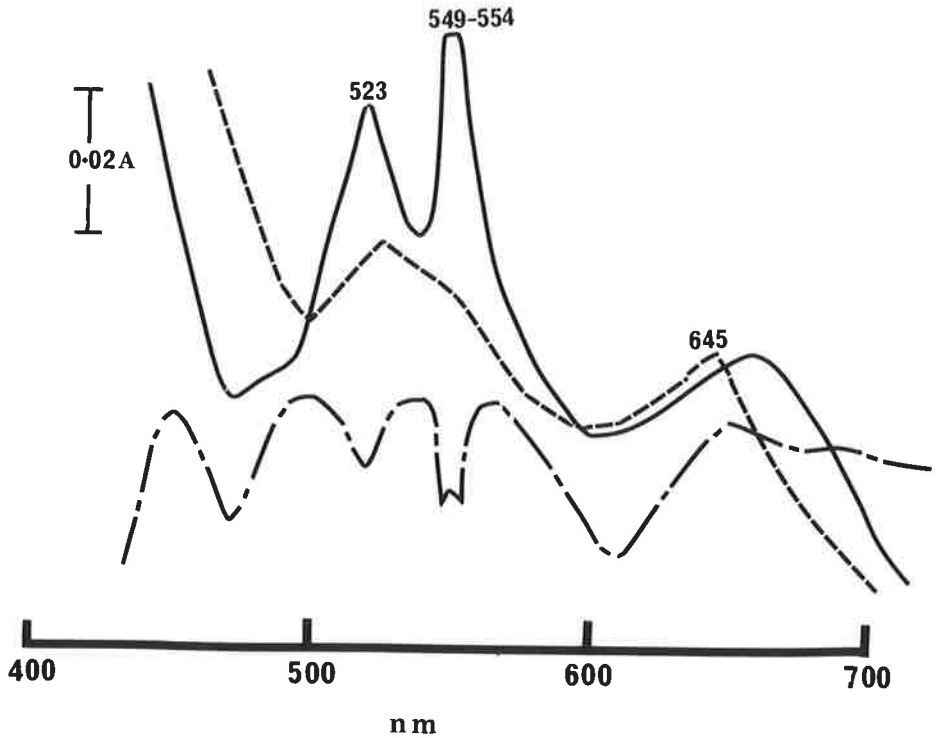


Figure 49. Spectra of purified nitrite reductase in the presence of alkaline pyridine and sodium dithionite.

The method of Newton (1969) described in Section 2.7.4 was used for examining the formation of pyridine haemochromogen. 250 µl of purified enzyme (Fraction V, Table 34) in 0.025 M Tris-HCl buffer (pH 7.5) was made 25% (v/v) and 0.1 M with respect to pyridine and NaOH respectively. This mixture (5 ml) was then dispensed into two 1 cm cuvettes. To the reference cuvette, a crystal of $K_3Fe(CN)_6$ was added and the difference spectra was recorded (– – – – –). Difference spectra was recorded again after adding $Na_2S_2O_4$ to the sample cuvette (————————).

Figure 50. Absolute and sulphide-reduced *versus* oxidized spectra of SDS-treated nitrite reductase.

Purified nitrite reductase (12.5 mg protein/ml) was incubated with 1.5% (w/v) SDS at 30^{o} for 30 min. The absolute spectrum of SDS-treated enzyme (– – – – –) and difference spectra, after reducing with sulphide (————————), were recorded as described in Section 2.6.3.

Figure 49

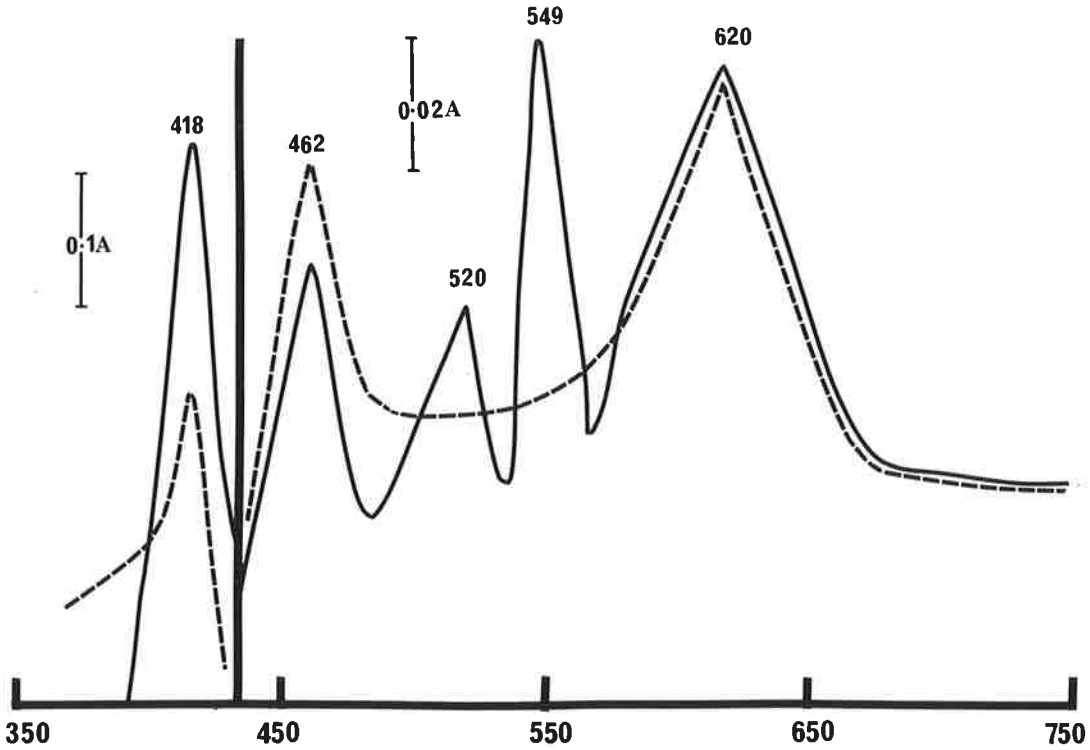
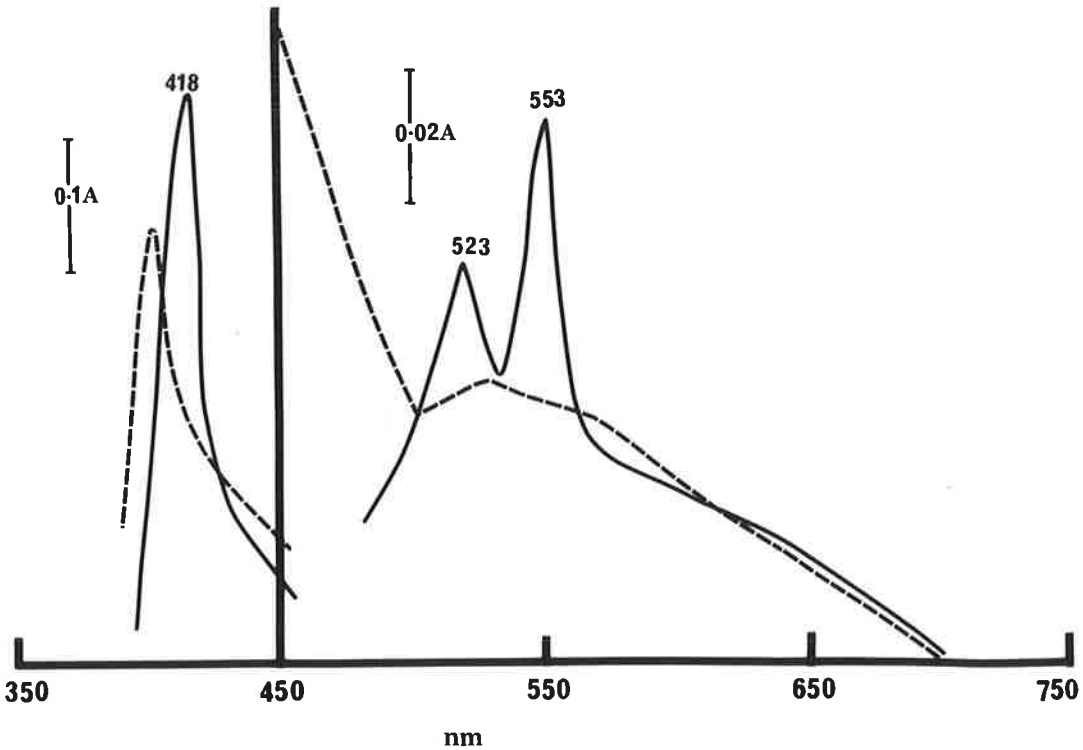


Figure 50



pyridine.

3.2.11 Dissociation of purified nitrite reductase into cytochrome *c* and haem *d*

Cytochrome *c* and haem *d* were dissociated from the purified nitrite reductase on incubating it with SDS. The optimum concentration of SDS required to dissociate these components from the enzyme ranged between 1.0 to 1.5 mg SDS per mg protein.

The absorption spectra of the SDS-treated enzyme after its reduction with sulphide is illustrated in Figure 50. The appearance of absorption bands at 418, 523 and 553 nm indicated that only the cytochrome *c* component of the SDS-treated enzyme was reduced by sulphide. Thus the absorption bands of reduced cytochrome *d* were not detected on reducing the SDS-treated enzyme with sulphide.

Gel-filtration, through a Sephadex G-100 column, resolved the SDS-treated enzyme into two distinct components, one pink and the other green coloured fraction as shown in Figure 51.

The pink fraction, containing the cytochrome *c* moiety, was eluted first from the column followed by dark green cytochrome *d*-containing fractions. The absorbance of these two fractions at 280 nm indicated that bulk of the protein was associated with cytochrome *c* containing fractions (Figure 52).

3.2.11.1 Spectral properties of dissociated cytochrome *c*

Absorption spectra of the cytochrome *c* containing component, recovered after passage of the SDS-treated enzyme through a Sephadex G-100 column, is illustrated in Figure 53. It had absorption bands

Figure 51. Separation of cytochromes *c* and *d* on Sephadex G-100 column.

Purified nitrite reductase (Fraction V, Table 34), after incubation with 1.5% (w/v) SDS at 30^{o} for 30 min as described in Section 2.5.8 was loaded on to a Sephadex G-100 column (2.5 x 40 cm). Separation of SDS-treated nitrite reductase into red (containing cytochrome *c*) and green (cytochrome *d*) fractions is illustrated in the Figure.

Figure 51

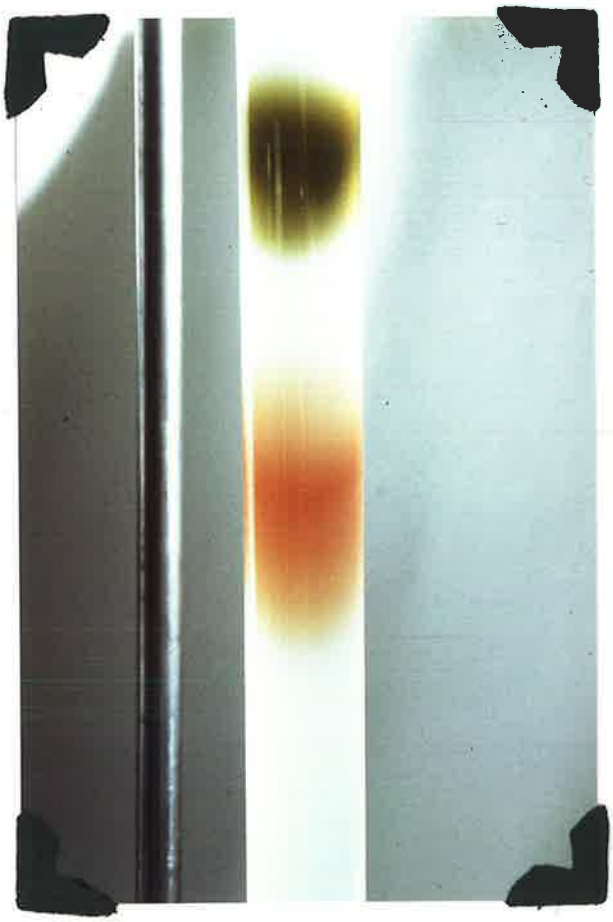


Figure 52. Elution profile of cytochromes *c* and *d* from Sephadex G-100 column.

Purified nitrite reductase (Fraction V, Table 34) was incubated with 1.5% (w/v) SDS at 30° for 30 min and loaded on to a Sephadex G-100 column (2.5 x 40 cm) which had been equilibrated, at room temperature, with 0.025 M Tris-HCl buffer (pH 7.5). The sample was eluted with the same buffer at a flow rate of 15 ml/hr. 3 ml fractions were collected and the absorbance at 405 nm and 280 nm were measured.

□- - - - - - - -□ E_{280}

■———————■ E_{405}

Figure 52

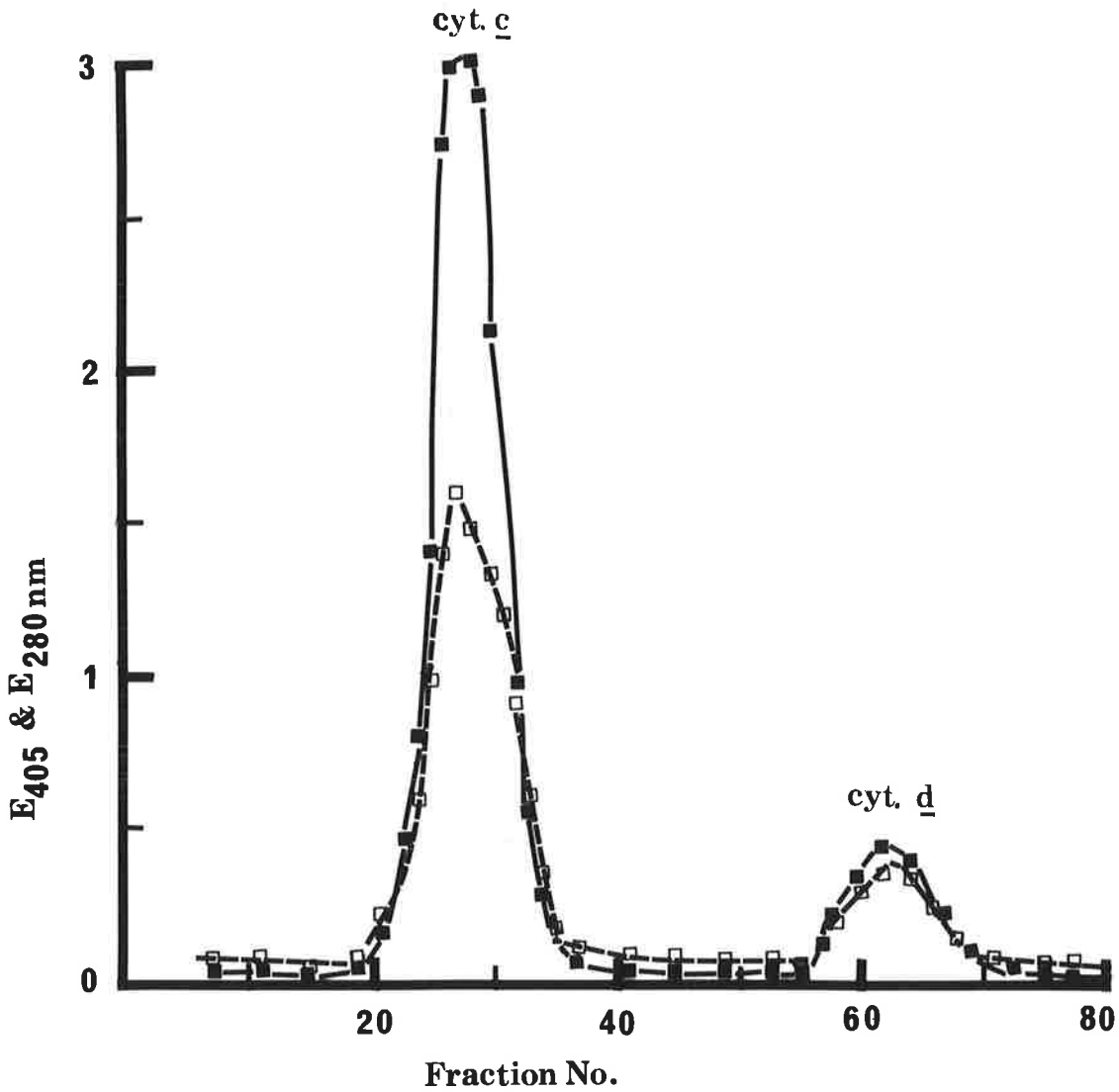


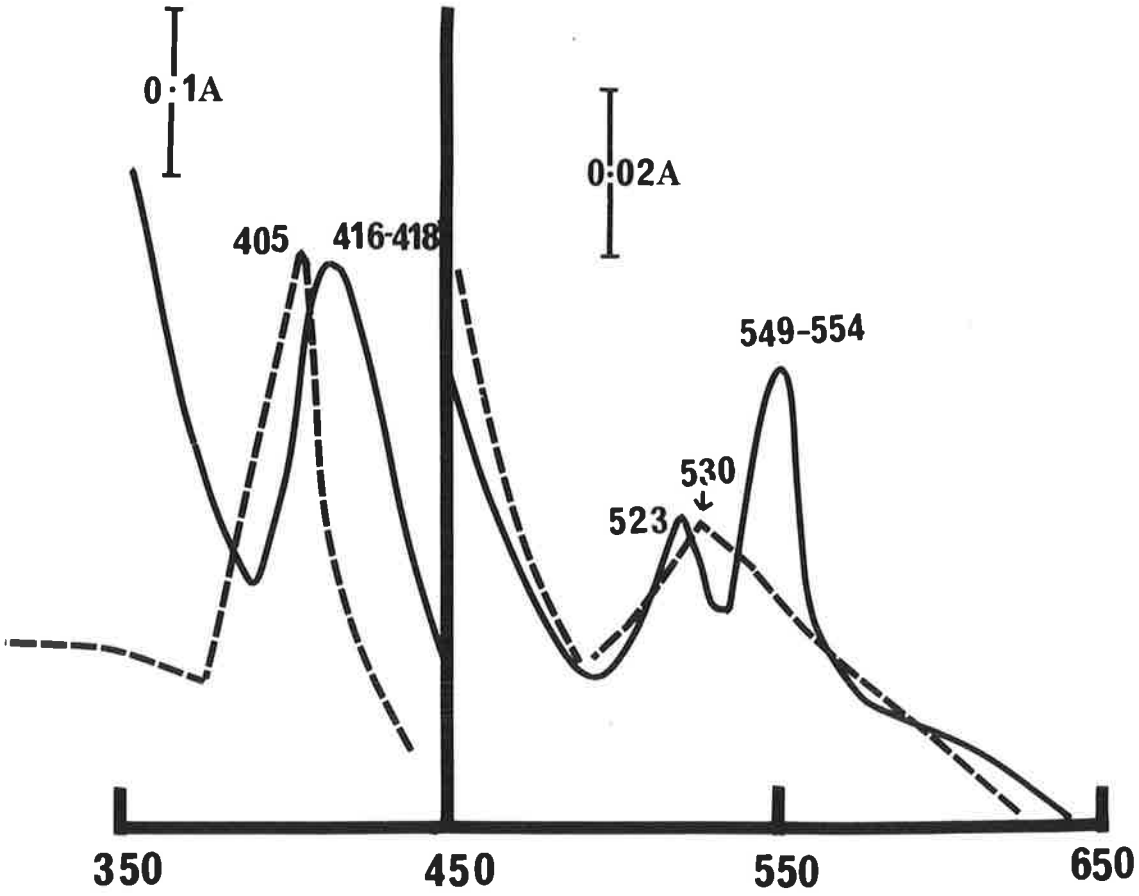
Figure 53. Absolute and dithionite-reduced *versus* oxidized, difference spectra of dissociated cytochrome *c*.

The sample and the reference cuvettes each, in a final volume of 2.5 ml, contained dissociated cytochrome *c* (1.6 mg protein) and 0.025 M Tris-HCl buffer (pH 7.5). To the sample cuvette, a few crystals of $Na_2S_2O_4$ were added and absorption spectra were recorded as described in Section 2.6.3.

– – – – – – – – – Absolute spectra of dissociated cytochrome *c*

——————— Difference spectra, $Na_2S_2O_4$-reduced *versus* oxidized.

Figure 53



at 405 and 530 nm and on reduction with sodium dithionite at 416-418, 523 and 549-554 nm. This spectra was almost identical with that obtained for cytochrome *c* of the undissociated reduced enzyme (Figure 42) except that bifurcation of α-band was not very marked in the dissociated cytochrome *c*. The ratio of α/β bands of the separated cytochrome *c* was 0.81 which was lower than the value of 1.0 for the undissociated enzyme.

On adding sulphide to the dissociated cytochrome *c*, absorption spectra similar to that after reduction with sodium dithionite was observed except that the rate of reduction was much slower. Although only a slight bifurcation of the α-band of the either dithionite- or sulphide-reduced cytochrome *c* was observed, it became more pronounced on adding nitrite (Figure 54). In the presence of nitrite, the α/β ratio of the cytochrome *c* was lowered to 0.42. Reduction of dissociated cytochrome *c* with sulphide, in the presence of 1mM KCN, showed a sharp peak at 551 nm and the addition of nitrite did not result in the bifurcation of the α-peak as was observed in the absence of KCN. This bifurcation of α-peak of the reduced cytochrome *c* was caused specifically by nitrite since nitrate did not produce this effect. The addition of nitrite to the dithionite-reduced cytochrome c_{551}, which was isolated from *T. denitrificans* (Section 3.2.7.2, Figure 37) did not affect its α-peak. The dissociated cytochrome *c* was not reduced by sulphite.

The spectrum for the pyridine haemochromogen of dissociated cytochrome *c* (Figure 55) was identical to that of the cytochrome *c* of the undissociated enzyme (Figure 49).

3.2.11.2 Spectral properties of haem *d*

The haem *d* component separated from the SDS-treated purified

Figure 54. Absorption spectra of sulphide-reduced dissociated cytochrome *c*.

Experimental details are as in Figure 53 except that 10 µmoles of sulphide, instead of $Na_2S_2O_4$, was added and the reaction mixture was incubated for 20 min. After recording the spectra, 20 µmoles of $NaNO_2$ was added to the sample cuvette.

A. ———————— S^{2-}-reduced *versus* oxidized, difference spectra

B. – – – – – – – – – After adding $NaNO_2$ to A.

Figure 54

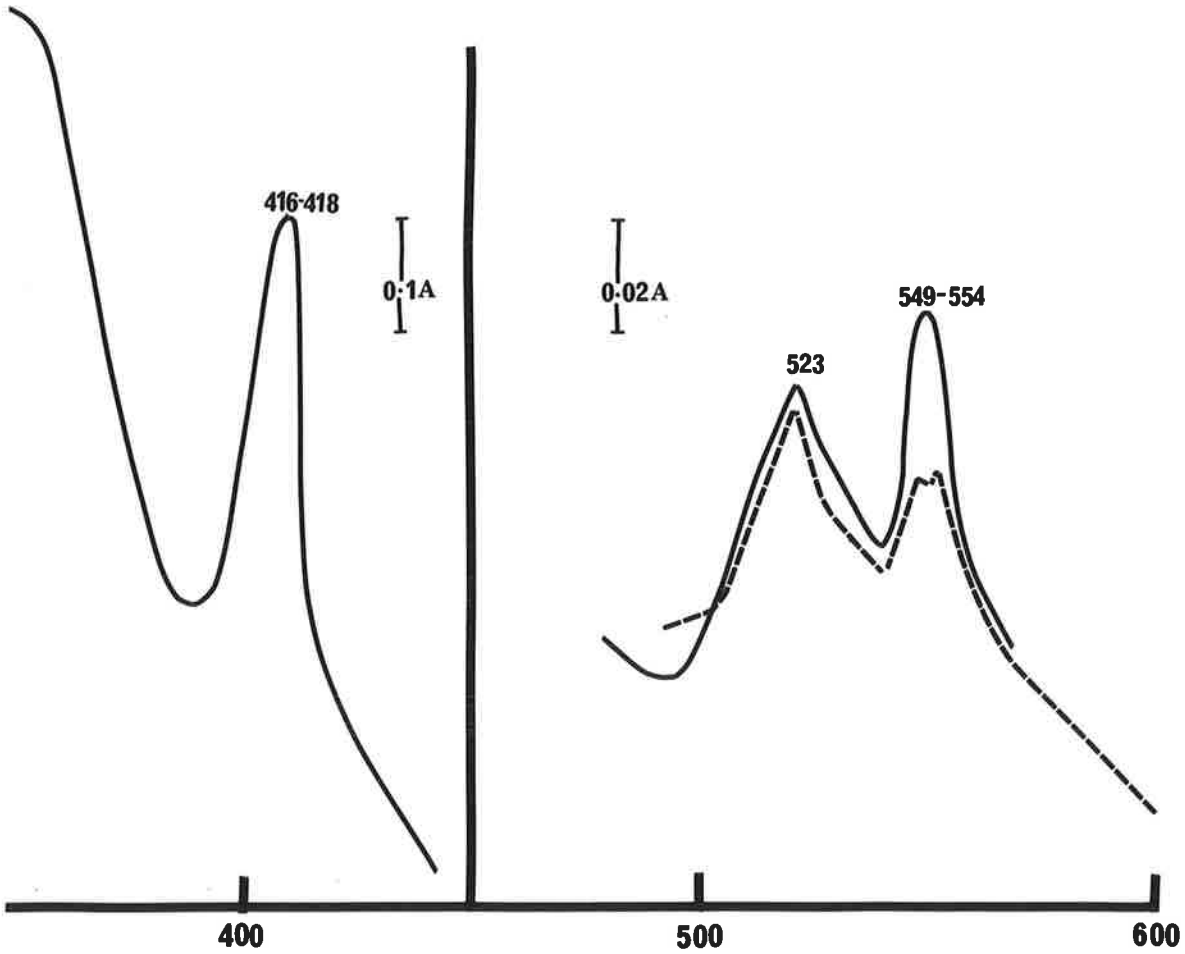
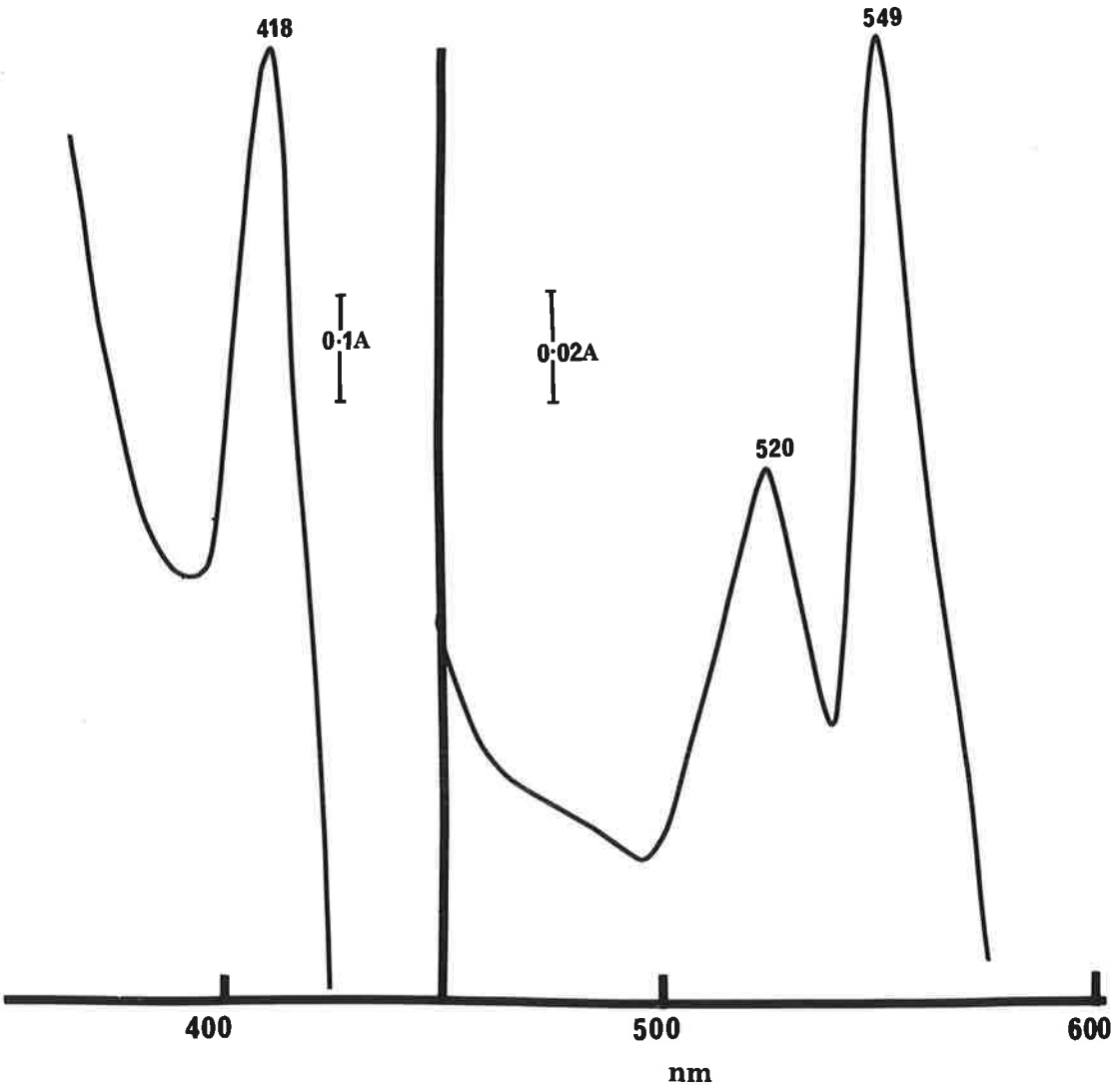


Figure 55. Difference spectra of alkaline pyridine haemochromogen of cytochrome *c*, dissociated from cytochrome *cd* complex.

The method of Newton (1969), given in Section 2.7.4, was used for the preparation of pyridine haemochromogen. The cytochrome *c* (3.2 mg protein) in 0.025 M Tris-HCl buffer (pH 7.5) was made 25% (v/v) and 0.1 M with respect to pyridine and NaOH respectively. This mixture was dispensed equally into two cuvettes. After adding a small amount of $Na_2S_2O_4$ to the sample cuvette and a crystal of $K_3Fe(CN)_6$ to the reference cuvette, the difference spectra was recorded.

Figure 55



nitrite reductase, showed absorption bands at 405 and 680 nm with a shoulder around 480 nm (Figure 56). On reduction with sodium dithionite, two broad peaks at 460 and 610-620 nm were observed. Under anaerobic conditions, the haem *d* component was reduced slowly by sulphide and it showed absorption band at 457 nm and a broad peak at 610-640 nm. The ratio of absorbance at 457 and 610-640 nm was 2.2. Haem *d* was readily autooxidizable in air (Figure 57).

3.2.12 Products of nitrite reduction by purified enzyme

The reduction products of ^{15}N-labelled nitrite by the purified nitrite reductase were examined in a mass spectrometer. The data in Figure 58 show that both NO (mass 31) and N_2O (mass 45) were produced. During the first 30 min, N_2O was formed in relatively larger amounts than NO, however beyond this period, N_2O content remained constant whereas NO continued to increase for a further 120 min incubation period. Only traces of N_2 gas (mass 29) were detected during the reduction of nitrite by the purified enzyme.

3.2.13 Effect of NO gas on purified nitrite reductase

3.2.13.1 Formation of NO- enzyme complex

On bubbling NO gas for 20 sec through the purified nitrite reductase, which had been previously reduced anaerobically with sulphide, a small but distinct peak appeared at 573 nm (Figure 59). At the same time, the height of the absorption bands of the reduced cytochromes *c* and *d* decreased. As demonstrated earlier (Figure 46), this band at 573 nm was also observed during the oxidation of the sulphide-reduced nitrite reductase enzyme with nitrite.

Figure 56. Absolute and dithionite-reduced *versus* oxidized spectra of haem *d*.

Details of the procedure are as in Figure 53 except that haem *d*, isolated from purified nitrite reductase (Fraction V, Table 34) as described in Section 2.5.8, was used in place of dissociated cytochrome *c*.

———————— Absolute spectra

– – – – – – – – $Na_2S_2O_4$-reduced *versus* oxidized, difference spectra.

Figure 56

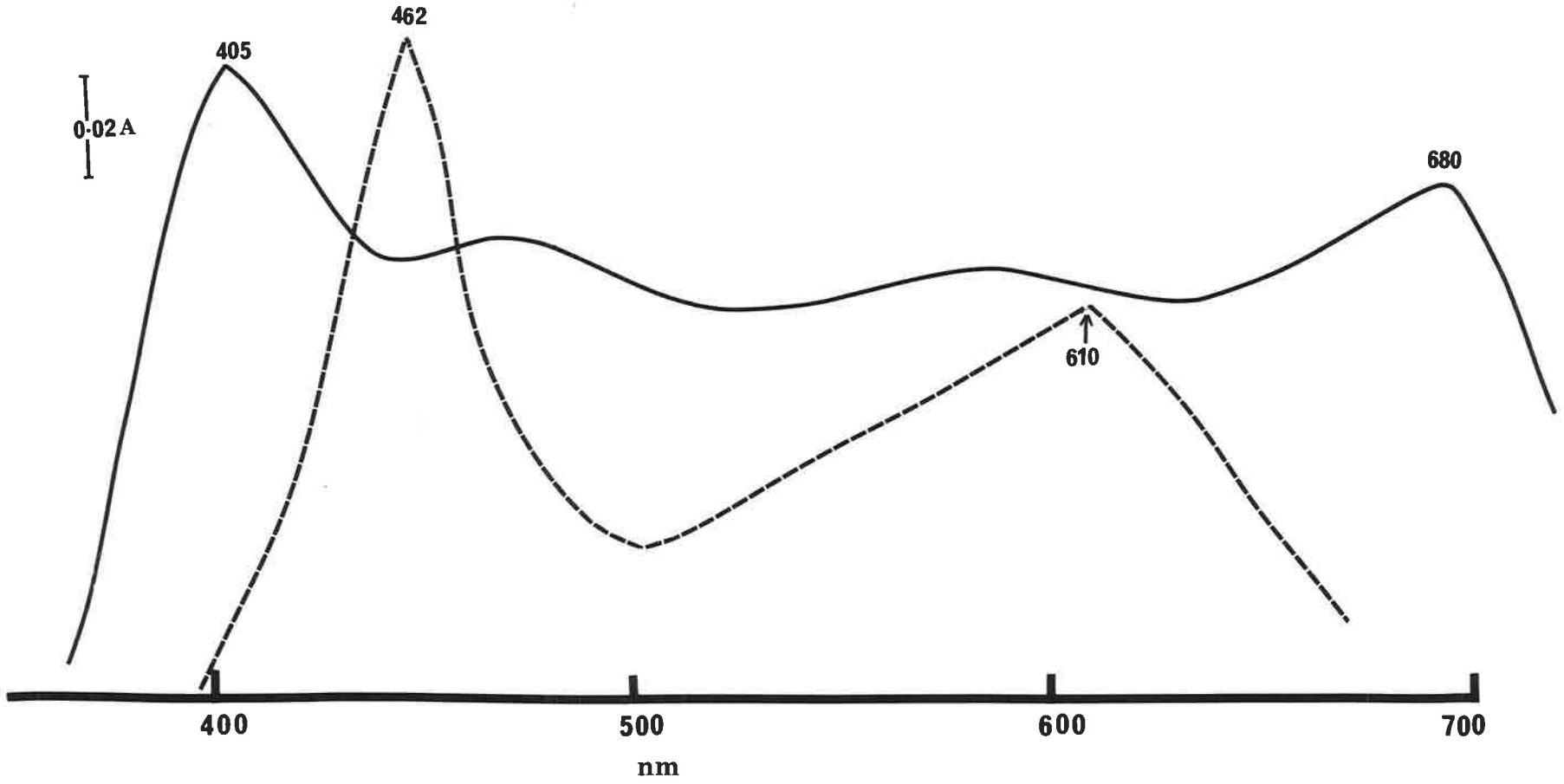


Figure 57. Sulphide-reduced *versus* oxidized difference spectra of haem *d*.

Haem *d*, 250 µl (3.25 µM) and 0.025 M Tris-HCl buffer (pH 7.5), in a total volume of 2.5 ml, were dispensed into both sample (Thunberg) and reference cuvettes. Side arm of the sample cuvette contained 10 µmoles of sulphide solution. The sample cuvette was thoroughly evacuated and flushed with high purity N_2 gas. This procedure was repeated three times. Sulphide solution was then tipped into the cuvette from the side arm and after 30 min incubation at room temperature, difference spectra was recorded.

A. ———————— Sulphide reduced *versus* oxidized difference spectra

B. – – – – – – – – After bubbling air for 30 sec through A.

Figure 57

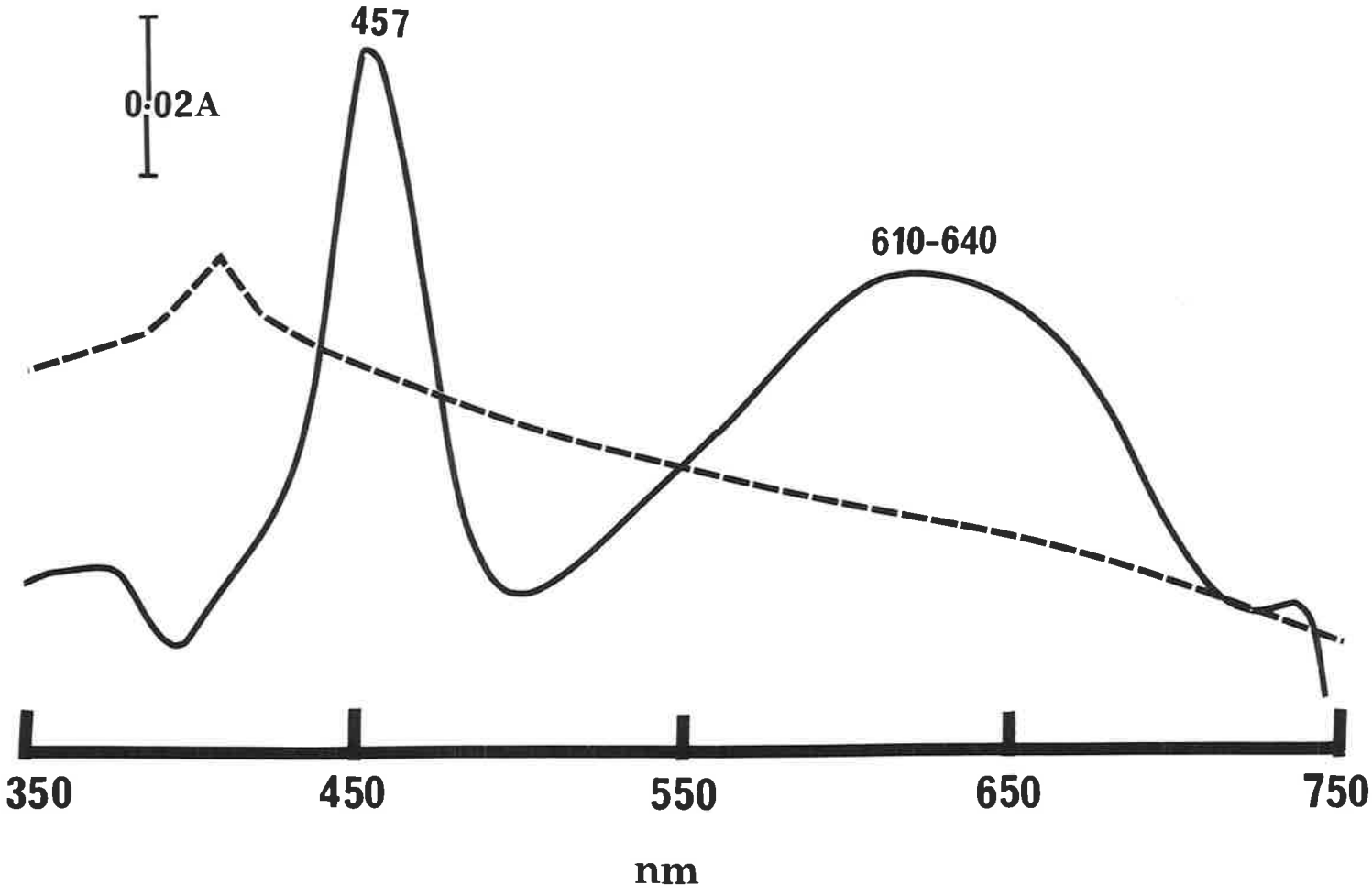
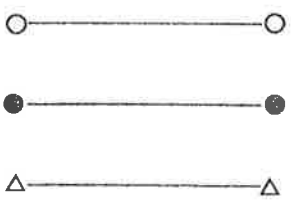


Figure 58. Effect of incubation time on the production of various nitrogenous gases from $^{15}NO_2^-$ by purified nitrite reductase (Fraction V, Table 34).

The experimental details are given in Section 2.6.7. The reaction mixture in Warburg flasks, in a final volume of 1 ml, contained the following: Tris-HCl (pH 7.5), 35 µmoles; $Na^{15}NO_2$ (27 atom % excess), 2 µmoles and 0.2 ml of purified nitrite reductase enzyme (2.4 mg protein). The reaction was started by injecting 5 µmoles of sulphide solution into the side arm via subaseal. At specified time, the reaction was stopped by injecting 250 µl of 10 M KOH into the flasks. The gaseous products were identified in an A.E.I. mass spectrometer.

The results are expressed as relative peak heights (proportional to concentrations) and have been corrected for control values (without the enzyme).



NO (mass 31)

N_2O (mass 45)

N_2 (mass 29).

Figure 58

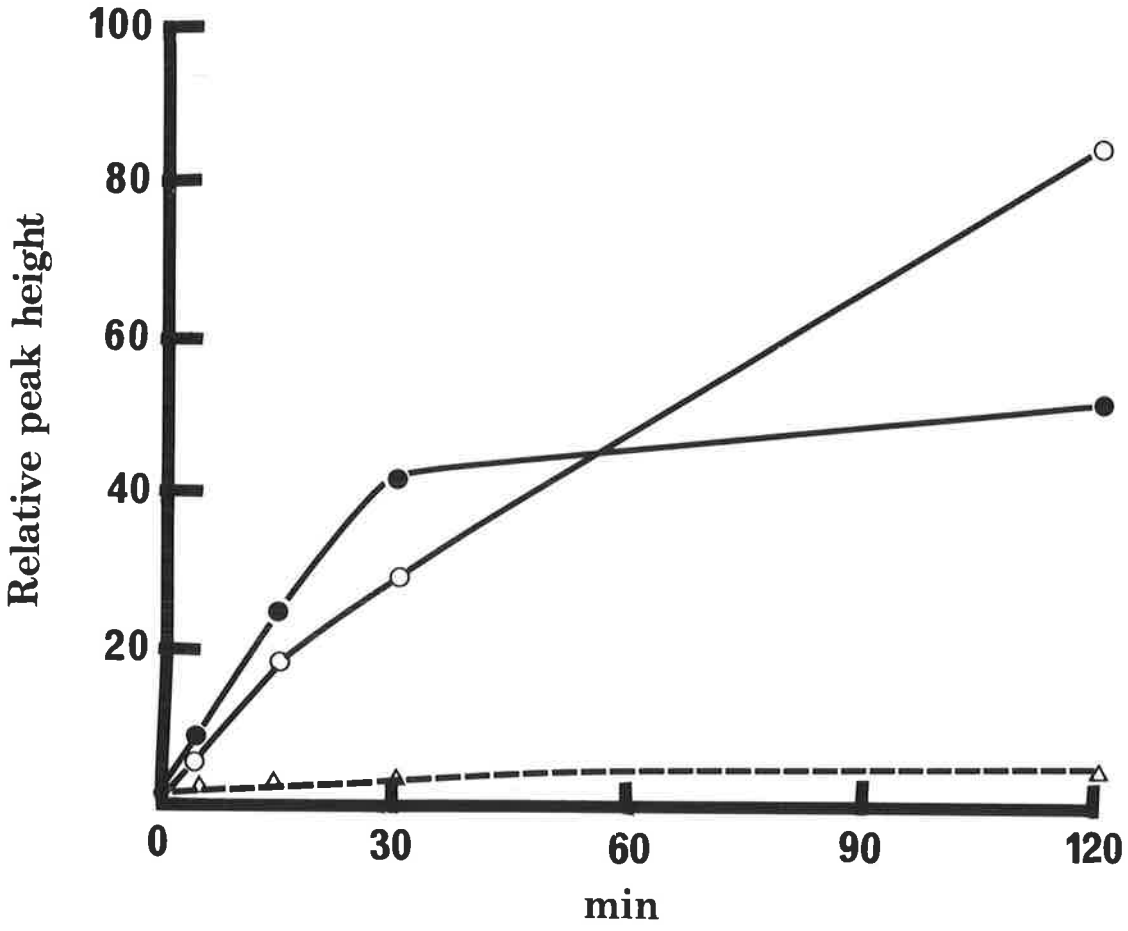


Figure 59. Effect of NO gas on the spectra of purified nitrite reductase reduced with sulphide.

Purified enzyme (200 µl of Fraction V, Table 34) and 0.05 M Tris-HCl buffer (pH 7.5), in a final volume of 2.5 ml, were dispensed into both sample (Thunberg) and reference cuvettes. The side arm of the sample cuvette contained 10 µmoles of freshly prepared sulphide solution. The cuvette was made anaerobic as described in Section 2.6.3. Sulphide was mixed with the contents of the cuvette and incubated for 10 min. Then NO gas was bubbled through the reaction mixture for 20 sec. The cuvette was then immediately closed under the stream of N_2 gas and the difference spectra recorded after 2 min.

Figure 60. Effect of sulphide and dithionite on the spectra of purified nitrite reductase treated with NO gas.

Purified nitrite reductase (200 µl) and 0.05 M Tris-HCl buffer (pH 7.5) were dispensed into both sample and reference cuvettes. After bubbling NO gas through the sample cuvette for 15 sec, the enzyme was reduced with either 10 µmoles S^{2-} or 2 mg $Na_2S_2O_4$ and the following spectra were recorded:

A. ———————— NO-treated enzyme

B. — — — — — — — S^{2-} added to A

C. — — — — — — — $Na_2S_2O_4$ added to A.

Figure 59

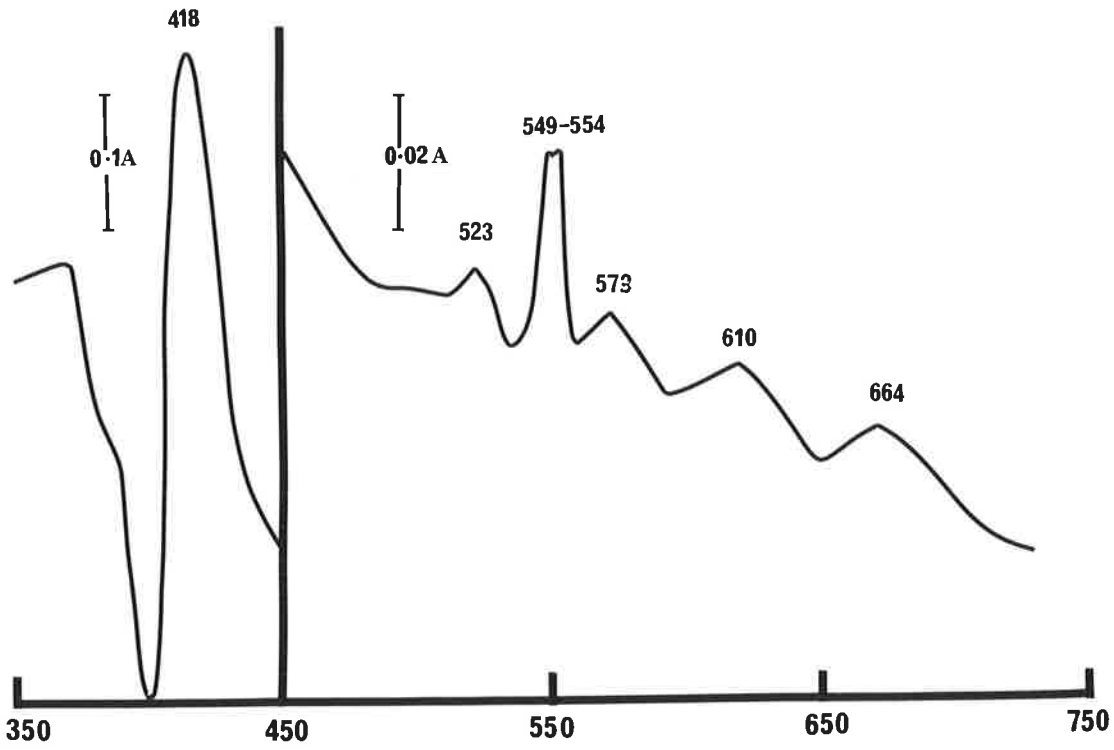
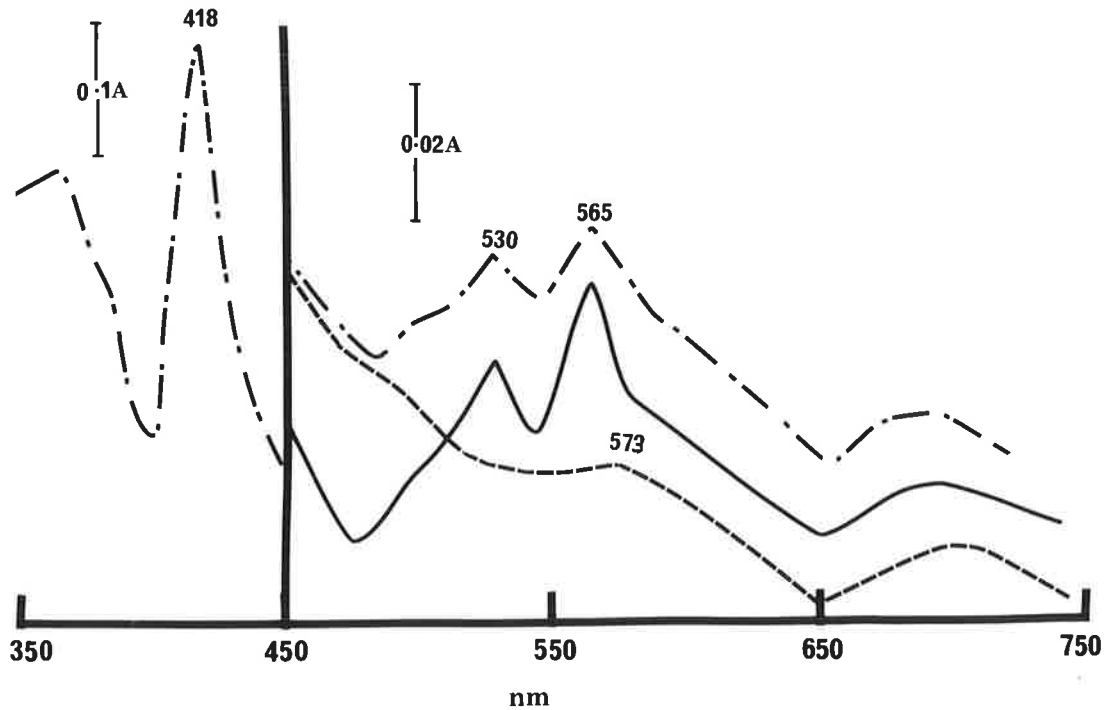


Figure 60



In subsequent experiments, the purified nitrite reductase was treated with NO gas for 15 sec in the absence of the reductant, viz. sulphide, to ascertain whether prior reduction of the enzyme was essential for the formation of the NO- enzyme complex. Under these conditions the absorption peak at 573 nm was not detected (Figure 60). The absorption bands at 525 and 645 nm, associated with the purified nitrite reductase were, however, replaced by peaks at 530 and 565 nm after treatment with NO gas. The addition of sulphide to this NO-treated enzyme did not change the pattern of the spectra except that the peak heights were lowered. On adding sodium dithionite to the NO-treated enzyme, the peaks at 530 and 565 nm disappeared and a small shoulder appeared at 573 nm.

3.2.13.2 Dissociation of the NO-enzyme complex

The spectra in Figure 61 indicate that the absorbance at 573 nm, due to the formation of a complex between NO and the sulphide-reduced enzyme, decreased with time. It was accompanied by the oxidation of both cytochromes *c* and *d*. After a 30 min incubation, the absorbance bands of reduced cytochrome *c* and *d* and that at 573 nm disappeared and two prominent peaks at 530 and 565 nm appeared. As mentioned earlier similar peaks were observed on treating the enzyme with NO gas in the absence of sulphide. The colour of the enzyme preparation at this stage, had turned yellow and the pH of the reaction mixture was very acid (pH 3.5).

3.2.13.3 Effects of pH, CO, CN^- on the formation and dissociation of the NO-enzyme complex

As noted in the preceding experiment, the pH of the reaction mixture was markedly lowered after the treatment with NO gas. The

Figure 61. Effect of incubation time on the formation of complex between NO and sulphide-reduced purified nitrite reductase (Fraction V, Table 34).

Experimental details as in Figure 59 except that the difference spectra were recorded at the following times after bubbling NO gas through the reaction mixture:

———————— After 3 min

— — — — — — — After 10 min

— — — — — — — — After 30 min

Figure 62. Formation of complex between NO and sulphide-reduced purified nitrite reductase at pH 10.2.

Experimental details are as in Figure 59 except that 0.1 M Tris-HCl buffer (pH 10.2) was used and difference spectra were recorded at the following times after bubbling NO gas through the reaction mixture:

——— ———————— After 3 min

— — — — — — — — After 1 hr.

Figure 61

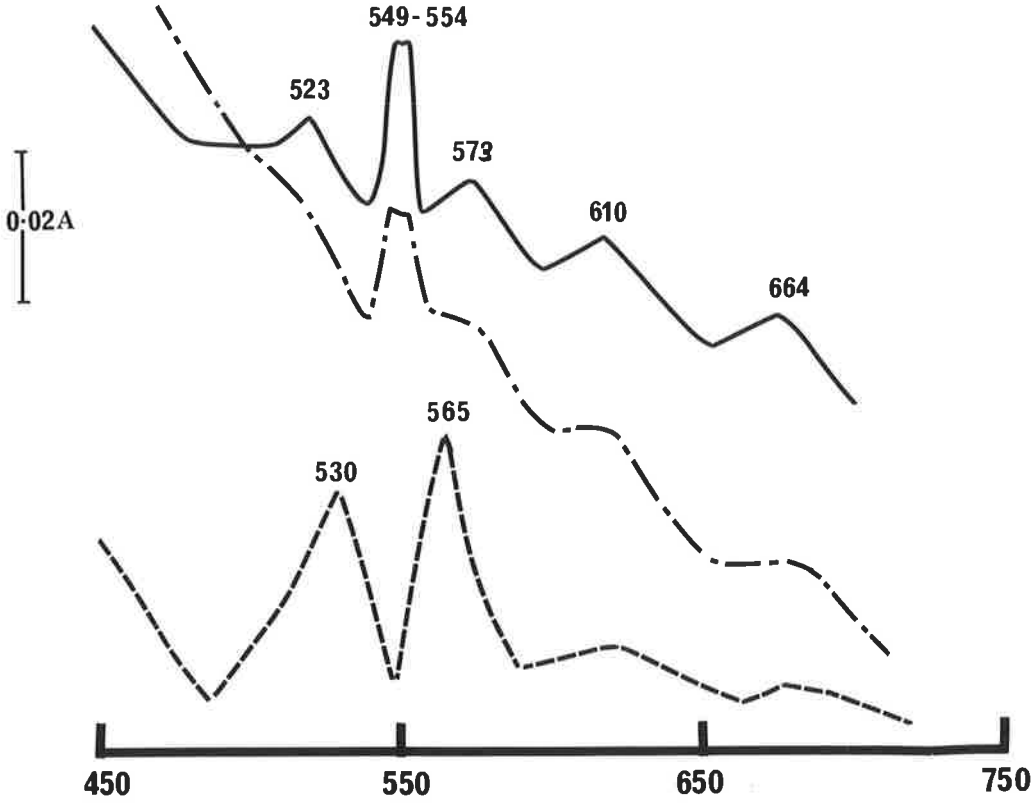
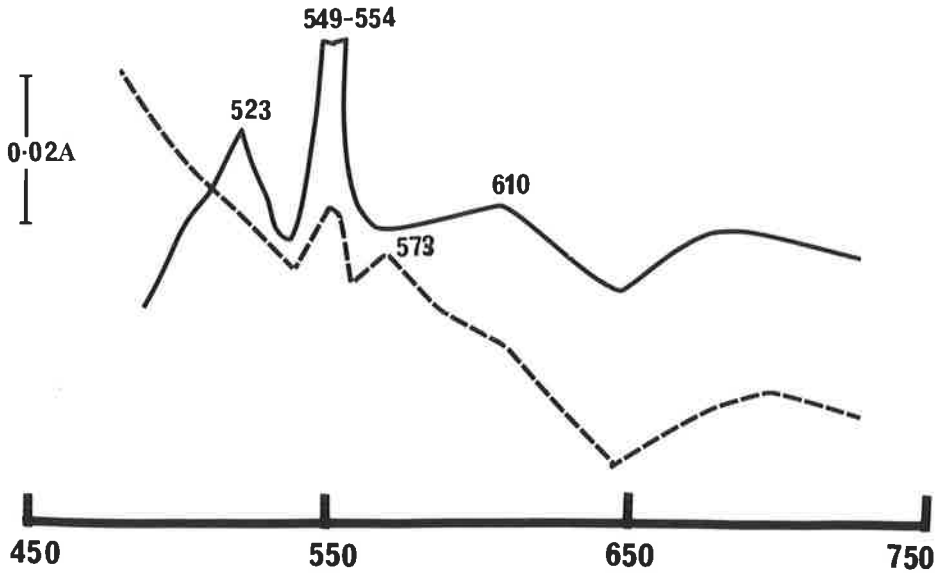


Figure 62



effect of NO gas was, therefore, investigated in the presence of a buffer of a higher ionic strength (0.1M Tris-HCl instead of 0.05M) at pH 10.2. On bubbling NO gas through the sulphide-reduced enzymes, the peak at 573 nm did not appear immediately (Figure 62). After 1 hr, the absorbance bands of cytochromes *c* and *d* were markedly diminished and this was accompanied by the appearance of a peak at 573 nm. The pH of the reaction mixture at this stage was 7.6.

From these results, it is apparent that the formation of the NO- enzyme complex, at 573 nm, occurred at the neutral pH, while the peaks at 530 and 565 nm appeared at acid pH (below 6.5). As previously mentioned, when NO gas was bubbled through the enzyme (without reductant) absorption bands appeared at 530 and 565 nm and the pH of the reaction mixture was lowered to 2.3. However on raising the pH to 6.5 with 1N NaOH, the peak heights at 530 and 565 nm were markedly reduced and at pH 7.8, they disappeared altogether (Figure 63). These bands reappeared on lowering the pH of the reaction mixture to 3.3 with N HCl.

Pre-treatment of the sulphide-reduced enzyme with CO for 5 min, did not affect the formation of the peak at 573 nm when NO gas was subsequently bubbled through this preparation. CO, however, delayed the disappearance of the 573 nm band (Figure 64) by about 1 hr.

Cyanide had no effect on either the appearance or disappearance of the absorption band at 573 nm (Figure 65).

3.2.13.4 Effect of NO on dissociated cytochrome *c* and haem *d*

The cytochrome *c* dissociated from purified nitrite reductase was reduced with sulphide and then treated with NO gas. Instead of the

Figure 63. Effect of pH on the formation of complex between NO and the purified nitrite reductase.

Purified enzyme (200 µl of Fraction V, Table 34) and 0.1 M Tris-HCl buffer (pH 7.5) were dispensed into both sample (Thunberg) and reference cuvettes. No gas was bubbled through the sample cuvette for 20 sec. pH of the NO-treated sample was 3.4. After recording the difference spectra, pH of the reaction mixture was progressively adjusted to 4.0, 6.5 and 7.8 with 2N NaOH. The absorption spectra at each pH value was recorded. pH of the reaction mixture was then lowered with 2N HCl from 7.8 to 3.3 and the absorption spectra recorded.

A. ———————— pH 3.4 or 4.0

B. — — — — — — — — pH 6.5

C. — — — — — — — — — pH 7.8

D. —Δ—Δ—Δ—Δ—Δ— pH of C lowered to 3.3.

Figure 64. Effect of CO on the formation and dissociation of complex between NO and sulphide-reduced purified enzyme (Fraction V, Table 34).

Experimental details are as in Figure 59 except that CO was bubbled through the reaction mixture for 5 min prior to treating it with NO gas for 20 sec. After the latter treatment, the difference spectra was recorded at the following time intervals:

———————— After bubbling CO gas through the reaction mixture

— — — — — — — — 1 hr after bubbling NO gas

— — — — — — — — 2 hr after bubbling NO gas.

Figure 63

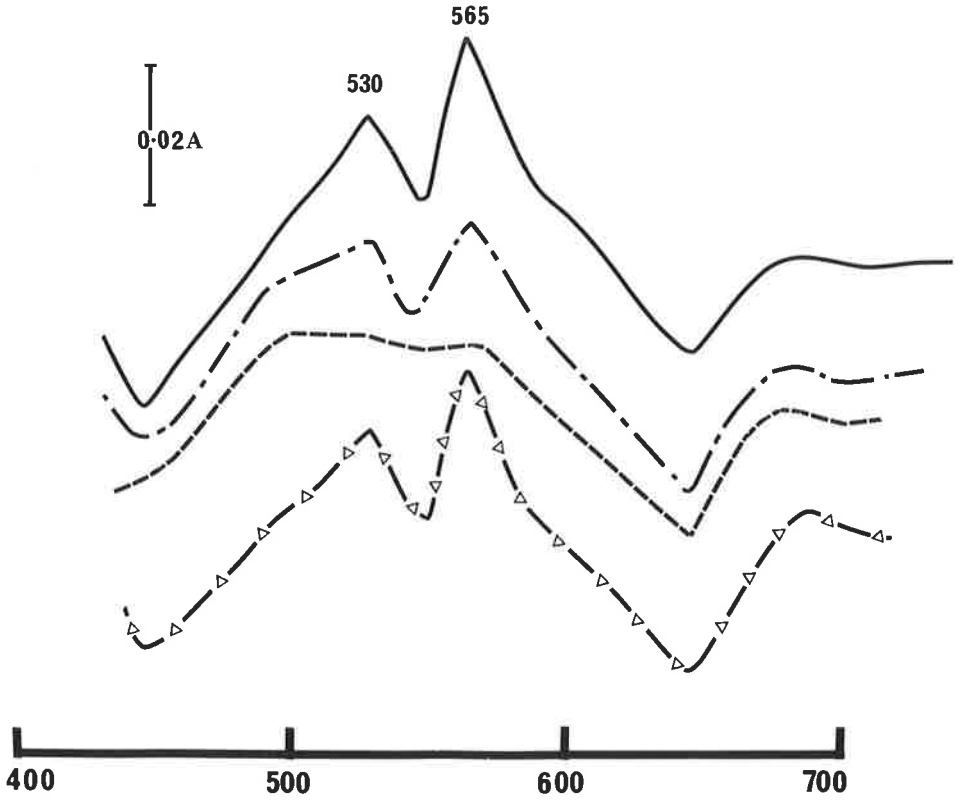


Figure 64

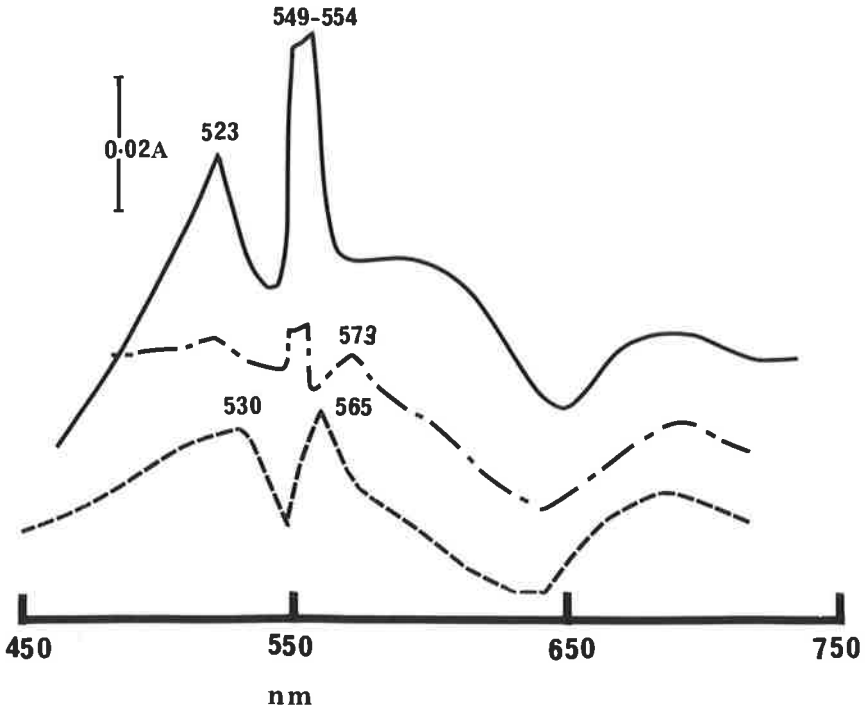


Figure 65. Effect of cyanide on formation and dissociation of the complex between NO and sulphide-reduced purified nitrite reductase.

Experimental details are as in Figure 59 except that 1 mM KCN was also included in the reaction mixture. Difference spectra at following times, after bubbling NO through reaction mixture for 20 sec, were recorded:

———————— After 2 min

– – – – – – – – – After 30 min.

Figure 66. Effect of NO on spectra of sulphide-reduced cytochrome *c*, dissociated from the *cd* complex of purified nitrite reductase.

0.2 ml (3.6 mg protein) of cytochrome *c* component, isolated from purified nitrite reductase by the procedure given in Section 2.5.8, was dispensed into a cuvette containing 0.05 M Tris-HCl buffer (pH 7.5). After reducing it with S^{2-} as described in Figure 54, NO gas was passed through the reaction mixture for 15 sec.

A. ———————— S^{2-}-reduced spectra of dissociated cytochrome *c*.

B. – – – – – – – – – 2 min after treatment of A with NO gas.

Figure 65

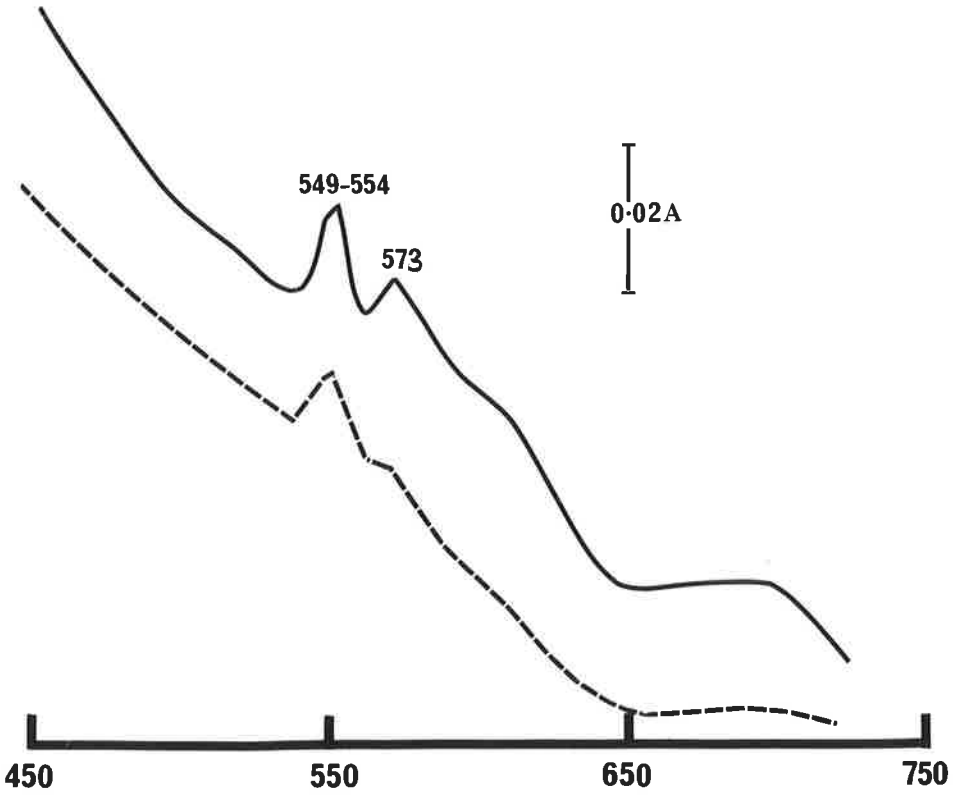
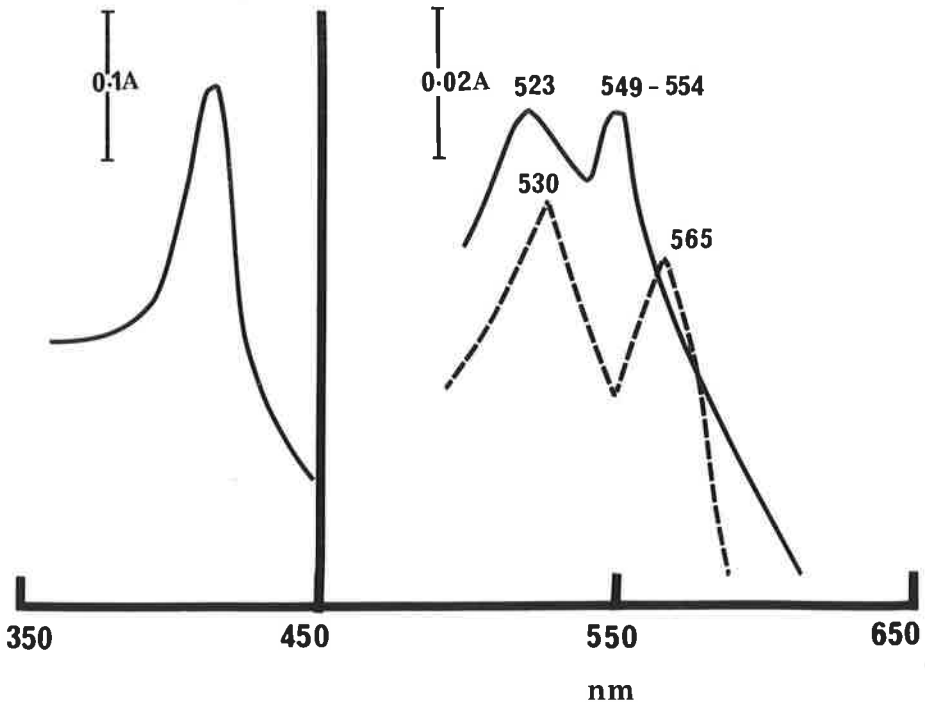


Figure 66



normal absorption bands at 523 and 549-554 nm, two peaks appeared at 530 and 565 nm (Figure 66). A similar experiment with haem *d* showed that NO gas abolished the spectral bands at 405 and 680 nm.

4. DISCUSSION

4.1 Nitrate reductase

Nitrate reductase is the first enzyme involved in both assimilatory and dissimilatory nitrate reduction in microorganisms. Since in the latter process nitrate serves as an alternate terminal oxidant to O_2, dissimilatory nitrate reductase is generally integrated with the respiratory chain of the organism. The association of nitrate reductase in *T. denitrificans* with cell-membranes, shown in the present investigation, confirms the earlier work of Adams *et al.* (1971) and is in accord with its role in cell respiration under anaerobic conditions. With the exception of *Spirillum itersonii* (Gauthier *et al.*, 1970) and *Clostridium perfringens* (Chiba and Ishimoto, 1973, 1977), respiratory nitrate reductases from various bacteria, *Escherichia coli* (Iida and Taniguchi, 1959), *Pseudomonas aeruginosa* (Fewson and Nicholas, 1961B), *Micrococcus denitrificans* (Lam and Nicholas, 1969A, B), *Aerobacter aerogenes* (Van't Riet and Planta, 1969), *Pseudomonas denitrificans* (Radcliffe and Nicholas, 1970) and *Bacillus stearothermophilus* (Kiszkiss and Downey, 1972) have been shown to be associated with the cell membranes.

Dissimilatory nitrate reductase in *T. denitrificans* differs from nitrate reductase in other organisms because the reduction of nitrate in this chemolithotrophic bacterium is linked to the oxidation of sulphur compounds (Baalsrud and Baalsrud, 1954; Peeters and Aleem, 1970; Adams *et al.*, 1971; Aminuddin and Nicholas, 1974B). The work presented here confirms that nitrate was reduced, both in the P100 fraction and in the partially purified enzyme, when sulphite was the electron donor. In addition, as reported by Adams *et al.* (1971), NADH was also an effective

reductant for nitrate reduction in the P100 fraction.

In the present investigation, the properties of sulphite- and NADH-linked nitrate reductase activities were compared. The enzyme activity with sulphite and NADH as electron donors had different pH optima at 8.0 and 7.0 respectively. These values are similar to the pH optima reported by Adams *et al.* (1971). The sulphite-linked nitrate reductase but not the NADH-linked enzyme activity was markedly suppressed in phosphate buffer. It is likely that this anion affected the sulphite-linked enzyme by acting as a pseudo end product because of its chemical similarity with sulphate. This mechanism was proposed for the inhibition by phosphate of the sulphite : cytochrome *c* oxido-reductase from *Thiobacillus thioparus* by Lyric and Suzuki (1970A).

The NADH-linked nitrate reductase activity was relatively more labile than the sulphite-linked enzyme during storage and also at elevated temperatures. Restoration of the NADH-dependent activity of the stored enzyme by GSH indicates the involvement of -SH groups and its greater sensitivity to thiol binding reagents, viz. p-CMB and iodoacetamide, than the sulphite-dependent activity further supports this conclusion. The very marked inhibition of the sulphite-linked activity by NEM was probably due to the formation of an NEM-sulphite complex (Ellis, 1964) rather than a direct effect on the enzyme. The requirement of -SH groups for the activity of dissimilatory nitrate reductases has been demonstrated in *P. aeruginosa* (Fewson and Nicholas, 1961B), *M. denitrificans* (Lam and Nicholas, 1969B) and *P. denitrificans* (Radcliffe and Nicholas, 1970). Highly purified nitrate reductases from *M. halodenitrificans* (Rosso *et al.*, 1973), *E. coli* (Forget, 1974) and *M. denitrificans* (Forget, 1971), all contained labile sulphide.

The results presented herein indicate that both the sulphite- and NADH-linked nitrate reductase activities in *T. denitrificans* were affected by the inhibitors of electron transport chain. The differential sensitivity of the nitrate reductase activity with these reductants towards flavoprotein inhibitors suggests the involvement of two different flavoproteins or dehydrogenases for the transfer of electrons from sulphite and NADH to nitrate. The isolated FAD from the solubilized sulphite-linked nitrate reductase, as reported herein, suggests that it functions as an electron carrier for the enzyme. A requirement for flavins for dissimilatory nitrate reductases has been demonstrated in *E. coli* (Taniguchi *et al.*, 1956), *Achromobacter fisheri* (Sadana and McElroy, 1957), *P. aeruginosa* (Fewson and Nicholas, 1961B) and *M. denitrificans* (Lam and Nicholas, 1969B). Assimilatory nitrate reductases from various organisms are also flavoprotein (Nason, 1962).

Downey (1962) has shown that U.V. light destroys vitamin K type electron carriers. The diminished activities of both the sulphite- and NADH-linked nitrate reductase after exposure to U.V. light thus indicates that a quinone is required for nitrate reduction in *T. denitrificans*. This was confirmed by the inhibition of both these activities by a quinone antagonist, dicumarol. The failure of exogenously supplied ubiquinone to restore nitrate reductase activity in the U.V.-irradiated P100 fraction of *T. denitrificans* may well be due to the damaged quinone remaining tightly bound to the membranes (Knook and Planta, 1971A). Ubiquinone has been implicated in the reduction of nitrate in other gram-negative bacteria including *E. coli* (Itagaki, 1964; Enoch and Lester, 1974), *A. aerogenes* (Knook and Planta, 1971B).

As reported herein, nitrate reduction in P100 fraction with either sulphite or NADH as reductant was inhibited by antimycin A which blocks the transfer of electrons between cytochromes *b* and *c*. A role for cytochromes *b* and *c* in sulphite-linked nitrate reduction has been inferred earlier by Peteers and Aleem (1970), Adams *et al.* (1971), and Aminuddin and Nicholas (1974B). As shown in the current investigations, spectral changes in the P100 fraction, on its reduction with either sulphite or NADH and its subsequent oxidation with nitrate provides further evidence for cytochrome *c* as an electron carrier in the reduction of nitrate. Several other observations confirm this view. Thus a cytochrome c_{554}, isolated from the solubilized nitrate reductase, was reduced by sulphite in the presence of catalytic amounts of nitrate reductase. This reduced cytochrome c_{554} served as effective electron donor for nitrate reduction. The low K_m value of purified nitrate reductase for the reduced cytochrome c_{554} indicates a high affinity of the enzyme for this electron carrier. Because the redox-potential of nitrate/nitrite couple at pH 7.0 is +421 mV (Burton, 1957), the reduction of nitrate *via* cytochrome c_{554} (E'_o = +223 mV) is thermodynamically possible.

The molecular weight and the redox-potential of the cytochrome c_{554} isolated from the solubilized nitrate reductase from *T. denitrificans* was similar to that of mammalian cytochrome *c* (Dutton *et al.*, 1970). A cytochrome of the *c* type, with a redox-potential of +270 mV, prepared from *T. denitrificans* also mediated electron flow between thiosulphate or sulphite and nitrate (Aubert *et al.*, 1958; Milhaud *et al.*, 1958).

The participation of cytochrome *c* in nitrate reduction in *T. denitrificans*, as reported in the present work, contrasts with many

of the other facultative anaerobic bacteria where electrons are diverted to nitrate from cytochrome *b* of the respiratory chain. Thus cytochrome *b* has been shown to be the penultimate electron donor for nitrate reduction in *E. coli* (Itagaki *et al.*, 1962; Ruiz-Herrera and DeMoss, 1969; Enoch and Lester, 1974; Garland *et al.*, 1975), *M. denitrificans* (Scholes and Smith, 1968; Lam and Nicholas, 1969B), *P. denitrificans* (Radcliffe and Nicholas, 1970), *B. stearothermophilus* (Kiszkiss and Downey, 1972A) and *A. aerogenes* (Van't Riet *et al.*, 1972; Knook *et al.*, 1973). Only in *P. aeruginosa* (Fewson and Nicholas, 1961B) and in *E. coli* (Gray *et al.*, 1963; Fujita and Sato, 1966) does cytochrome *c* function in nitrate reduction. The physiological significance of the oxidation by nitrate of reduced cytochrome c_{552} in *E. coli* has however been questioned since this cytochrome is believed to be involved in nitrite but not in nitrate reduction. A purified nitrate reductase from *A. fisheri* showed cytochrome *c* type absorption spectra (Sadana and McElroy, 1957).

The requirement of molybdenum for nitrate reduction with either sulphite or NADH as the reductant was indicated in the present studies by the inhibitory effects of dithiol and thiocyanate which are known to chelate this micronutrient (Meriwether *et al.*, 1966). Molybdenum is a constituent of purified nitrate reductase in *M. denitrificans* (Lam and Nicholas, 1969B) and its participation in nitrate reduction was confirmed by ESR studies with the enzyme from *P. aeruginosa* (Fewson and Nicholas, 1961B) and *M. denitrificans* (Forget and Dervartanian, 1972).

Inhibition of nitrate reductase from *T. denitrificans* by azide and cyanide with either sulphite or NADH as the reductant reported in this thesis is in agreement with the results for the enzyme in *E. coli*

(Iida and Taniguchi, 1959; Forget, 1974) and *M. denitrificans* (Forget, 1971). However the assimilatory nitrate reductases are less sensitive to azide (Payne, 1973). The competitive inhibition of nitrate reductase by azide and the non-competitive effect of cyanide with respect to nitrate, are among some of the properties which distinguish this enzyme from the assimilatory type (Pichinoty, 1964).

The competitive inhibition by chlorate with respect to nitrate, observed in this work, suggests that it acts as an alternate substrate for the enzyme. This is in accord with the effect of chlorate on respiratory nitrate reductases from several other microorganisms (Fewson and Nicholas, 1961B; Hackenthal and Hackenthal, 1965; Payne, 1973).

The particulate nitrate reductase from *T. denitrificans* was solubilized by sodium deoxycholate treatment. After treatment of the P100 fraction with the detergent, the specific activity of the sulphite-linked nitrate reductase increased, whereas the NADH-dependent enzyme was inactivated. A similar loss in NADH-linked nitrate reductase after its dissociation from cell-membranes has been reported in *M. denitrificans* (Lam and Nicholas, 1969B), *P. denitrificans* (Radcliffe and Nicholas, 1970), *B. stearothermophilus* (Kiszkiss and Downey, 1972A). This loss in enzyme activity has been attributed to the disruption of electron flow from NADH to nitrate (Kiszkiss and Downey, 1972B). In *B. stearothermophilus*, the activity of the solubilized enzyme was restored on adding divalent cations especially Mg^{2+} (Kiszkiss and Downey, 1972B). In the present studies, the NADH-linked nitrate reductase of the deoxycholate treated P100 fraction was partially restored on adding PMS and almost completely with menadione. In addition, menadione alleviated the effects of various inhibitors, viz. atabrine, rotenone, HQNO and antimycin A on the NADH-

linked but not the sulphite-linked nitrate reductase activity. This effect is probably associated with an active NADH : menadione reductase which in the presence of menadione transfers electrons from NADH directly to cytochrome *c*. In fact in *E. coli*, Medina and Heredia (1958) have described an NADH-dependent system coupled to menadione reductase which utilized vitamin K_3 as an electron carrier for the reduction of nitrate. The presence of NADH-dependent menadione-reductases has been demonstrated in *E. coli* (Bragg, 1965) and in *Mycobacterium phlei* (Asano *et al.*, 1965).

From the results presented in this thesis, it may be concluded that the reducing equivalents from either sulphite or NADH are transferred to nitrate *via* the electron transport chain composed of flavins, quinone, cytochromes *b*, *c* and finally to the Mo-containing nitrate reductase. Nitrate reductase activities with sulphite and NADH respectively as the reductants, however, showed some important differences: the greater dependence of NADH-linked activity on -SH groups, inactivation by deoxycholate treatment of the NADH system only, differential effects of flavin inhibitors and difference in their pH optima, indicate that the electrons from these two reductants are donated at separate sites in the electron transport chain. Similar inhibitory effects of dicumarol, U.V. irradiation, antimycin A, metal chelators and the observed involvement of cytochrome c_{554} in both the sulphite- and NADH-linked nitrate reductase activities indicate that the components of the electron transfer chain beyond the flavins are common to both systems.

During the present investigation, a comparison was made between the sulphite oxidizing systems with nitrate and O_2 as terminal electron acceptors. Sulphite-linked O_2 uptake and nitrate reduction was maximal in the P100 fraction, indicating that both systems were associated with

similar cell membranes. Kodama and Mori (1968) found that the sulphite oxidizing system linked to O_2 was associated with cell membranes in *Thiobacillus thiooxidans*.

In the present work it is shown that as with nitrate reductase, sulphite and NADH were effective reductants for O_2 uptake. The inhibition of sulphite-linked O_2 uptake by thiol-binding agents, reported herein, has been observed for sulphite oxidizing systems in *Thiobacillus novellus* (Charles and Suzuki, 1966) and *Thiobacillus thioparus* (Lyric and Suzuki, 1970A). However, a particulate sulphite oxidase from *T. thiooxidans* was insensitive to -SH group inhibitors (Kodama and Mori, 1968).

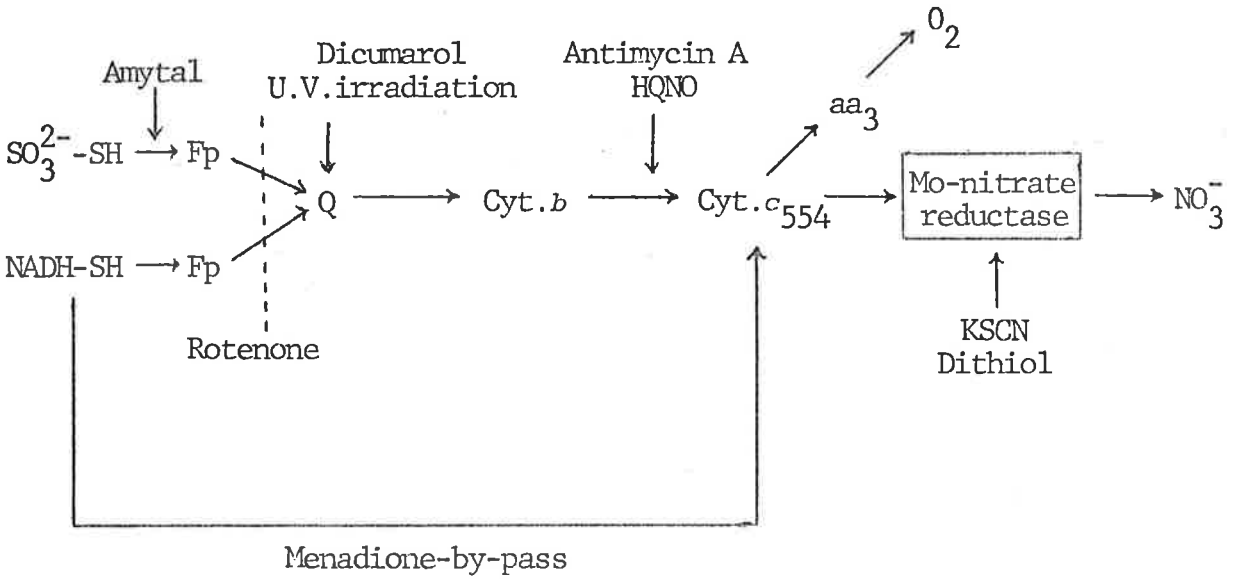
Sulphite-linked O_2 uptake and nitrate reduction are shown herein to be affected to about the same extent by various inhibitors of the electron transport chain, viz. rotenone, atabrine, dicumarol, HQNO and antimycin A. Peeters and Aleem (1970) however observed aerobic oxidation of sulphite in *T. denitrificans* was markedly less sensitive to rotenone than was the anaerobic oxidation of sulphite.

In the work presented here, the sulphite-dependent O_2 uptake is more susceptible to cyanide than the sulphite-linked nitrate reductase whereas the reverse was true for azide.

The association of O_2 uptake and nitrate reduction with the same cellular fraction, their identical requirements for the reductants and comparable sensitivities to the various inhibitors of the respiratory chain indicate that as in the other nitrate dissimilatory bacteria, the reducing equivalents were transferred from sulphite and NADH to either O_2 or nitrate *via* a common respiratory chain.

On the basis of these observations, the following scheme is proposed for the transfer of electrons from sulphite and NADH respectively

to nitrate in *T. denitrificans*.



The inhibitory effects of NAD^+ and 5'-AMP on the NADH-linked nitrate reductase reported herein, are probably important in conserving NADH for the anabolic reactions of the cell. In chemolithotrophic bacteria including *T. denitrificans*, the incorporation of CO_2 into carbon compounds requires a supply of ATP and NADH. In these bacteria, ATP is produced from the oxidation of inorganic compounds whereas, NADH is generated through an ATP-driven reverse electron flow (Aleem *et al.*, 1963). The present investigation shows that in *T. denitrificans* NADH is also oxidized *via* the respiratory chain by either nitrate or O_2. Therefore the observed effects of NAD^+ and 5'-AMP on the NADH-linked nitrate reductase activity are probably important in regulating the rate of NADH oxidation. The positive cooperative effect of NADH on the NADH-linked nitrate reductase in the S18 fraction indicates that this nucleotide would be utilized at an accelerated rate only when present at relatively high concentrations. Whenever its concentration

in the cell declines, the accumulated NAD^+ besides inhibiting the NADH-linked enzyme would also suppress the positive cooperative effect of NADH on nitrate reductase. Thus under conditions of a limited supply of NADH, the accumulated NAD^+ would restrict the utilization of NADH for nitrate reduction and conserve it for anabolic reactions.

The inhibition of the NADH-linked nitrate reductase by 5'-AMP suggests that its activity is also controlled by the energy status of the bacterium. Whenever the concentration of ATP in the cell diminishes, elevated concentrations of 5'-AMP, produced from the action of adenylate kinase on ADP, would decelerate the utilization of NADH by nitrate. Such a regulation in chemolithotrophic bacteria, in which NADH is generated *via* an ATP-driven reverse electron flow, is essential particularly when the concentration of ATP in the cell is low. These findings are pertinent in view of the proposal by Aleem (1977) that the functioning of electron transport chain with respect to the forward and reverse electron flow could be dependent on the phosphorylation state of the adenine nucleotides. Thus the control of NADH-linked nitrate reductase by NAD^+ as well as energy charge of the bacterium may well be of physiological importance. Respiratory NADH-oxidase in *Mycobacterium tuberculosis* (Worcel *et al.*, 1965) and *Mycobacterium phlei* (Bogin *et al.*, 1969) and NADH-dehydrogenase from *E. coli* (Dancey and Shapiro, 1976) are also known to be regulated by NAD^+, AMP and Pi.

4.2 Nitrite reductase

The association of nitrite reductase with the cell membranes in *T. denitrificans* reported in the present investigation contrasts with

the soluble dissimilatory nitrite reductases in the other denitrifying bacteria (Radcliffe and Nicholas, 1968; Lam and Nicholas, 1969C; Newton, 1969; Iwasaki and Matsubara, 1972; Cox and Payne, 1973). The present work also shows that the P100 fraction from *T. denitrificans* contained an active sulphide-linked O_2 uptake system in addition to the sulphide-linked nitrite reductase. Thus the oxidation of sulphide in this fraction was linked to either nitrite or O_2 as a terminal oxidant.

The activity of nitrite reductase in this bacterium was also linked to the oxidation of sulphide. None of the other sulphur compounds examined was effective in reducing nitrite. Although the intact cells reduce nitrite with thiosulphate as a reductant (Baalsrud and Baalsrud, 1954; Ishaque and Aleem, 1973), in the present investigations, thiosulphate was not effective for nitrite reduction in the P100 fraction. As pointed out by Aminuddin (1974), it is likely that an enzyme system, probably the cleaving enzyme, required for the oxidation of thiosulphate *via* nitrite reductase, was either inactivated or removed during the preparation of the P100 fraction.

Besides sulphide, nitrite reductase in P100 fraction functioned with either NADH plus menadione or with ascorbate in conjunction with various electron carriers, viz. PMS, DCIP, TMPD as donor systems. The purified nitrite reductase although active with sulphide and ascorbate plus PMS did not utilize NADH plus menadione as a reductant. This effect was probably due to either the inactivation or removal of the NADH-dependent menadione reductase which mediated the transfer of electrons from NADH *via* menadione to nitrite reductase.

During these investigations, the effects of various inhibitors on the sulphide-linked nitrite reduction and O_2 uptake were examined.

Both activities were inhibited by p-CMB, iodoacetamide and NEM. The more pronounced inhibition by NEM was probably due to the formation of a complex between sulphide and NEM (Ellis, 1964). The effect of these inhibitors indicates a requirement for -SH groups for the two activities and is in accord with the properties of dissimilatory nitrite reductases from *P. aeruginosa* (Walker and Nicholas, 1961) and *P. denitrificans* (Radcliffe and Nicholas, 1968).

The results presented in this thesis show that the inhibitors of the respiratory chain did not affect the sulphide-linked nitrite reductase or O_2 uptake. The insensitivity of these reactions towards inhibitors of the electron transport chain (except dicumarol) indicates the non-involvement of some electron carriers of the respiratory chain for the transfer of electrons from sulphide to either nitrite or O_2. Ishaque and Aleem (1973) have also observed that some inhibitors of the electron transport chain did not affect nitrite reductase activity in the cells of *T. denitrificans*. They attributed this to the modification in the path of electron transfer in the presence of excess of nitrite and proposed that the electrons were transferred directly from a flavoprotein system to nitrite. However the work reported herein precludes the involvement of either flavin or cytochrome *b* in the sulphide-dependent nitrite reduction in the P100 fraction or in the purified enzyme.

The inhibition of the sulphide-dependent nitrite reductase and O_2 uptake in the P100 fraction by cyanide, 2,2'-bipyridyl, O-phenanthroline and 8-hydroxyquinoline indicates participation of transition metal(s). During these studies, the purified nitrite reductase has been shown to contain iron but not copper. These effects of the metal chelating agents on the nitrite reductase are similar to those reported for the respiratory nitrite reductases from other bacteria (Walker and Nicholas, 1961;

Radcliffe and Nicholas, 1968; Lam and Nicholas, 1969C; Iwasaki and Matsubara, 1972).

The combined results on the nature of the reductants required and effects of the inhibitors of the electron transport chain on the sulphide-linked nitrite reductase and O_2 uptake activities indicate that the reducing equivalents are transferred from sulphide to either nitrite or O_2 *via* the same electron transfer chain. Lam and Nicholas (1969C) also concluded that in *M. denitrificans*, the oxidation of NADH and succinate with either O_2 or nitrite as the terminal electron acceptor was mediated *via* the same electron transport system.

During the investigation reported herein, the membrane-bound, sulphide-linked nitrite reductase was dissociated from the membranes of cells treated with cold acetone. The solubilized enzyme was purified twenty-fold and had a molecular weight of 120,000 daltons. Although this value is slightly higher than that of 90,000 daltons of the crystalline enzyme from *Alcaligenes faecalis* (Iwasaki and Matsubara, 1971) it is in agreement with the molecular weight of 120,000 daltons for nitrite reductase from *M. denitrificans* (Lam and Nicholas, 1969C; Newton, 1969) and of 121,000 daltons of *P. aeruginosa* (Gudat *et al.*, 1973).

As with the enzyme from *M. denitrificans* (Lam and Nicholas, 1969C) and *P. aeruginosa* (Horio *et al.*, 1961A; Gudat *et al.*, 1973) the purified nitrite reductase from *T. denitrificans* did not contain copper. However, as for *P. aeruginosa* (Gudat *et al.*, 1973) iron was present in the purified enzyme from *T. denitrificans*.

The results presented in this thesis show that on adding sulphide anaerobically to either P100 fraction or the purified enzyme, a composite spectra for cytochromes *c* and *d* were obtained. The spectra of the

cytochrome *c* component of the *cd* complex of the purified enzyme, however, differed from that of mammalian cytochrome *c*. A bifurcated α-band of the bacterial cytochrome *c* was produced on adding reductant and it had a lower α/β absorbance ratio than mammalian cytochrome *c*. The spectra of cytochrome *d* of the reduced enzyme showed that the absorbance of its γ-band was much smaller. The absorbance ratio of α/γ bands of cytochrome *d* was markedly influenced by pH of the reaction mixture. The cytochrome *d* component of nitrite reductase was reduced by alkaline pyridine even in the absence of a reductant. Thus cytochrome *d* of the *cd* complex showed characteristic features of a chlorin haem. Cytochrome *d* of the enzyme from *P. aeruginosa* (Yamanaka and Okunuki, 1963C) and *M. denitrificans* (Newton, 1969) have also been identified as a chlorin derivative of haem *d*.

As reported herein, the sulphide-reduced cytochromes *c* and *d* of either the P100 fraction or the purified nitrite reductase were oxidized with nitrite but not with nitrate. These results signify that the sulphide-reduced cytochromes *c* and *d* participate in the reduction of nitrite. Nitrite reductases from *P. aeruginosa* (Horio *et al.*, 1961A), which was initially studied as a cytochrome oxidase but referred to as nitrite reductase in a later communication (Yamanaka and Okunuki, 1974) and from *M. denitrificans* (Lam and Nicholas, 1969C; Newton, 1969) and *A. faecalis* (Iwasaki and Matsubara, 1971) also contain cytochromes *c* and *d*. As with the enzyme from *P. aeruginosa* (Horio *et al.*, 1961A) and *A. faecalis* (Iwasaki and Matsubara, 1971), it is shown in this thesis that the purified nitrite reductase from *T. denitrificans* contained cytochromes *c* and *d* in 1 : 1 molar ratio. The results for the reduction and reoxidation of cytochromes *c* and *d* of the purified nitrite reductase, reported herein, indicate that the

reducing equivalents from sulphide are transferred first to cytochrome *c* and then to cytochrome *d* which acted as an immediate electron donor for the reduction of nitrite. A similar sequence of reduction of cytochromes *c* and *d* was observed in *P. aeruginosa* (Wharton *et al.*, 1973; Shimada and Orii, 1976). The proposed sequence of electron transfer for *cd* complex from *T. denitrificans* was further confirmed by a more positive redox-potential of cytochrome *d* than for cytochrome *c*. However, the redox-potential of cytochrome *d* of the enzyme from *P. aeruginosa* was more negative than that of cytochrome *c* (Horio *et al.*, 1961B; Wharton *et al.*, 1973; Shimada and Orii, 1976).

During purification of nitrite reductase, cytochrome c_{551} and cytochrome c_{554} were isolated. Cytochromes c_{551} and c_{554} from *P. aeruginosa* (Horio *et al.*, 1958) and cytochrome c_{552} from *M. denitrificans* (Smith *et al.*, 1966) have also been separated. In addition to these species of cytochrome *c*, a blue protein was also recovered from *P. aeruginosa* (Horio, 1958; Gudat *et al.*, 1973). This blue protein, which in its reduced state functioned as a reductant for cytochrome oxidase and nitrite reductase in *P. aeruginosa* (Yamanaka and Okunuki, 1963A; Gudat *et al.*, 1973), was however not detected during the purification of nitrite reductase from *T. denitrificans*.

Catalytic amounts of cytochrome c_{551}, isolated from *T. denitrificans* during the present investigation, significantly enhanced the sulphide-linked nitrite reductase activity. This effect was quite specific since neither cytochrome c_{554} nor mammalian cytochrome *c* stimulated enzyme activity. Thus cytochrome c_{551} is involved in transferring electrons from sulphide to nitrite. This was further supported by the observation that reduced cytochrome c_{551} but not mammalian cytochrome *c* served as an effective reductant for the purified

nitrite reductase. Reduced forms of cytochrome c_{551}, isolated from *P. aeruginosa* (Horio *et al.*, 1961A; Yamanaka and Okunuki, 1963A) and cytochrome c_{552} from *M. denitrificans* (Smith *et al.*, 1966) have also been shown to serve as electron donors for purified nitrite reductases and cytochrome oxidases in these microorganisms. In addition as shown herein for nitrite reductase from *T. denitrificans*, the specificity of the enzyme towards cytochrome c_{551} as a reductant was also observed in *P. aeruginosa* (Yamanaka and Okunuki, 1961A).

The purified nitrite reductase from *T. denitrificans* also exhibited a sulphide-linked cytochrome oxidase activity. As with nitrite reductase, this cytochrome oxidase activity was also stimulated by cytochrome c_{551} and in its reduced state it was an effective reductant for cytochrome oxidase activity.

The results presented in this thesis indicate that nitrite reductase and cytochrome oxidase have several common properties. These include their identical requirements for reductants, similar effects of various inhibitors of the respiratory chain in P100 fraction, involvement of cytochromes *c* and *d* in these reactions and a functional role for cytochrome c_{551} for both these activities. From these results it can be concluded that as in *P. aeruginosa* (Yamanaka and Okunuki, 1963A) and *M. denitrificans* (Lam and Nicholas, 1961C; Newton, 1969), these two activities are associated with the same enzyme which functions with either O_2 or nitrite as a terminal electron acceptor. The purified cytochrome *cd* complex of *A. faecalis* was also reported to possess both nitrite reductase and cytochrome oxidase activities (Iwasaki and Matsubara, 1971). In the present investigation, since the bacterium

were grown anaerobically with nitrate, the physiological function of this enzyme is for nitrite reduction. This is confirmed by a higher specific activity of the purified enzyme for nitrite reduction than for cytochrome oxidase activity. On the basis of the turnover numbers for nitrite reductase and cytochrome oxidase activities of the purified enzyme from anaerobically grown cells of *P. aeruginosa* (Yamanaka and Okunuki, 1963A) and *M. denitrificans* (Lam and Nicholas, 1969C; Newton, 1969), it was concluded that in these bacteria also the physiological function of the enzyme was for the reduction of nitrite.

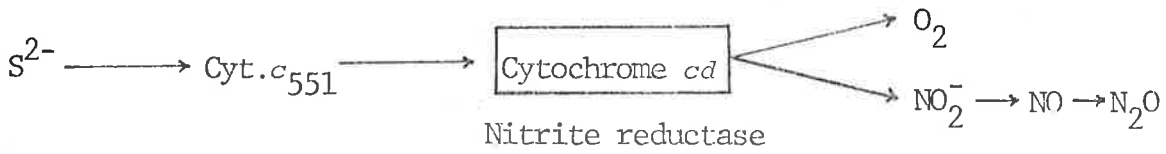
As reported earlier, the sulphide-reduced, purified nitrite reductase was oxidized by either nitrite or O_2. On oxidation of the sulphide-reduced enzyme with nitrite, a prominent shoulder appeared at 573 nm which was not observed when O_2 was the oxidant. The appearance of this band was probably associated with the formation of a complex between the enzyme and a product of nitrite reduction. A similar peak was obtained on flushing the sulphide-reduced enzyme with NO gas but not by either N_2O, N_2 or nitrate. Thus this spectral effect was produced by an interaction between NO and the reduced enzyme. The decrease in the absorbance at 573 nm with time, noted during the present investigation, suggests that NO was reduced further by the purified nitrite reductase. Rowe *et al.* (1977) also recorded an absorption peak at 572 nm on passing NO through cell-free extracts of *P. aeruginosa* and they also demonstrated that its disappearance was associated with the production of N_2O gas. The formation of similar peak at 572 nm was also reported during the oxidation of a reduced nitrite reductase from *P. aeruginosa* by nitrite (Yamanaka *et al.*, 1961; Yamanaka and Okunuki, 1963B) or after passing NO gas through a purified preparation of nitrite reductase (Yamanaka and Okunuki, 1963B; Shimada and Orii, 1975).

The results of present investigations with *T. denitrificans* also show that in addition to the NO complex with the sulphide-reduced enzyme, two absorption peaks at 535 and 565 nm were produced when NO was passed through the purified enzyme in the absence of sulphide. These peaks were observed at acid pH only (6.5 to 4). These spectral changes were associated with the formation of a complex between the oxidized enzyme and NO, because in the absence of the gas these effects were not observed. Shimada and Orii (1975) also reported the appearance of twin peaks at 529 and 560 nm at acid pH with the oxidized enzyme from *P. aeruginosa*, treated with NO. Similar bands were obtained during the present studies, on passing NO through the dissociated cytochrome *c* of the *cd* complex of *T. denitrificans*. Thus the absorption peaks at 530 and 565 nm result from an interaction of NO with the cytochrome *c* component of the *cd* complex.

The products of nitrite reduction by the purified enzyme from *T. denitrificans* were NO and N_2O. In contrast, the purified nitrite reductase from *P. aeruginosa* (Walker and Nicholas, 1961), *P. denitrificans* (Radcliffe and Nicholas, 1968), *M. denitrificans* (Lam and Nicholas, 1969C) and *P. perfectomarinus* (Payne *et al.*, 1971) produced NO only. However, highly purified nitrite reductase from *A. faecalis* (Matsubara and Iwasaki, 1972) reduced nitrite to NO and N_2O, in agreement with the results reported herein. The reduction of NO to N_2O by the purified nitrite reductase from *A. faecalis* may not be physiologically significant in view of the presence of another particulate nitric oxide reductase with a lower K_m for NO. Whether a separate nitric oxide reductase occurs in *T. denitrificans* is not known. In *P. perfectomarinus* nitrite, NO and N_2O reducing fractions have been isolated (Payne *et al.*, 1971).

As reported herein, cytochrome *c* and haem *d* were cleaved from the purified nitrite reductase by treating the enzyme with SDS. The purified enzymes from *P. aeruginosa* (Yamanaka and Okunuki, 1963C) and *M. denitrificans* (Lam and Nicholas, 1969C) were dissociated into haem *d* and a protein containing cytochrome *c* components by an acid-acetone treatment of the enzyme. In the present investigation neither the separated cytochrome *c* nor haem *d*, either singly or when mixed together, showed nitrite reductase or cytochrome oxidase activity. This contrasts with a report by Kijimoto (1968) that a cytochrome *d* fraction, obtained after sucrose-density gradient centrifugation of the SDS-treated nitrite reductase from *P. aeruginosa*, catalyzed the reduction of nitrite. Since their cytochrome *d* preparation contained appreciable amounts of cytochrome *c*, it cannot be concluded that the former component alone functions as a nitrite reductase.

On the basis of the properties of nitrite reductase described in this thesis, the following scheme is proposed for the sulphide-linked nitrite reduction in *T. denitrificans*:-

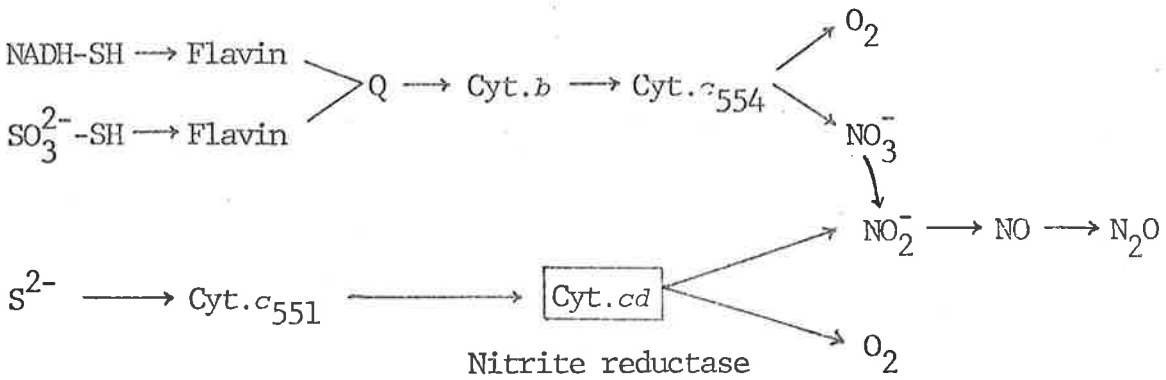


The present investigation indicates that nitrate and nitrite reductases in *T. denitrificans* have some enzymic properties that are similar to those in other denitrifying bacteria. The main important differences are associated with the reduced sulphur compounds which serve as physiological electron donors for these enzymes. This unusual requirement for sulphite and sulphide as the reductants for nitrate and nitrite reductases respectively correlates well with the

unique physiology of this chemolithotrophic bacteria which couples the oxidation of these reduced inorganic sulphur compounds with denitrification.

From the results presented in this thesis, it is concluded that the reducing equivalents from sulphite and sulphide to nitrate and nitrite respectively are transferred *via* different electron carriers.

An integrated electron transfer scheme coupling the oxidation of sulphite and sulphide to the reduction of nitrate and nitrite respectively, involving different carrier systems, is as follows:-



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